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Protective effect of serofendic acid on ischemic injury induced by occlusion of the middle cerebral artery in rats

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ABSTRACT

We previously reported that a sulfur-containing neuroprotective substance named serofendic acid purified and isolated from fetal calf serum prevented glutamate neurotoxicity in rat cortical cultured neurons. In the present study, we investigated the effect of serofendic acid on ischemic injury induced by a transient occlusion of the middle cerebral artery in rats. Serofendic acid was intracerebroventricularly administered 30 min after the onset of the occlusion. Serofendic acid (30 nmol) significantly reduced total infarct volume, similar to edaravone (30 nmol), a free radical scavenger. Treatment with serofendic acid (1–30 nmol) reduced the infarct volume in a dose-dependent manner. Moreover, serofendic acid (30 nmol) improved neurological deficit scores. These results suggest that intracerebroventricular administration of serofendic acid prevents the neurodegeneration induced by a transient focal cerebral ischemia and reperfusion.

1. Introduction

The neurotoxicity induced by the excessive activation of glutamate receptors and production of large amounts of free radicals is associated with various neurological disorders including ischemic brain injury (Clemens, 2000; Fujimura et al., 2005). Reactive oxygen species (ROS) such as the hydroxyl radical and nitric oxide (NO) have been considered important mediators of the brain damage caused by cerebral ischemia (Coyle and Puttfarcken, 1993; Dirnagl et al., 1999; Loh et al., 2006). Following a transient ischemia, free radicals produced during reperfusion may contribute to a disturbed membrane function, resulting in a critical intracellular calcium accumulation. The production of free radicals is considered to become prominent during reperfusion because reperfusion causes a resupply of oxygen in the penumbral cortex (Morimoto et al., 1996; Kuroda and Siesjo, 1997). Therefore, free radicals may play a role in the pathophysiologic cascade leading to ischemic brain injury with reperfusion. Moreover, a role for apoptotic mechanisms has been recently proposed in neuronal cell death following ischemic brain injury, although it had traditionally been considered that ischemic neuronal cell death involved necrotic mechanisms (MacManus and Linnik, 1997). Several studies have suggested that apoptotic mechanisms were initiated at the molecular level in ischemic or post-ischemic neurons, especially those in penumbral regions (Charriaut-Marlangue et al., 1996a,b; Rupalla et al., 1998; Sasaki et al., 2000).

We previously found a novel neuroprotective substance, serofendic acid in a lipophilic extract of fetal calf serum (Kume et al., 2002). Serofendic acid is a low-molecular-weight (mw.382) atisane-type diterpenoid. Atisane-derivatives have not been found in animals, although a few atisane-derivatives of plant origin, distinct from serofendic acid, have been reported (Appendino et al., 2000). Serofendic acid is a 15-hydroxy-17-methylsulfinylatisan-19-oic acid, a sulfur-containing atisane-type diterpenoid, which is an epimeric mixture having the opposite configuration in the sulfoxide group (Kume et al., 2002; Terauchi et al., 2002; Akaike et al., 2003). The compound exhibited potent protective action against neurotoxicity induced by glutamate, NO, and oxidative stress without inhibiting glutamate receptors in cultured cortical, striatal, and spinal cord neurons (Kume et al., 2002, 2005; Taguchi et al., 2003; Osakada et al., 2004). The molecular mechanisms of the neuroprotection remain to be determined, but it was demonstrated that serofendic acid inhibited the generation of the hydroxyl radical, a presumed executor radical in the neurotoxic cascade (Kume et al., 2002). We also reported that serofendic acid prevented acute glutamate neurotoxicity, which probably caused necrotic neuronal death (Taguchi et al., 2003). Moreover, serofendic acid inhibits mitochondrial membrane depolarization and caspase-3 activation (Kume et al., 2006). However, it remains unclear whether serofendic acid has protective effects on focal cerebral ischemia in vivo. Therefore, in this present study, we investigated the effect of serofendic acid on ischemic brain injury induced by a transient occlusion of the middle cerebral artery and subsequent reperfusion in rats.

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2. Materials and methods

2.1. Animals

Experiments were carried out using 10-week-old male Sprague–Dawley rats weighing 300–320 g, which were purchased from Nihon SLC (Shizuoka, Japan). The animals were treated in accordance with the guidelines of the Kyoto University animal experimentation committee, and the guidelines of the Japanese Pharmacological Society.

