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KR-31378 protects neurons from ischemia–reperfusion brain injury by attenuating lipid peroxidation and glutathione loss

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Abstract

Neuronal hyperexcitability and oxidative stress play critical roles in neuronal cell death in stroke. Therefore, we studied the effects of (2*S*,3*S*,4*R*)-*N*″-cyano-*N*-(6-amino-3,4-dihydro-3-hydroxy-2-methyl-2-dimethoxymethyl-2*H*-benzopyran-4-yl)-*N*′ -benzylguanidine (KR-31378), possessing both antioxidant and K^+ channel-modulating activities, on brain ischemia–reperfusion injury models. Treatment with KR-31378 (30 mg/kg, i.v.) significantly reduced infarct area and edema by 24% and 36%, respectively, in rats subjected to 2 h of middle cerebral artery occlusion and 22 h of reperfusion with significant attenuation of elevated lipid peroxidation (99% of normal) and glutathione loss (60% of normal) in ischemic hemisphere. We further studied its neuroprotective mechanism in fetal rat primary mixed cortical culture. Incubation of cortical neurons with KR-31378 protected $FeSO_4$-induced cell death in a concentration-dependent manner (IC_{50} = 12 μM). Its neuroprotective effect was neither mimicked by other K^+ channel openers nor abolished in the presence of ATP-dependent K^+ channel (K_{ATP}) blockers, indicating that its effect was not related to K^+ channel opening activity. The mechanism of protection is rather attributable to the antioxidant property of KR-31378 since it suppressed the intracellular accumulation of reactive oxygen species and ensured lipid peroxidation by 120% and 80%, respectively, caused by $FeSO_4$. We further studied its effect on antioxidant defense, enzymatic and nonenzymatic systems. Treatment of neurons with $FeSO_4$ resulted in decrease of catalase (8% of control) and glutathione peroxidase (14% of control) activities, which were restored by KR-31378 treatment (70% and 57% of control, respectively). In addition, it attenuated the depletion of glutathione contents (60% of control) caused by $FeSO_4$. These results suggest that KR-31378 exerts a beneficial effect in focal ischemia, which may be attributed to its antioxidant property.

Keywords: Brain ischemia; Oxidative neuronal injury; KR-31378; Neuroprotection; Antioxidant; K^+ channel opener

1. Introduction

Cerebral ischemia is caused by a deficiency in blood supply to a part of brain, which in turn triggers various pathophysiological changes. It has been accepted recently that ischemic cell injury arises from complex interactions between multiple electrophysiological, hemodynamic, and biochemical cascades, which include disturbances in energy metabolism, modifications in synaptic transmission, production of reactive oxygen species, stimulation of the inflammatory process, and endothelial dysfunction (Dirnagl et al., 1999). All neuroprotective agents so far targeting a specific pathway in ischemic cascade failed to demonstrate clinical efficacy (Keyser et al., 1999; Legos et al., 2002). Therefore, the search for novel therapeutic approaches is even more critical. In this context, KR-31378 (Fig. 1), a novel benzopyran derivative possessing both antioxidant (Kim et al., 2001; Hong et al., 2002) and K^+ channel-modulating activities (Yoo et al., 2001; Lee et al., 2001), was designed as a new therapeutic strategy for neuroprotection to obtain better clinical efficacy and a wider therapeutic window.

Previously, we have demonstrated that KR-31378 has good cardioprotective activity in the rat and dog models of ischemic myocardium, which appeared to be mediated by K_{ATP} opening activity (Yoo et al., 2001; Lee et al., 2001). ATP-sensitive K^+ channel has been known to be

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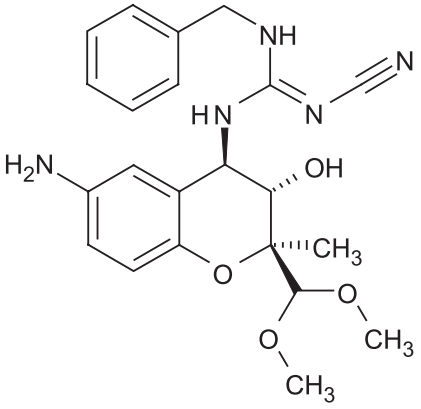

Fig. 1. The chemical structure of KR-31378.

involved in the protection against ischemia–reperfusion injury both in the heart and the brain, presumably through "ischemic preconditioning," an endogenous protective mechanism (Li et al., 1992; Liu et al., 1992; Shake et al., 2001). Several K^+ channel openers have been shown to protect cardiac myocytes against ischemic injury and cultured hippocampal neurons against excitotoxicity, presumably due to their ability to hyperpolarize the plasma membrane and reduce calcium influx (Abele and Miller, 1997).

In addition, KR-31378 showed cortical neuroprotection via inhibition of antiapoptotic cell death in focal ischemic brain rat model (Hong et al., 2002). We also reported that KR-31378 prevented lipopolysaccharide-induced apoptosis in endothelial cells in association with its reactive oxygen species-scavenging activity and reduction of tumor necrosis factor-α production (Kim et al., 2002). Increased formation of reactive oxygen species, which induced lipid peroxidation, protein oxidation, and DNA damage, and also served as signaling molecule in the apoptotic process, has been postulated to play a critical role in neuronal injury (Chan, 1994). Neuroprotective antioxidants are considered to be a promising approach to limit the extent of neuronal loss in ischemic brain injury. Several agents with antioxidant properties such as tirilazad (Peters et al., 1996), 2-phenyl-1,2-benzisoselenazol-3(2*H*)-one (ebselen) (Parnham and Seis, 2000), disodium 2,4-disulfophenyl-*N*-*tert*-butylnitrone (NXY-059) (Lees et al., 2001), and 3-methyl-1-phenyl-2-pyrazolin-5-one (MCI-186) (Wu et al., 2000) have been studied in experimental models and evaluated clinically.

