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Hydroxyl radical generation after the third hour following ischemia contributes to brain damage

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Abstract

The purpose of the present study was to determine after what time period hydroxyl radical formation contributes most to ischemic brain damage in focal ischemia, using a hydroxyl radical scavenger, EPC-K_1, L-ascorbic acid 2-[3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethyl-tridecyl)-2*H*-1-benzopyran-6yl-hydrogen phosphate] potassium salt. Focal ischemia was produced by thrombotic occlusion of the left middle cerebral artery in rats. After evaluation of the pharmacokinetics of EPC-K_1 in the brain tissue and plasma following 10 mg/kg intravenous bolus treatment of conscious rats, we investigated the neuroprotective effect of EPC-K_1 in the middle cerebral artery occlusion model. A single intravenous bolus of EPC-K_1 was given immediately, 3 or 6 h after ischemia, and cerebral brain damage was measured 24 h after ischemia. When EPC-K_1 was injected 3 h after ischemia, a significant ($P < 0.01$) reduction of cerebral brain damage was observed. EPC-K_1 delivered by intravenous infusion that started immediately after ischemia and lasted for 24 h, also significantly ($P < 0.05$) reduced brain damage, but the efficacy of the neuroprotective effect was the same as that of the 3 h after ischemia bolus treatment. These results may indicate that the period of hydroxyl radical formation most critical for ischemic brain damage is a few hours after the third hour following ischemia in this model.

Keywords: Hydroxyl radical; EPC-K_1; Cerebral focal ischemia; Middle cerebral artery occlusion; Thrombosis middle cerebral artery; (Rat)

1. Introduction

It is known that oxygen free radicals contribute to ischemic brain damage. Among oxygen free radicals, the hydroxyl radical is highly reactive and oxidizes essential cellular lipids, protein, and nucleic acids, leading to cell damage and ultimately to cell death (Oliver et al., 1990; Floyd and Carney, 1992). The hydroxyl radical is generated from superoxide or hydrogen peroxide in the presence of ferrous iron (Repine et al., 1981). The hydroxyl radical can also be generated from the degradation of peroxynitrite, a product of the reaction between superoxide and nitric oxide (Beckman et al., 1990). Under physiological conditions, superoxide and hydrogen peroxide are constantly scavenged by superoxide dismutase, and by glutathione peroxidase and catalase, respectively. But in ischemia, the overproduction of oxygen free radicals perturbs the antioxidative defense mechanisms, and hydroxyl radicals are generated.

In several animal models of focal ischemia, an increase in the release of hydroxyl radicals has been reported (Morimoto et al., 1996; Kil et al., 1996). Studies have shown that radical scavengers and inhibitors of lipid peroxidation can ameliorate ischemic neuronal damage (Clemens et al., 1993; Cao and Phillis, 1994; Umemura et al., 1994; Zhao et al., 1994; Pahlmark and Siesjö, 1996; Takasago et al., 1997) and brain edema (Abe et al., 1988; Nishi et al., 1989; Cao and Phillis, 1994). Iron depletion or chelation, prevention of hydroxyl radical generation, reduces ischemia/reperfusion-induced edema in gerbil brain (Patt et al., 1990). In studies of focal ischemia using transgenic and knockout mutant mice, the ischemic infarct volume was significantly reduced in superoxide dismutase 1 transgenic mice (Kinouchi et al., 1991) and in neuronal nitric oxide synthase knockout mice (Huang et al., 1994).

The purpose of the present study was to find the period of hydroxyl radical formation most critical for ischemic brain damage, and how long the antioxidative defense mechanisms persist after ischemia. We investigated the neuroprotective effect of a hydroxyl radical scavenger, EPC-K_1, L-ascorbic acid 2-[3,4-dihydro-2,5,7,8-tetra-

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methyl-2-(4,8,12-trimethyl-tridecyl)-2*H*-1-benzopyran-6yl-hydrogen phosphate] potassium salt (Mori et al., 1989), on middle cerebral artery thrombotic occlusion in rats. Based on the results of measurement of EPC-K_1 in the brain tissue after a single intravenous bolus treatment, the optimum time of post-occlusion treatment was sought for. The most critical period of hydroxyl radical formation for ischemic brain damage is discussed.