2.2. Surgical procedure and drug administration

The middle cerebral artery was occluded for 1.5 h then reperfused for 48 h using an intraluminal suture technique, modified as described in detail previously (Nagasawa and Kogure, 1989). Briefly, rats were anesthetized with halothane (4% for induction, 1% for maintenance, Takeda Pharmaceutical, Co, Ltd. Osaka) in a mixture of 70% nitrogen and 30% oxygen during surgery. After median incision of the neck skin, the left common and external carotid arteries were carefully exposed and ligated using 4-0 nylon surgical thread. A 19-mm length of 4-0 nylon surgical thread was transiently inserted into the left internal carotid artery for 1.5 h to occlude the left middle cerebral artery at its origin. While the animals were anesthetized, rectal temperatures were maintained at 37.5±0.5 °C. Regional cerebral blood flow in the left middle cerebral artery territory (5 mm lateral and 2 mm posterior to the bregma) was measured at each time point (0 h, 0.5 h, and 1 h after the onset of middle cerebral artery occlusion and 0.5 h, and 1 h after reperfusion) using a Laser-Doppler flowmetry FLO-N1 (Omegawave, Japan) in several animals, as described in detail previously (Amemiya et al., 2005). Sham-operated animals underwent the same procedures except for a transient occlusion. Animals were randomly divided into serofendic acid, edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one, Nacalai Tesque, Kyoto, Japan), and vehicle-treated groups. Serofendic acid and edaravone were dissolved in 1 N NaOH, titrated to pH7.5, and diluted with 50 mM PBS to doses of 1, 10, and 30 nmol. Rats were intracerebroventricularly administered each dose of serofendic acid, edaravone, or 50 mM PBS (vehicle group) after 30 min of occlusion. Serofendic acid was synthesized as described previously (Terauchi et al., 2002) and supplied by Eisai Co. Ltd. (Tsukuba, Japan).

2.3. Evaluation of infarct volume by TTC staining

Infarct volumes were evaluated as described previously (Zhao et al., 2005). Briefly, the brain was quickly removed and chilled in ice-cold saline. Eight 2-mm thick coronal sections were cut with a brain slicer and the slices were immerse in a saline solution containing 2.0% 2,3,5-triphenyltetrazolium chloride (TTC, Nakalai Tesque, Kyoto, Japan) at 37 °C for 20 min and fixed by immersion in 10% neutral formalin isotonic fluid. The sections were quantified using the public-domain Scion image program (Scion Co, Ltd. version beta 4.0.2). Total infarct volume was determined by summing the infarcted area of the eight sections and infarct volume in the cortex or the striatum was determined by summing the section number of 3, 4, 5 or 3, 4, respectively. They were presented as a percentage of the ipsilateral to the contralateral hemispheric volume.

2.4. Assessment of neurological deficit scores

Neurological symptoms after 48 h of reperfusion were assessed (n=9–12) using neurological deficit scores (grade 0–4), modified as described in detail previously (Murakami et al., 1998). Rats were suspended by the tail, and forelimb flexion and body twisting were evaluated as 0 (no observable neurological deficit), 1 (failed to extend right forepaw), 2 (circled to the right), 3 (failed to move the right), and 4 (could not walk spontaneously).

2.5. Body weight loss

Animals were weighed before ischemia and 48 h after reperfusion. Body weight loss is presented as a percentage of preischemic body weight.

2.6. Statistics

Data were expressed as means±S.E.M. Statistical analyses were performed using GraphPad Instat (Graph Pad Software, San Diego, USA). Regional cerebral blood flow, physiological parameters and infarct volume in the cortex or the striatum were analyzed using two-way analysis of variance. The effect of serofendic acid on the infarct volume or on the infarct volume in a dose-dependent manner was analyzed using one-way analysis of variance followed by Tukey's test or Dunnet two-tailed test, respectively. A Mann Whitney U test was used for neurological deficit scores. Statistical significance was defined as a probability value of less than 5%.