Therefore, in the present study, the neuroprotective effect of KR-31378 was evaluated in middle cerebral artery (MCA) occlusion–reperfusion rat model, the primary site of many strokes, which is considered to more closely mimic the clinical situation (Macrae, 1992). Its neuroprotective mechanism was further studied in oxidative stress-induced neuronal injury model, and we describe the network of antioxidant defense system involved in the neuroprotective action of KR-31378 against oxidative injury.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats purchased from Orient (Seoul, Korea) were group-housed in polypropylene cages (38 × 23 × 10 cm) with no more than five animals. They were maintained under standard temperature (22 ± 2 °C) and relative humidity (50 ± 10%) control with 12 h of light–dark cycle and allowed free access to protein rodent diet (Harlan Teklad, USA) and tap water ad libitum. All experimental procedures described in rats were reviewed by the Animal Care Committee and performed in compliance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

2.2. Materials

Dulbecco's modified Eagle's medium (DMEM), DMEM/F12, minimum essential medium (MEM), Hank's balanced salt solution (HBSS), fetal bovine serum, horse serum, and laminine were purchased from Gibco BRL (Rockville, MD, USA). Catalase, glutathione, β-NADPH, poly-D-lysine, superoxide dismutase, glutathione peroxidase, glutathione reductase, and cytosine arabinoside were obtained from Sigma (St. Louis, MO, USA). Lactate dehydrogenase (LDH) assay kit was from Promega (Madison, WI, USA) and 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) was from Molecular Probes (Eugene, OR, USA). KR-31378 was prepared as previously published (Yoo et al., 2001), and (3*S*-*trans*)-*N*-(4-chlorophenyl)-*N*′-cyano-*N*″-(6-cyano-3,4-dihydro-3-hydroxy-2,2-dimethyl-2*H*-1-benzopyran-4-yl) guanidine (BMS-180448) and (3*R*)-*trans*-4-(4-chlorophenyl)-*N*-(1*H*-imidazol-2-yl-methyl)dimethyl-2*H*-1-benzopyran-6-carbonitrile monohydrochloride (BMS-191095) were synthesized in the Korea Research Institute of Chemical Technology (Daejeon, South Korea). Glibenclamide was from Molecular Probes; trolox, a hydrosoluble vitamin E analogue, was from Sigma-Aldrich (St. Louis, MO, USA); and 5-hydroxydecanoic acid, diazoxide, and dizocilpine (MK-801) were obtained from Sigma-RBI (St. Louis, MO, USA).

2.3. Transient focal ischemia in rat

Transient ischemia was induced in isoflurane (1.5–2.5% in 70% N_2/30% O_2)-anaesthesized male Sprague–Dawley rats (265–270 g) by 2-h occlusion of the right middle cerebral artery by the procedure of Chen et al. (1992) with minor modifications. Briefly, 1.7-cm length of a 4-0 surgical nylon suture (Nitcho Kogyo, Japan) coated with silicone (Bayer Dental, Germany) was advanced from the external carotid artery into the lumen of the internal carotid artery to occlude the origin of the middle cerebral artery. After 2 h, the filament was retracted to allow reperfusion of the ischemic region. Core body temperature was monitored

and maintained around 37 °C through the surgical procedure using a heating lamp and heating pad (Harvard Apparatus, Holliston, USA). The arterial blood pressure was monitored through the femoral artery with a blood pressure transducer (Harvard Apparatus) and physiological variables, including blood gas, pH, and glucose, were checked before and during middle cerebral artery occlusion and 15 min after reperfusion with an automatic pH/blood gas analyzer (Roche, Basel, Swiss) and glucose-E kit (Yeongdong Pharm, Korea).

Twenty-four hours after middle cerebral artery occlusion, the brains were sliced into 2-mm coronal sections and stained with 2% solution of 2,3,5-triphenyltetrazolium chloride in saline at 37 °C for 30 min. The size of infarct was calculated with an image analysis system (Image-Pro Plus; Media Cybernetics, Silver Spring, MD, USA) and expressed as the percentage of infarcted tissue in reference to the ipsilateral hemisphere. To evaluate the contribution of brain edema to the effect of a treatment, edema, expressed in percentage as the difference between left and right hemisphere volumes to the total hemisphere, was calculated in control and treated groups (Golanov and Reis, 1995). KR-31378 was administered as an intravenous bolus (30 mg/kg, 2 ml/kg) in 50% polyethylene glycol (PEG) 400 at 0, 4, and 8 h after reperfusion. MK-801 (2 mg/kg, i.p.) was injected before the onset of ischemia and 0 and 2 h after reperfusion. Sham-operated control rats received a bolus injection of 50% PEG 400.

2.4. Cortical neuron culture

Primary mixed glial–neuronal cultures were prepared from the brains of 16- to 18-day-old rat embryos (Choi et al., 1987) with the following modifications. After dissection, the cortical tissue was placed in trypsin–EDTA–HBSS solution at 37 °C for 15 min. The trypsin solution was removed and the tissue was gently dissociated in MEM, 5% fetal bovine serum, 5% horse serum, and 2 mM glutamine, and then plated onto 96-well plate or Petri dishes coated with poly-D-lysine and laminine. After 2 days in vitro, cultures were exposed to 0.5 μM cytosine arabinoside for 48 h to prevent non-neuronal cell division. Fresh media was added to cultures after cytosine arabinoside exposure and cells were maintained in the MEM supplemented with 28 mM $NaHCO_3$, 22 mM glucose, 2 mM glutamine, 5% horse serum, and 5% fetal bovine serum at 37 °C in a 5% CO_2-humidified incubator. Half of the medium was replaced with fresh medium every 3 days. All experiments were performed in 8- to 12-day-old cultures.

2.5. Astrocyte culture

Astrocytes were prepared from embryonic Sprague–Dawley rats by dissecting the cortices, removing the meninges, and dissociating the tissue through trituration using a fire-polished Pasteur pipette. Cells were suspended and plated in 75-cm^2 tissue culture flasks at a concentration of 1.5×10^7 cells in 11 ml of modified DMEM/F12 culture medium. Incubating the flasks at 37 °C in a 5% CO_2 incubator for 48–72 h before moving allowed the cells sufficient time to adhere and begin multiplying. The medium was changed at this time and every 48–72 h until the cells were ready to be used for culture on plates. After incubating the primary cultures for 2 weeks, the medium was changed completely, and the caps were tightened. Flasks were wrapped in plastic, placed on a shaker platform in a horizontal position with the medium covering the cells, and were shaken at 350 rpm for 3 h at 35 °C to remove the microglia from the astrocytes. The cells were passed from the flasks into 96-well plates at a concentration of 2.5×10^4 cells.