2. Materials and methods

Male Sprague–Dawley rats (Japan SLC, Japan) weighing 280 to 300 g were used. All experiments were performed in accordance with institutional guidelines of Hamamatsu University School of Medicine.

2.1. Measurement of EPC-K_1 in brain tissue and plasma

The concentration of EPC-K_1 in brain tissue and plasma was determined by high performance liquid chromatography (HPLC). EPC-K_1 (10 mg/kg) was administered as a single bolus intravenous injection in conscious rats. Blood samples were collected 5 min, 0.5, 1 or 3 h after injection, and the plasma fractions were collected. For the brain tissue sample collection, rats were anesthetized with sodium pentobarbital 30 min, 1 or 3 h after injection, and perfused transcardially with 100 ml of saline at 100 mm Hg. The whole rat brain without the cerebellum was then removed.

2.2. Animal preparation

The rats were anesthetized with 4% halothane and subsequently maintained with 2% halothane in a 30% oxygen and 70% room air mixture. The middle cerebral artery was occluded according to the method of Umemura et al. (1993). A vertical incision was made between the left orbit and the external auditory canal. The temporalis muscle was reflected, and a subtemporal craniotomy without removal of the zygomatic arch was performed. The main trunk of the middle cerebral artery and the olfactory tract were observed through the dura mater. Photoillumination using green light (wavelength, 540 nm) was achieved by using a xenon lamp (model L-4887, Hamamatsu Photonics, Japan) over the main trunk of the middle cerebral artery at the olfactory tract through the dura mater. Rose bengal (20 mg/kg) was injected intravenously (i.v.). Photoillumination was performed for 10 min. The temporalis muscle and skin were then closed in layers and the anesthesia was discontinued. The surgical procedure was carried out within 20 min. During the operation, the body temperature of the animals was maintained at 37.5°C with a heating pad (K-module model K-20, American Pharmaseal, USA). About 24 h after the photoillumination, the animals were decapitated under pentobarbital sodium anesthesia. The brain was removed and six preselected coronal sections (from anterior 3.5 mm to anterior 13.5 mm) were made. Each section was stained with 1% 2,3,5-triphenyltetrazolium chloride in phosphate buffer (pH 7.4). Photographs of the sections were then taken. For each animal, the sum of the area of brain damage in cortex and in striatum, and the sum of the whole area of cerebrum in six sections were calculated by using a computerized image analysis system. After correction for swelling (Swanson et al., 1990), the brain damage in each animal was calculated by the ratio of the area of the brain damage to the whole area of the cerebrum.

2.3. Administration of EPC-K_1

EPC-K_1 was dissolved in saline. For the single intravenous bolus, EPC-K_1 was administered at three different time points. EPC-K_1 was administered immediately, 3 or 6 h after MCA occlusion, and the brain damage 24 h after occlusion was compared to that in the saline injected control group. In a separate experiment, saline and EPC-K_1 (10 mg kg^{-1} h^{-1}) were infused i.v. for 24 h, starting immediately after occlusion.

2.4. Statistical analysis

Data are presented as the means ± S.E.M. Statistical analysis was performed using an analysis of variance (ANOVA). Comparison of two groups was made by means of an unpaired Student's *t*-test. $P < 0.05$ was considered significant.

3. Results

Fig. 1 shows the concentration of EPC-K_1 in the plasma and brain tissue. The half-life ($t_{1/2}$) in the plasma was 32.2 min for the one compartment analysis. The concentration in brain tissue was maintained at about 4.4 μg/g of tissue for 1 h after administration. The limit of detection was 0.4 μg/g of tissue.

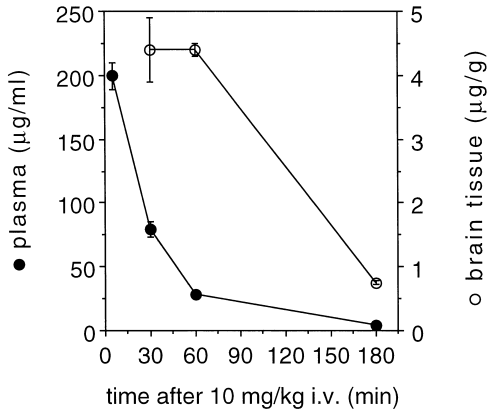

Fig. 1. Concentrations of EPC-K_1 in plasma and brain tissue.