3. Results

3.1. Physiological parameters and body weight

At 3 h after ischemia-reperfusion, the effects of vehicle or serofendic acid on physiological parameters such as blood pH, gas pressure, and blood contents were examined. The treatment with serofendic acid did not influence physiological parameters, and there were no significant differences both between vehicle-treated group and serofendic acid-treated group and between the sham-treated

Table 1
Physiological parameters in the serofendic acid- and vehicle-treated groups

Groups	pO_2 (mm Hg)	pCO_2 (mm Hg)	pH	Na^+ (mmol/L)	K^+ (mmol/L)	Cl^- (mmol/L)	Gluc(mg/dL)	Lac (mg/dL)	Hct (%)
Vehicle									
Sham	83.8±11.6	47.1±1.5	7.35±0.02	138.3±0.9	3.9±0.2	98.8±0.7	221±10	19.8±5.7	45.6±1.0
I/R	93.2±10.8	39.6±5.0	7.37±0.03	137.9±1.0	5.0±0.7	99.2±0.6	204±11	27.6±7.9	45.5±0.8
Serofendic acid									
Sham	80.5±9.1	46.3±3.2	7.37±0.02	136.4±1.4	4.1±0.4	98.7±0.8	208±14	18.0±5.0	44.2±1.7
I/R	81.5±8.7	45.8±3.8	7.36±0.03	138.0±0.7	4.1±0.3	99.4±0.4	218±13	24.0±5.2	45.8±1.3
Sham vs *I/R*	F=0.32	F=1.32	F=0.06	F=0.40	F=2.21	F=0.94	F=0.00018	F=0.25	F=0.27
	P=0.58	P=0.26	P=0.81	P=0.53	P=0.15	P=0.34	P=0.99	P=0.62	P=0.61
Vehicle vs	F=0.67	F=0.61	F=0.07	F=0.78	F=0.69	F=0.00095	F=0.075	F=1.63	F=0.34
Serofendic acid	P=0.42	P=0.43	P=0.80	P=0.39	P=0.42	P=0.98	P=0.79	P=0.22	P=0.57

Serofendic acid (30 nmol) was given 0.5 h after the ischemia. There were no statistically significant differences in any parameters among the groups. Both values of degrees of freedom (surgery and drug) were 1. *I/R*: 1.5 h ischemia+3 h reperfusion. n=5–8. Gluc: glucose, Lac: lactate, Hct: hematocrit.

Table 2
Regional cerebral blood flow in the serofendic acid- and edaravone-treated groups

	Treatment		
	Vehicle	Serofendic acid (30 nmol)	Edaravone (30 nmol)
Regional cerebral blood flow (%)			
Immediately after ischemia	24.4±2.7	25.3±5.1	20.2±2.2
0.5 h after ischemia	19.2±4.3	21.2±7.2	18.3±2.8
1 h after ischemia	16.6±3.6	26.5±5.1	18.6±1.9
0.5 h after reperfusion	72.9±13.7	86.5±3.8	77.9±11.5
1 h after reperfusion	70.8±10.9	89.7±0.6	73.1±9.2

There were no statistically significant differences in any parameters among the drugs. $n=3$. The values of variance ratio, degrees of freedom and probability level were $F_{drug}=3.284$, $df_{drug}=2$, $P_{drug}=0.051$, $F_{time}=105.58$, $df_{time}=4$, $P_{time}<0.001$.

group and ischemia/reperfusion-treated group (Table 1). The body weight decreased at 48 h after reperfusion in vehicle-, serofendic acid- and edaravone-treated groups. These reductions were not significantly different among all groups (vehicle 85.4±1.6, serofendic acid 88.5±1.6, edaravone 91.0±2.1, $n=8$–11).

3.2. Regional cerebral blood flow (rCBF)

In the three groups, the occlusion resulted in a reduction of cerebral blood flow. At all time points (immediately, 0.5 h, and 1 h after ischemia and 0.5 h and 1 h after reperfusion), regional cerebral blood flow showed no significant differences between the vehicle-treated group and the serofendic acid- or the edaravone-treated group (Table 2).