2.6. Model of oxidative stress-induced neurotoxicity

The cortical mixed cell cultures and pure astrocyte cultures were washed twice with MEM to reduce the serum concentration to 0.2% and were pretreated for 30 min with varying concentrations of test compounds. For the experiments, the test compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted in a medium. At this time, the final concentration of DMSO was not allowed to exceed 0.2%. For a control group, only vehicle was applied. After the pretreatment with test compounds or vehicle, $FeSO_4$ was added to a desired concentration, and the cultures were maintained for 24 h in a CO_2 incubator. The extent of neuronal damage was assessed by measuring the amount of LDH as a marker for membrane breakage and cell death (Koh and Choi, 1987). Neuronal cell protective effects of the test compounds were estimated by the reduction rate of LDH release in treatment group compared with that of vehicle control.

2.7. Measurement of neuronal oxidation

Intracellular accumulation of reactive oxygen species was determined with DCFH-DA. The amount of intracellular oxidants is proportional to the intensity of fluorescent DCF measured at excitation at 488 nm and emission at 510 nm (Goodman and Mattson, 1994). Oxidative stress was induced by adding 100 μM $FeSO_4$ and incubating for 4 h. Cultures were washed with DMEM and cells were incubated for 30 min in the presence of 15 μM of DCFH-DA. At the end of incubation, the cells were washed again and immediately processed for quantitative image measurements and fluorescence plate reader studies.

2.8. Inhibition of lipid peroxidation

For determination of lipid peroxidation in ischemic brain or cortical culture in the presence and absence of KR-31378 treatment, brains or cells were collected in 0.1 M

phosphate buffer (pH 7.4) and homogenized. The homogenates were centrifuged for 30 min at 17,400 × *g* or 3000 × *g*, respectively, at 4 °C, and the supernatant was used. The content of protein in the supernatant was determined by the bicinchoninic acid method using the BCA reagent (Pierce, Rockford, IL, USA). The resulting lipid peroxidation was evaluated by the formation of malondialdehyde—the main decomposition product of peroxides derived from polyunsaturated fatty acids (Esterbauer and Cheeseman, 1990). The content of malondialdehyde was determined using the chromogenic reagent *N*-methyl-2-phenylindole with maximal absorbance at 586 nm (Bioxytech® LPO-586 kit; Sigma).

2.9. Antioxidant enzyme activity

The supernatant prepared as described above was used for determination of the antioxidant enzyme activities, superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase. Glutathione reductase and glutathione peroxidase activities were determined according to the method described by Hopkins and Tudhope (1973). The final concentration in the reaction mixture was 100 mM K^+ phosphate buffer, pH 7.0, 1 mM EDTA, 1 mM GSSG, and 0.2 mM NADPH for glutathione reductase assay and 50 mM K^+ phosphate buffer, pH 7.0, 0.5 mM EDTA, 1 mM GSH, 0.5 mM NADPH, 0.5 mM sodium azide, 0.24 U/ml glutathione reductase, and 150 μM H_2O_2 for glutathione peroxidase assay. During both assays, the decrease in absorbance due to the oxidation of NADPH was recorded at 340 nm in a total volume of 200 μl at 30 °C. The catalase activity was measured according to the method of Beers and Sizer (1952) by monitoring the decomposition of H_2O_2 that was followed by decrease in absorbance at 240 nm. The difference in absorbance at 240 nm per unit time is a measure of the catalase activity. The superoxide dismutase activity was measured according to the method previously reported (Flohe and Otting, 1984). The reduction rate of cytochrome *c* by superoxide radicals was monitored at 550 nm by utilizing the xanthine–xanthine oxidase system as the source of O_2^-. Superoxide dismutase competes for superoxide and decreases the reduction rate of cytochrome *c*. The final concentration of reagents in the reaction mixture was 50 mM K^+ phosphate buffer, pH 7.0, containing 0.1 mM EDTA and cytochrome *c*, and the absorbance change at 550 nm was monitored.

2.10. Glutathione assay

The content of total glutathione (reduced and oxidized) in the supernatant of brain or cell homogenate was measured using the glutathione assay kit (Cayman Chemical, Ann Arbor, MI, USA) in microtiter plate assay, which utilizes an enzymatic recycling method, using glutathione reductase, for the quantification of glutathione. The sulfhydryl group of glutathione reacted with 5,5′ -dithiobis-2-nitrobenzoic acid (DTNB) and produced a yellow-colored 5-thio-2-nitrobenzoic acid (TNB), which was measured at 405 nm. The rate of TNB production is directly proportional to this recycling reaction, which is in turn directly proportional to the concentration of glutathione in the sample.

2.11. Statistical analysis

Data were expressed as the mean ± S.E.M. Analysis of variance (ANOVA) with post-hoc comparison (Bonferroni correction) was used for statistical analysis. For the neuroprotective experiment in a model of oxidative stress-induced neurotoxicity, neuronal cell protection and LDH release were compared with Student's *t* tests. $P < 0.05$ represents the level of significance.