Physiological variables during each operation were within the normal range. Middle cerebral artery occlusion in the rats led to brain damage that affected both the cortex and the striatum.

A single bolus treatment with EPC-K_1 immediately after occlusion slightly reduced the total brain damage (control: 19.6 ± 2.3%, $n = 8$; EPC-K_1: 15.5 ± 1.9%, $n = 8$) and the cortical brain damage (control: 13.6 ± 1.9%; EPC-K_1: 10.2 ± 1.7%) (Fig. 2A).

Fig. 2B shows the neuroprotective effect of EPC-K_1 when a single bolus treatment was performed 3 h after ischemia. The total and the cortical brain damage in the control group ($n = 8$) was 20.8 ± 0.9% and 14.4 ± 0.8%, respectively. The total and the cortical brain damage in the EPC-K_1 treated group ($n = 8$) was 12.9 ± 1.1% ($P < 0.01$) and 7.8 ± 1.1% ($P < 0.01$), respectively.

Treatment with EPC-K_1 6 h after occlusion had no effect on brain damage (control ($n = 8$): total 16.6 ± 1.4%, cortex 11.6 ± 1.0%; EPC-K_1 ($n = 8$): total 15.5 ± 1.6%, cortex 10.2 ± 1.6%) (Fig. 2C).

Fig. 2D shows the effect of EPC-K_1 on brain damage when it was infused i.v. starting immediately after occlusion and continuing for 24 h. The total and the cortical brain damage of the control group ($n = 8$) was 19.0 ± 0.9% and 12.6 ± 0.8%, respectively. The total and the cortical brain damage in the EPC-K_1 (10 mg kg^{-1} h^{-1})-treated group ($n = 8$) was 13.0 ± 1.1% ($P < 0.05$) and 8.1 ± 0.7% ($P < 0.05$), respectively.

The dose-dependence of the neuroprotective effect of EPC-K_1 3 h after occlusion was examined (Fig. 3). A single bolus treatment with 1, 3, or 10 mg/kg EPC-K_1 was given 3 h after occlusion. The total and the cortical brain damage in the control group ($n = 9$) was 19.9 ± 1.2% and 14.4 ± 1.2%, respectively. An amount of 1 mg/kg of EPC-K_1 ($n = 9$) did not reduce the brain damage (total brain damage, 17.4 ± 1.4%; cortical brain damage, 12.3 ± 1.2%). A 3 or 10 mg/kg of EPC-K_1 reduced the brain damage. The total and the cortical brain damage in the group treated with 3 mg/kg EPC-K_1 ($n = 8$) was 14.7 ±

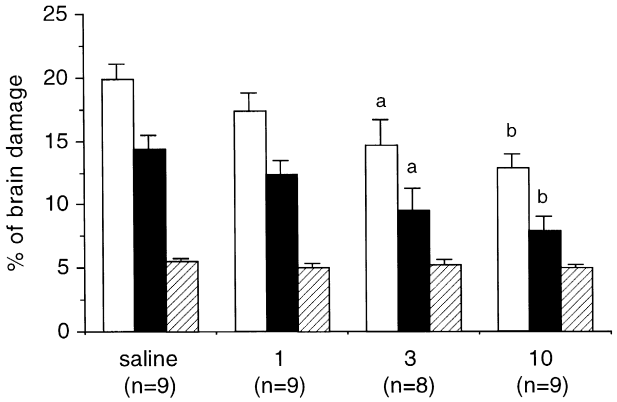

Fig. 3. Dose-dependent neuroprotective effect of EPC-K_1 on ischemic brain damage following middle cerebral artery occlusion in rats. EPC-K_1 (1, 3, or 10 mg/kg) was administered as a single bolus intravenous injection 3 h after occlusion. The open columns represent total brain damage, the dotted columns represent cortical brain damage, and the hatched columns represent striatal brain damage. Each column represents the mean for eight to nine animals, and the bars indicate the S.E.M. $^{a}P < 0.05$, $^{b}P < 0.01$ vs. saline group.