3.3. Effect of Serofendic acid on infarct volume

Fig. 1 shows representative images of protective effects on ischemic brain injury. Total infarct volume was significantly reduced in the serofendic acid-treated group compared to the vehicle-treated group (Figs. 1, 2). Fig. 2 shows dose-dependent effects of serofendic acid on ischemic infarct volume. Serofendic acid (1–30 nmol) reduced the volume in a dose-dependent manner. Serofendic acid significantly exhibited protective effect in cerebral cortex, but not in striatum (Fig. 3). Moreover, infarct volume was significantly reduced in the edaravone-treated group (30 nmol) compared to the vehicle-treated group (Fig. 4).

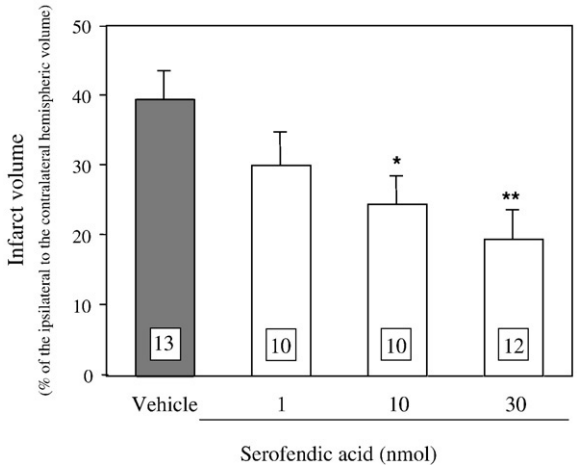

Fig. 2. Dose-dependent effect of serofendic acid on infarct volume induced by focal cerebral ischemia. An intracerebroventricular administration of serofendic acid (1–30 nmol) was given 0.5 h after the onset of the occlusion. Numbers in the columns indicate the number of experiments. *$P<0.05$, **$P<0.01$, compared with vehicle.

3.4. Neurological deficit scores

Fig. 5 demonstrates the neurological deficit scores. The treatment with serofendic acid significantly improved neurological deficit scores

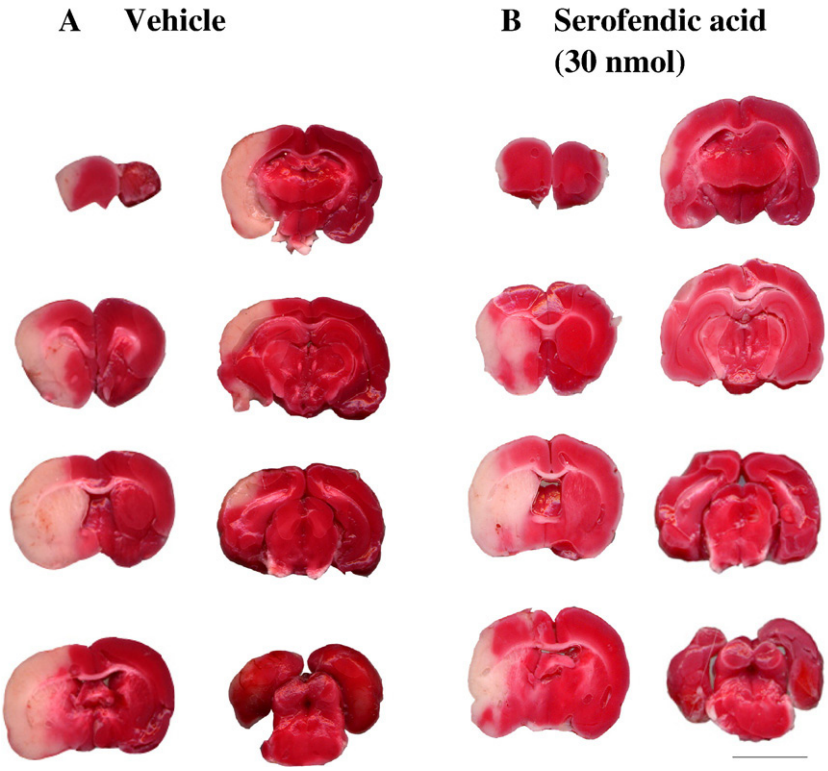

Fig. 1. Representative photographs of brain sections from rats injected with vehicle (A) or serofendic acid (B). The left middle cerebral artery was occluded for 1.5 h with subsequent reperfusion. After 48 h of reperfusion, the brain was sliced into coronal sections for TTC staining. An intracerebroventricular administration of serofendic acid was given 0.5 h after the onset of the occlusion. Calibration bar=1 cm.