3. Results

3.1. KR-31378 treatment reduced infarct size and edema with attenuation of elevated lipid peroxidation and glutathione loss in focal ischemia

KR-31378 treatment (30 mg/kg, i.v.) initiated after the completion of 2 h of ischemia significantly reduced the infarct size and edema by 24% and 36%, respectively. Similar protective effects were achieved by MK-801, a noncompetitive *N*-methyl-D-aspartate (NMDA) receptor antagonist dizocilpine (Fig. 2A and B). The physiological variables such as arterial blood pressure, blood gas, pH, glucose, and rectal body temperature were within normal range and not different in KR-31378- or vehicle-treated animals (Table 1). The mechanism of the neuroprotective effect of KR-31378 is likely to involve decreased oxidative damage in ischemic hemisphere. Therefore, we assessed brain malondialdehyde and brain levels of glutathione as oxidative stress markers. The levels of malondialdehyde in ipsilateral hemisphere after 24 h of middle cerebral artery occlusion were significantly increased in the vehicle-treated rats (30.7 ± 1.6 nmol/mg protein) than in the sham-operated rats (18.6 ± 2.9 nmol/mg protein, $P < 0.01$) while no changes were observed in the contralateral hemisphere. In the KR-31378-treated group, the levels of malondialdehyde (18.4 ± 3.0 nmol/mg protein, $P < 0.01$) were dramatically decreased to the levels seen in the normal control (Fig. 3A). Similarly, levels of glutathione in the ipsilateral hemisphere of ischemic rats (1.68 ± 0.59 nmol/mg protein) were significantly decreased compared to that in the normal rats (10.16 ± 0.66 nmol/mg protein, $P < 0.005$) while no changes were observed in the contralateral hemisphere. KR-31378 treatment (6.15 ± 0.24 nmol/mg protein, $P < 0.005$) significantly attenuated the reduced levels of glutathione in the ipsilateral hemisphere (Fig. 3B).

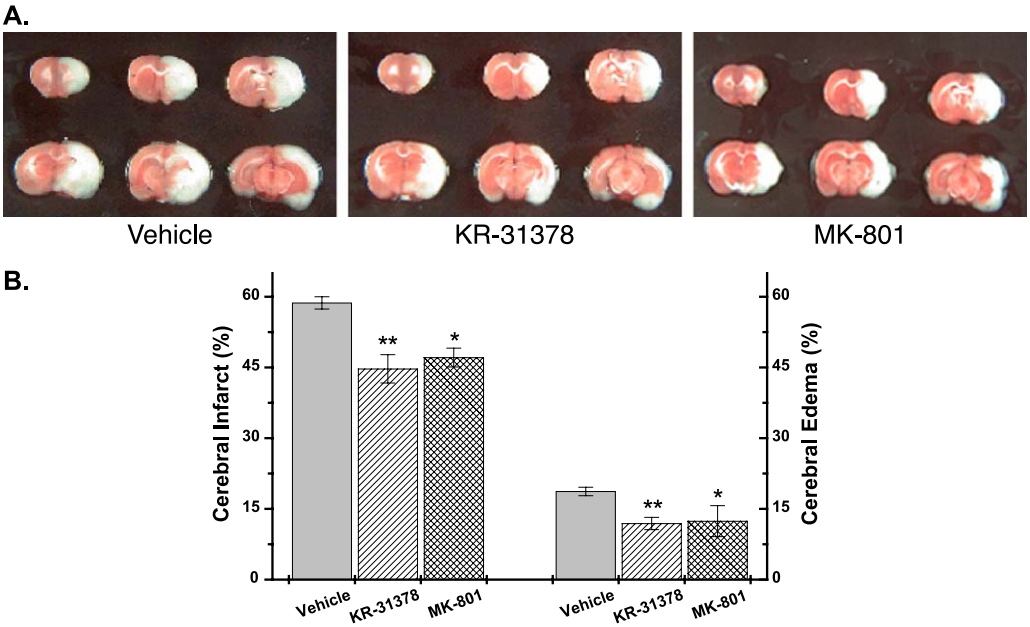

Fig. 2. Effect of KR-31378 on the infarct size and edema in rats subjected to 2 h of ischemia/22 h of reperfusion. Animals received KR-31378 (30 mg/kg, i.v., $n=7$) at 0, 4, and 8 h after the completion of 2 h of ischemia. MK-801 (2 mg/kg, i.p., $n=6$) was injected before the onset of ischemia and 0 and 2 h after reperfusion. Brains were quickly removed 24 h after ischemia. (A) Illustrative cornal sections showing infarct area in the cerebral hemisphere as a distinct pale-stained area in the rats subjected to 2 h of ischemia/22 h of reperfusion (vehicle) and attenuation of infarct area by treatment with KR-31378 and MK-801. (B) The infarct size and edema were quantified as described. *$P<0.005$, **$P<0.001$ versus vehicle.

3.2. KR-31378 protected $FeSO_4$-induced cell death in cultured rat cortical neurons

To further explore its neuroprotective mechanism, we have conducted neuronal survival studies to evaluate whether KR-31378 could protect oxidative stress-induced cell death in primary cortical mixed culture. Cultures contained approximately 40% of neurons and 60% of astrocytes, which were assessed by immunostaining cultures with antibodies against microtubule-associated protein II and glial acidic fibrillary acidic protein (data not shown). To evaluate the concentration dependence of $FeSO_4$ toxicity of neurons and astrocytes, we treated mixed cortical cultures and pure astrocyte cultures with varying concentrations of $FeSO_4$ for 24 h and measured neuronal viability. The sensitivity of mixed cortical and astrocyte cultures to the neurotoxic action of $FeSO_4$ was dependent on the concentration of $FeSO_4$. Incubation of cultured mixed cortical neurons or pure astrocytes with graded concentrations of $FeSO_4$ for 24 h provoked a dose-dependent increase of the cell death with the highest degree of toxicity at the concentration of 300 and 2000 μM, respectively. With KR-31378 treatment, cortical neurons and astrocytes were well preserved at all the tested $FeSO_4$ concentrations. Since half of the cells died (half-maximal neuronal injury) in mixed cortical cultures while nearly all cells were not injured in pure astrocyte cultures at 100 μM $FeSO_4$, this concentration was employed in all subsequent experiments to obtain maximal neuronal injury without affecting astrocytes (Fig. 4).