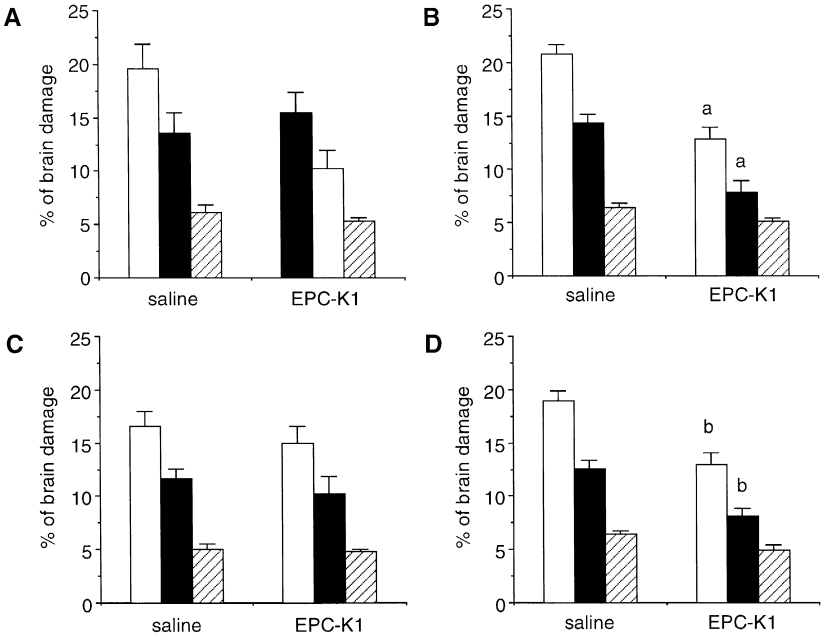

Fig. 2. Effect of EPC-K_1 on ischemic brain damage following middle cerebral artery occlusion in rats. EPC-K_1 (10 mg/kg) was administered as a single bolus intravenous injection immediately (A), 3 h (B), 6 h (C), or after occlusion. EPC-K_1 (10 mg kg^{-1} h^{-1}) was infused i.v. starting immediately after occlusion and continuing for 24 h (D). The open columns represent total brain damage, the dotted columns represent cortical brain damage, and the hatched columns represent striatal brain damage. Each column represents the mean for eight animals, and the bars indicate the S.E.M. $^{a}P < 0.05$, $^{b}P < 0.01$ vs. saline group.

2.0% ($P < 0.05$) and 9.5 ± 1.7% ($P < 0.05$), respectively, and those in the group treated with 10 mg/kg EPC-K_1 ($n = 9$) was 12.9 ± 1.1% ($P < 0.01$) and 7.9 ± 1.1% ($P < 0.01$), respectively.

4. Discussion

As shown in Fig. 1, the EPC-K_1 concentration in the brain tissue was maintained for 1 h, and was slightly over the limit of detection (0.4 μg/g of tissue) 3 h after administration. Therefore, in this study, we performed three courses of a single bolus treatment, immediately, 3 or 6 h after ischemia, and a continuous infusion for 24 h, as a sufficient treatment. In vitro studies showed that EPC-K_1 scavenges the same amount of hydroxyl radicals (1:1 in mol) generated by the iron-catalyzed reactions, but cannot scavenge superoxide generated from the hypoxanthine–xanthine oxidase system (Mori et al., 1989).