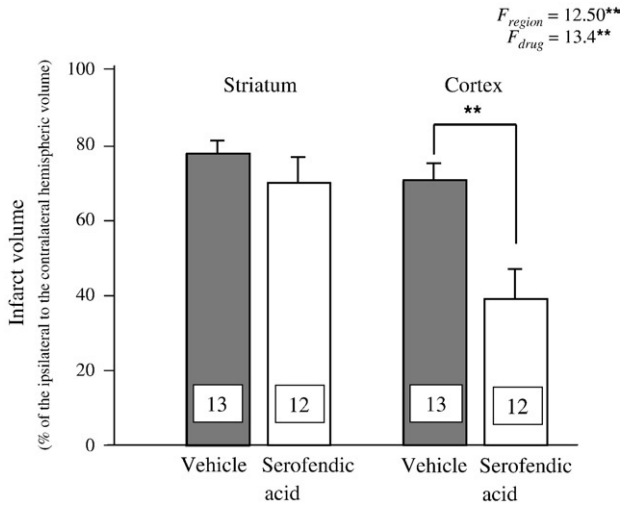

Fig. 3. The effect of serofendic acid on infarct volume in the cortex or striatum. Infarct volume in each area is expressed as the percentage of the ipsilateral to the contralateral hemispheric volume. Numbers in the columns indicate the number of experiments. The values of degrees of freedom were $df_{region} = 1$, $df_{drug} = 1$. $^{**}P<0.01$.

(Fig. 5A), compared to the vehicle, although scores of the edaravone group were not improved (Fig. 5B).

4. Discussion

In the present study, we investigated the effect of serofendic acid on focal cerebral ischemia using a transient middle cerebral artery occlusion in rats. Serofendic acid dose-dependently reduced infarct volume and improved neurological deficit scores, without influencing physiological parameters, body weight, or regional cerebral blood flow.

We demonstrated that serofendic acid reduced total infarct volume. As shown in Fig. 1 and Fig. 3, serofendic acid exhibited a neuroprotective action in the cortical area rather than in the striatum. Cerebral cortex contained a large volume of ischemic penumbra in an experimental focal ischemic model (Sommer et al., 2006). The neurodegenerative process in the penumbral region was related to delayed apoptotic neuronal cell death (Culmsee et al., 2005). Thus, serofendic acid may inhibit apoptotic neuronal death in the penumbral region. The opening of the mitochondria permeability transition pore also has a pivotal role in focal ischemic brain injury (Andrabi et al., 2004; Schinzel et al., 2005). Previous studies have

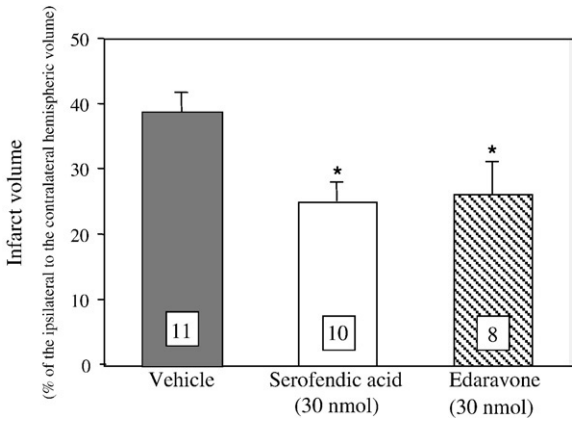

Fig. 4. Effects of serofendic acid and edaravone on infarct volume induced by focal cerebral ischemia. An intracerebroventricular administration of edaravone or serofendic acid was given 0.5 h after the onset of the occlusion. Numbers in the columns indicate the number of experiments. $^{*}P<0.05$, compared with vehicle.