Next, in order to see its protective effect upon posttreatment, the level of protection afforded by 30 μM KR-31378 treatment at preinsult, coinsult, or postinsult was compared. For each treatment time, LDH release was assayed 24 h after $FeSO_4$ addition. Fig. 5 shows statistically significant attenuation of $FeSO_4$ toxicity up to 5 h postinsult treatment of KR-31378. The 5-h posttreatment paradigm proved to be

Table 1
Physiological variables for vehicle or KR-31378 pretreatment in transient focal cerebral ischemia

Parameter	Before ischemia		1 h of ischemia		15 min after reperfusion	
	Vehicle	KR-31378	Vehicle	KR-31378	Vehicle	KR-31378
BP_a (mm Hg)	94 ± 4	90 ± 5	99 ± 4	89 ± 2	99 ± 7	91 ± 4
pH_a	7.45 ± 0.04	7.41 ± 0.01	7.43 ± 0.06	7.46 ± 0.03	7.41 ± 0.03	7.43 ± 0.05
p_aCO_2 (mm Hg)	40 ± 5	44 ± 3	43 ± 10	37 ± 2	45 ± 7	40 ± 7
p_aO_2 (mm Hg)	172 ± 6	190 ± 9	173 ± 2	182 ± 14	179 ± 2	173 ± 10
Glucose (mg/dl)	195.1 ± 20.3	209.9 ± 35.9	177.3 ± 22.6	168.8 ± 9.0	186.4 ± 14.3	177.8 ± 17.5

Physiological variables were measured before (15 min) and during (1 h) middle cerebral artery occlusion and after reperfusion (15 min) in Sprague–Dawley rats. All values are mean ± S.D. ($n=3$). BP_a = arterial blood pressure; pH_a = arterial pH; p_aCO_2 = arterial carbon dioxide partial pressure; p_aO_2 = arterial oxygen partial pressure.

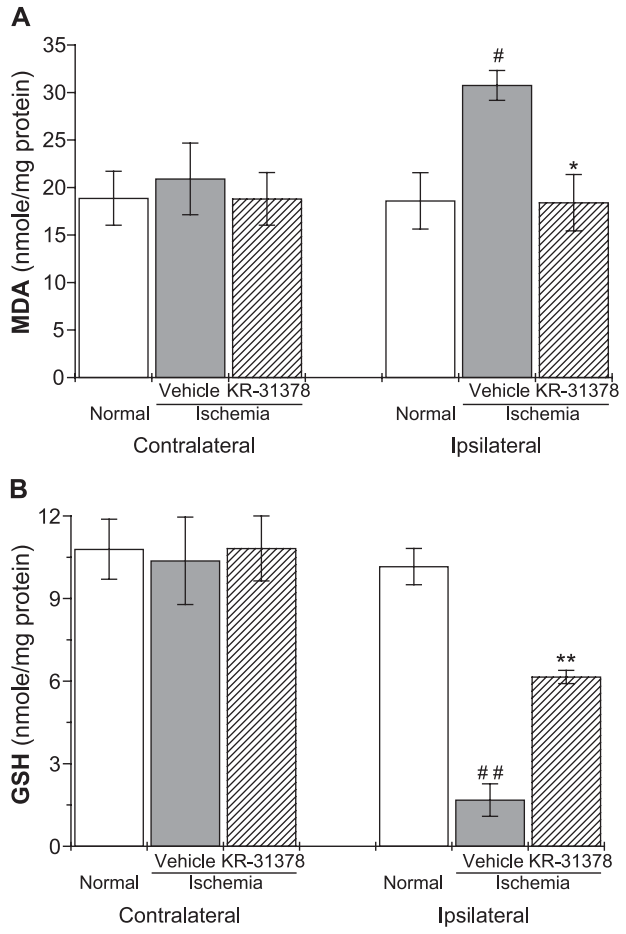

Fig. 3. Effect of KR-31378 on the level of malondialdehyde (A) and total glutathione (B) in rats subjected to 2 h of ischemia/22 h of reperfusion. Animals were treated with KR-31378 (30 mg/kg, i.v., $n=4$) or vehicle ($n=4$) and brains were removed for determination of malondialdehyde and total glutathione as described. $^{\#}P<0.01$, $^{\#\#}P<0.005$ vs. normal brain; $^{*}P<0.01$, $^{**}P<0.005$ vs. ischemic brain, respectively.

less efficacious as pretreatment or cotreatment, but nonetheless, it still greatly attenuated $FeSO_4$ toxicity (approximately 40% of neuronal cell protection). But no protection was observed in the 6-h posttreatment group. This result implies that KR-31378 can be effective even upon delayed treatment (Fig. 5).

3.3. Protective effect of KR-31378 on the oxidative stress-induced neuronal death was independent of its K^+ channel opening activity

To investigate the mechanism of KR-31378 involved in protecting neurons against oxidative insults, its neuroprotective effect was compared with an antioxidant, trolox, and other K_{ATP} openers. As shown in Fig. 6, coincubation of cortical neurons with $FeSO_4$ and varying concentrations of KR-31378 for 24 h induced a dose-dependent increase in the neuronal cell protection with IC_{50} of 12 μM. Cell death was also reduced in a similar fashion when the exposure to $FeSO_4$ was carried out in the presence of the antioxidant

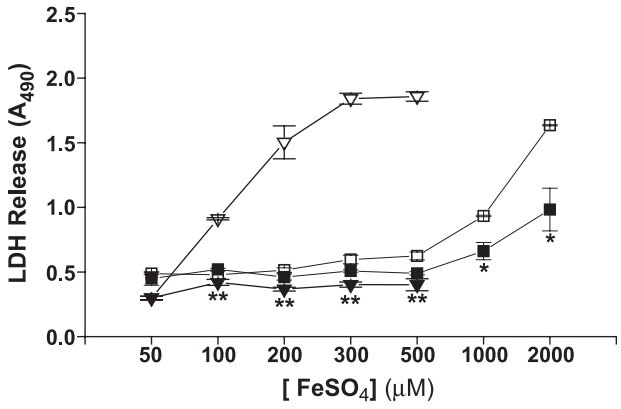

Fig. 4. Effect of $FeSO_4$ concentration on cell injury in mixed cortical cultures or pure astrocyte cultures and protective effect of KR-31378. Cultures were pretreated with 30 μM KR-31378 (▼: cortical culture; ■: astrocyte culture) or vehicle (▽: cortical culture; □: astrocyte culture) and then exposed to increasing concentrations of $FeSO_4$. Cell injury was quantified 24 h after $FeSO_4$ exposure by measurement of LDH in the medium. Values represent the mean ± S.E.M. of three separate cultures. $^{*}P<0.05$, $^{**}P<0.005$ vs. vehicle-treated cultures.

trolox, a water-soluble homologue of vitamin E. However, the addition of other K^+ channel openers such as BMS-180448, BMS-191095, and diazoxide before $FeSO_4$ exposure was not protective (Fig. 6).