In the case of the single intravenous bolus, 10 mg/kg of EPC-K_1 attenuated brain damage 3 h, but not immediately or 6 h after middle cerebral artery occlusion. These results may indicate that EPC-K_1 scavenged hydroxyl radical which was generated in a few hours after the third hour after ischemia, and attenuated brain damage 24 h after ischemia. Brain damage was also attenuated by a continuous infusion of 10 mg kg^{-1} h^{-1} EPC-K_1 for 24 h. We expect that this treatment reduced brain damage more efficiently than when carried out 3 h after ischemia. But the efficacy of the neuroprotective effect of a continuous infusion (32% reduction of damaged area, brain damage in control group, 19.0 ± 0.9%; brain damage in EPC-K_1-treated group, 13.0 ± 1.1%) was the same as that of a single bolus administration 3 h after ischemia (38% reduction of damaged area: brain damage in control group, 20.8 ± 0.9%; brain damage in the EPC-K_1-treated group, 12.9 ± 1.1%). These results may indicate that it was enough to scavenge hydroxyl radicals which were generated in a few hours after the third hour after ischemia in order to reduce brain damage 24 h after ischemia in this model. Therefore, we examined the minimum effective dose of EPC-K_1 when it was given 3 h after ischemia. EPC-K_1 reduced brain damage in a dose-dependent manner, and the minimum effective dose was 3 mg/kg.

Neuroprotective effects of α-phenyl-*N*-*tert*-butylnitrone, a spin trapping agent, on a model of focal cerebral ischemia have previously been shown (Cao and Phillis, 1994; Zhao et al., 1994; Pahlmark and Siesjö, 1996). Cao and Phillis (1994) reported that this compound was effective when it was given either prior to or for up to 12 h after ischemia. The differences in important periods concerning radical formation between previous and our results may depend on the differences in models and the selectivity of compounds used. Because we used a selective hydroxyl radical scavenger with short activity, we could investigate the most important period of hydroxyl radical formation clearly in this model.

It is generally considered that reoxygenation during reperfusion provides oxygen as a substrate for numerous enzymatic oxidation reactions that produce reactive oxidants. Therefore, several studies involving oxygen free radicals have been done in ischemia–reperfusion models. Reports on permanent ischemic models of focal cerebral ischemia are rare, but neuroprotective effects of U-74006F (Park and Hall, 1994), α-phenyl-*N*-*tert*-butylnitrone (Cao and Phillis, 1994), and ebselen (Takasago et al., 1997) have been reported. In the present study, we used the rat middle cerebral artery thrombosis model. In this model, the spontaneous recanalization of middle cerebral artery occurred about 8 h after occlusion (data not shown). Therefore, until about 8 h after ischemia, this model may resemble a permanent occlusion model. But, during middle cerebral artery occlusion, fibrin-rich microthrombi formation is observed in our model (Kawai et al., 1995). Microthrombi formation and degradation by endogenous thrombolytic substances may occur consecutively in microvessels, and consecutive ischemia–reperfusion-like phenomena may occur in this model.

Recent reports show the possible interactions between oxygen free radicals and excitatory amino acids in ischemic neuronal death. Bondy and Lee (1993) have reported that the activation of ionotropic receptors by excitatory amino acids generates oxygen free radicals, but that activation of metabotropic receptors cannot generate them in vitro. Hammer et al. (1993) have reported that *N*-methyl-D-aspartate receptor activation generates hydroxyl radicals in vivo. In our model, glutamate release during ischemia has been reported (Umemura et al., 1996). Morimoto et al. (1996) measured hydroxyl radical and glutamate concentrations during ischemia and reperfusion in a transient (2 h) middle cerebral artery occlusion model in rats. According to their data, glutamate levels increased immediately after ischemia; in contrast, hydroxyl radical levels increased slowly, and 2 h after ischemia, hydroxyl radical concentration reached a level about 2-fold that in control rats. This delayed generation of hydroxyl radicals may be in line with our present data. In the acute phase of ischemia, oxygen free radicals generated by glutamate may be scavenged by endogenous antioxidative defense mechanisms, such as superoxide dismutase, glutathione peroxidase, catalase, ascorbic acid and vitamin E. But prolongation of ischemia may perturb the antioxidative defense mechanisms, and as a result, hydroxyl radical, an extremely active oxidant, may be generated.

In conclusion, in the middle cerebral artery thrombotic occlusion model in rats, the most critical period of hydroxyl radical formation for ischemic brain damage is a few hours after the third hour following ischemia. The antioxidative defense mechanisms persist for a few hours after ischemia in this model.

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