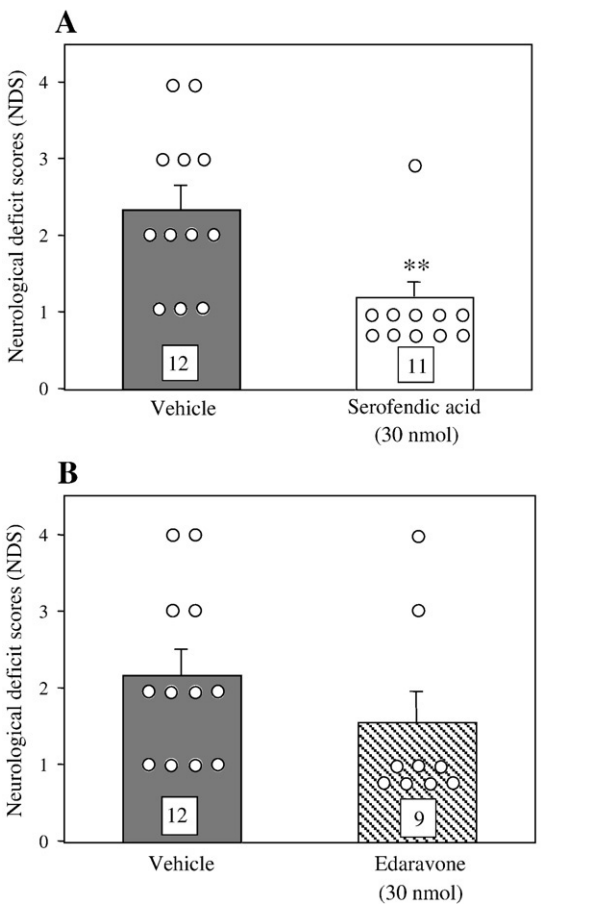

Fig. 5. Effects of serofendic acid (A) and edaravone (B) on neurological deficit scores after 48 h of reperfusion following a transient occlusion of the middle cerebral artery. An intracerebroventricular administration of edaravone and serofendic acid was given 0.5 h after the onset of the occlusion. Numbers in the columns indicate the number of experiments. $^{**}P<0.01$, compared with vehicle.

shown that the inhibition of the permeability transition pore in mitochondria may essentially contribute to its anti-apoptotic effects in transient brain ischemia. As we previously reported, serofendic acid has an anti-apoptotic effect on glutamate-induced neuronal cell death in cultured cortical neurons (Kume et al., 2006). Also, we reported that the protective mechanism of serofendic acid might involve the opening of mitoK_{ATP} channels (Takeda et al., 2006). Taken together, one of the protective mechanisms of serofendic acid in transient middle cerebral artery occlusion might be related to anti-apoptotic effects by inhibition of the opening of permeability transition pores in mitochondria.

We also showed that edaravone, a free radical scavenger, also ameliorated the ipsilateral infarct volume in rodent models of transient middle cerebral artery occlusion. These findings are in full agreement with results presented in other previous studies (Mizuno et al., 1998; Nakashima et al., 1999; Amemiya et al., 2005). Free radicals caused cytosolic deterioration during prolonged cerebral ischemia-reperfusion in a transient cerebral ischemia model (Siesjo and Siesjo, 1996; Fiskum et al., 2004). Previous studies reported that hydroxyl radical formation was increased in the penumbral cortex during middle cerebral artery occlusion (Morimoto et al., 1996). Edaravone is considered to protect cerebral cortex containing a large penumbral area which may be pharmacologically rescued even after the ischemic event. We previously demonstrated that serofendic acid inhibited the formation of hydroxyl radicals (Kume et al., 2002). Therefore, serofendic acid may have a protective effect by scavenging free radicals, which is similar to the effect of edaravone.

The intracerebroventricular administration of serofendic acid reduced the ipsilateral infarct volume. These results were related to an improvement of neurological deficit scores. In contrast, the effect of edaravone did not reach statistical significance. It remains to be resolved why edaravone did not significantly improve neurological deficit scores in the present study. Previous studies have shown that the intravenous administration of edaravone improved neurological deficit scores in transient brain ischemia (Amemiya et al., 2005; Zhang et al., 2005). The cause may be the intracerebroventricular injection of edaravone in our studies resulting in a different absorption and distribution of the drug.

In conclusion, our studies show that serofendic acid had protective effects on ischemic brain injury induced by a transient middle cerebral artery occlusion in rats. Serofendic acid is a low-molecular-weight substance with anti-apoptotic and anti-radical effects, so it may inhibit apoptosis of neurons. Our results raise the possibility that serofendic acid and its analogs may be of therapeutic use in protecting against ischemic brain injury.

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