In order to further address whether its protection against iron-induced oxidative insult involves K_{ATP} channel activation, glibenclamide, a sarcolemnal K_{ATP} blocker, and 5-hydroxydecanoic acid, a mitochondrial K_{ATP} blocker, were used. Cultures were preincubated in the presence of 100 μM glibenclamide and 5-hydroxydecanoic acid, alone or in combination with KR-31378 (30 μM), and then exposed to $FeSO_4$. KR-31378 provided high protection against iron-induced toxicity even in the presence of glibenclamide or 5-

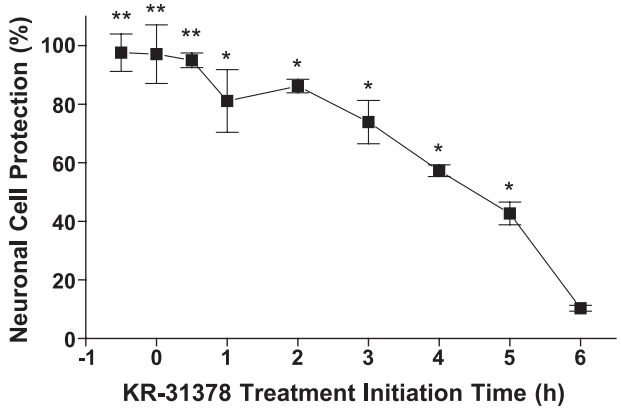

Fig. 5. Posttreatment effect of KR-31378 against $FeSO_4$-induced neurotoxicity. For posttreatment, the cortical cultures were exposed to $FeSO_4$, and KR-31378 (30 μM) was added at the indicated time. For pretreatment and cotreatment, KR-31378 was added 30 min before and simultaneously with iron exposure, respectively. Then cells were further incubated for 24 h and neuronal cell protection in KR-31378-treated culture was measured by LDH assay. Each value represents the mean ± S.E.M. of three experiments. $^{*}P<0.05$, $^{**}P<0.005$ vs. vehicle-treated cultures.

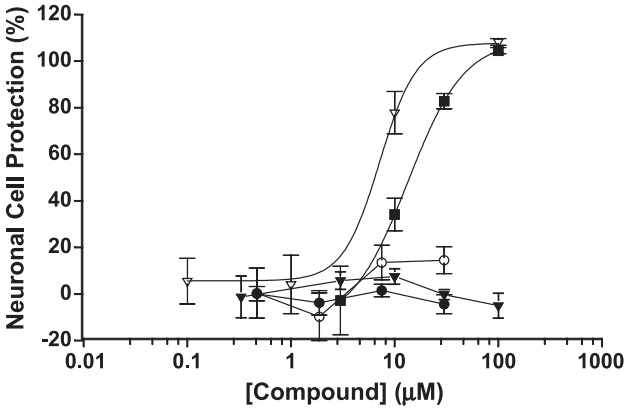

Fig. 6. Protective effect of KR-31378, trolox, and K^+ channel openers against $FeSO_4$-induced neurotoxicity. The cortical cell cultures were pretreated with varying concentrations of test compounds or vehicle. Then, $FeSO_4$ was added to a final concentration of 100 μM, and the cultures were maintained for 24 h in a CO_2 incubator. The neuronal toxicity was determined with LDH assay. KR-31378 (■), trolox (▽), BMS-180448 (○), BMS-191095 (▼), and diazoxide (●).

hydroxydecanoic acid, indicating that K_{ATP} channel opening activity was not related to its protective mechanism against $FeSO_4$ -induced toxicity in cortical culture (Fig. 7).

3.4. KR-31378 suppressed intracellular production of free radicals and lipid peroxidation

To determine if KR-31378 blocks toxicity by inhibiting free radical accumulation, neurons were treated with $FeSO_4$ for 24 h in the presence or absence of KR-31378, then the cells were loaded with DCFH-DA and levels of DCF fluorescence were determined by fluorescent reader and fluorescence microscope. The intracellular reactive oxygen species production measured by DCF fluorescence was not apparent in 1 h of $FeSO_4$ treatment and increased with incubation time, reaching a plateau at 4 h of treatment (data not shown). As illustrated in Fig. 8A, there was a threefold increase in reactive oxygen species generation relative to that found in the control at the end of $FeSO_4$ treatment for 4 h. This elevation was reduced by the addition of KR-31378 (30 μM) to a level slightly lower than that observed in the absence of $FeSO_4$. Similarly, trolox (30 μM) reduced intracellular levels of free radicals. These results indicate that KR-31378 acts as an antioxidant like trolox by suppressing intracellular reactive oxygen species accumulation (Fig. 8A).

Because free radicals cause lipid peroxidation, we checked whether KR-31378 could protect lipid peroxidation induced by $FeSO_4$. We determined the content of cellular malondialdehyde, a compound that is produced as a result of

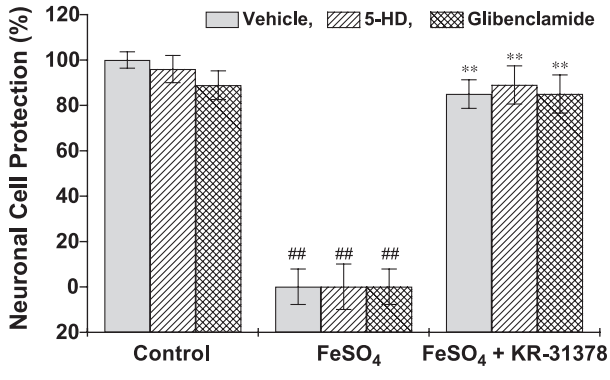

Fig. 7. KR-31378 protects $FeSO_4$ toxicity in the presence of K_{ATP} channel blockers. The cortical cell cultures were preincubated in the presence of 100 μM K_{ATP} channel blocker (glibenclamide or 5-hydroxydecanoic acid), alone or in combination with KR-31378 (30 μM), and then exposed to $FeSO_4$. Neuronal injury was assessed 24 h later by measurement of LDH. Values represent the mean ± S.E.M. of determination made in three separate cultures. $^{\#\#}P<0.005$ vs. control, ** $P<0.005$ vs. $FeSO_4$-treated cultures, respectively.

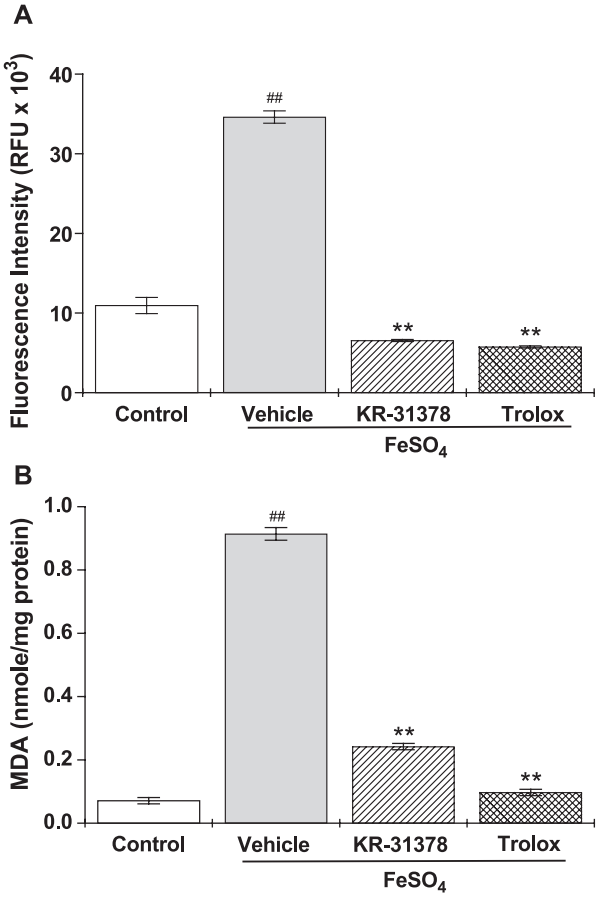

Fig. 8. Effect of KR-31378 on intracellular reactive oxygen species levels (A) and lipid peroxidation (B). (A) Cortical cultures were treated with KR-31378 (30 μM) and trolox (30 μM) and then exposed to $FeSO_4$ (100 μM) for 4 h. At the end of incubation, the cells were washed again and immediately processed for the fluorescence plate reader studies. DCF fluorescence was quantified using fluorescence plate reader. (B) For determination of iron-induced lipid peroxidation in cortical culture in the presence and absence of KR-31378 or trolox, cells were pooled and homogenized. The homogenate was centrifuged and the content of malondialdehyde in the supernatant was determined. Each value represents the mean ± S.E.M. of three experiments. $^{\#\#}P<0.005$ vs. control; $^{**}P<0.005$ vs. vehicle-treated cultures.

Table 2
Effect of KR-31378 on the antioxidant enzyme activities and total glutathione levels in $FeSO_4$-treated cortical cultures

	Superoxide dismutase (U/mg protein)	Catalase (U/mg protein)	Glutathione peroxidase (U/mg protein)	Glutathione reductase (U/mg protein)	Total glutathione (nmol/mg protein)
Control	1.64 ± 0.01	1028 ± 64.7	4.65 ± 0.52	0.025 ± 0.003	51.9 ± 1.2
$FeSO_4$	1.76 ± 0.07	86 ± 7.8[a]	0.65 ± 0.04[a]	0.025 ± 0.003	2.7 ± 0.7[a]
$FeSO_4$ + KR-31378	1.99 ± 0.25	723 ± 34.5[b]	2.67 ± 0.03[c]	0.017 ± 0.001	31.6 ± 1.9[c]
$FeSO_4$ + trolox	1.56 ± 0.15	606 ± 16.5[c]	3.00 ± 0.09[c]	0.011 ± 0.004	44.3 ± 1.1[c]

Cortical cultures were treated with 30 μM KR-31378 or trolox and then exposed to $FeSO_4$ (100 μM) for 24 h. After assessing the extent of neuronal damage, cells were harvested for the measurement of antioxidant enzyme activities and total cellular glutathione, as described in Materials and Methods. The values represent the mean ± S.E.M. of three experiments.

[a] $P < 0.005$ vs. control.

[b] $P < 0.05$ vs. $FeSO_4$-treated cultures.

[c] $P < 0.005$ vs. $FeSO_4$-treated cultures.

lipid peroxidation, in $FeSO_4$-treated cortical cultures. The results demonstrated that $FeSO_4$ treatment causes a 13-fold increase in malondialdehyde over untreated control, which indicates that extensive lipid peroxidation had taken place in these damaged cells. Parallel to the inhibition of reactive oxygen species formation, KR-31378 (30 μM) suppressed the formation of malondialdehyde, and neuronal survival was well preserved at the same time. Trolox (30 μM) also resulted in a similar suppressive effect on the formation of malondialdehyde (Fig. 8B). These results indicate that both the formation of reactive oxygen species and the resulting cell damage were attenuated by the addition of KR-31378 and suggest that the formation of reactive oxygen species plays an important role in causing or mediating neuronal cell injury.

3.5. KR-31378 affected on antioxidant enzyme activities and glutathione levels

It is becoming clear that oxidative stress induces an adaptive antioxidant response within the cell and the mode of antioxidant response appears to be dependent on the interaction between specific prooxidant and cell type (Mattson et al., 1997). Hence, we have determined the activity of the antioxidative enzymes, superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase, in $FeSO_4$-treated neurons in the absence and presence of KR-31378. As shown in Table 2, $FeSO_4$ treatment markedly reduced the activity of catalase and glutathione peroxidase by 92% and 86%, respectively, in cortical cultures, whereas no significant change in activities of superoxide dismutase and glutathione reductase was detected. Pretreatment with KR-31378 and trolox significantly attenuated the decrease in catalase and glutathione peroxidase activity.

The glutathione system is especially important for cellular defense against reactive oxygen species by serving as an electron donor in the reduction of peroxides catalyzed by glutathione peroxidase and also by reacting directly with radicals in nonenzymatic reactions. To determine if the mechanism of protection by KR-31378 is attributable to the altered glutathione metabolism, cortical neurons were treated with 100 μM $FeSO_4$ in the presence or absence of KR-31378 (30 μM) for 24 h and harvested, and then total cellular glutathione was determined. $FeSO_4$ treatment resulted in a marked reduction of total glutathione level (5% of control). As shown in Table 2, KR-31378, like trolox, attenuated glutathione loss caused by $FeSO_4$.

4. Discussion

We have previously reported that KR-31378 has good cardioprotective activity in the ischemic myocardium rat model, which appeared to be mediated by K_{ATP} opening (Yoo et al., 2001; Lee et al., 2001) as well as neuroprotective effect on focal ischemic brain rat model in association with inhibition of apoptotic cell death (Hong et al., 2002). The present study showed protective effects of KR-31378 on brain injuries induced by middle cerebral artery occlusion followed by reperfusion in rats and development of its neuroprotective mechanism of KR-31378 against oxidative stress-induced toxicity in cortical cultures as a model system.

We have used a model of transient focal cerebral ischemia in rats as a way of reproducing clinical situations since reperfusion can occur spontaneously through resolution of an embolus or clinical intervention, and of evaluating pharmacological neuroprotection against the deleterious effect of reperfusion injury (for review, see Macrae, 1992). Intravenous administration of 30 mg/kg KR-31378 affords a significant reduction in the infarct size and edema in a middle cerebral artery occlusion model, as assessed 22 h after reperfusion. Studies were also conducted to determine the brain concentration of KR-31378. Following intravenous administration, the overall brain concentration of KR-31378 corresponded to 10% of plasma concentration. The relatively poor brain penetration of KR-31378 was improved by ~2-fold following ischemic insult due to the disruption of the blood–brain barrier (data not shown). There is also some evidence that the antioxidant activity of plasma might be an important factor in neuroprotection (Leinonen et al., 2000).

It is well established that reactive oxygen species such as hydroxyl radical, superoxide anion, and hydrogen peroxide play a major role in the pathophysiology of ischemic stroke (Hall and Braughler, 1989) and that the oxidative damage to membrane lipids and proteins is increased during ischemia and reperfusion (Mason et al., 2000). Middle cerebral artery occlusion significantly increased lipid peroxidation in the ischemic hemisphere, which was measured by malondialdehyde as compared to levels in the normal brain. There was also a significant reduction in the level of glutathione in the ischemic hemisphere. Glutathione, an endogenous antioxidant found in animal cells, reacts with free radicals and provides protection from free radical damage. Both increased level of malondialdehyde and reduced glutathione level in the ischemic hemisphere suggest an increased load of free radicals during ischemia–reperfusion injury. Treatment with KR-31378 having benzopyran moiety in the molecule significantly attenuated the elevated level of malondialdehyde and glutathione loss (oxidative stress markers) in the ischemic hemisphere and thereby showed a neuroprotective effect against focal ischemia in rats.

Exploration of oxidative stress is important for neuroprotective strategies to enhance neuronal survival after cerebral ischemia–reperfusion. Neurons from the embryonic rat cortex provide a convenient in vitro preparation for examining the participation of oxidative stress in neuronal alterations and for studying the protective mechanism of KR-31378. In agreement with previous reports, we observed that exposure to $FeSO_4$ provoked the death of cortical neurons in a concentration-dependent manner (Goodman and Mattson, 1996). The neuronal death induced by $FeSO_4$ exhibited the characteristic features of apoptosis and necrosis, two distinct cell death pathways, which were effectively prevented by KR-31378 in a dose-dependent manner as determined by morphological features, mitochondrial function, and LDH release (data not shown). Mitochondria have been recognized as target organelles for the regulation and execution of cell death under pathological conditions (Budd and Nicholla, 1998; Kroemer et al., 1998), serving as the major source of metabolic energy as well as of free radicals in living cells. Hence, the pathophysiological reactive oxygen species exposure disrupts mitochondrial function such as Ca^{2+} uptake and ATP production, both of which could lead to neuronal death. In addition, we have shown that neuroprotective effect was maintained up to 5 h of posttreatment, which may imply that KR-31378 can be effective even upon delayed treatment. This result is significant in view of the fact that a drug that is to be used to treat stroke patients must have therapeutic value when given after the initial insult.

KR-31378 has been shown to possess K_{ATP} opening activity (Yoo et al., 2001; Lee et al., 2001) and antioxidant activity, both of which were considered to play important roles in neuroprotection (Hong et al., 2002). Free radicals are increasingly implicated as key mediators of neuronal injury and neuroprotective antioxidants are considered a promising approach to limit the extent of neuronal cell loss. It has also been accepted that hypoxic depolarization is a trigger signal for induction of neuronal cell death and the hypoxic hyperpolarization induced by the activation of K^+ channels is associated with a loss of neuronal activity and plays a role in cell survival during hypoxia (Nieber, 1999). Hyperpolarization may be caused by the opening of K_{ATP} channels (Zhang and Krnjevic, 1993; Fujimura et al., 1997). Mitochondrial K_{ATP} channels have been found in the inner membrane of mitochondria and represent a pharmacologically distinct population of K_{ATP} channels. There is increasing evidence about the diverse functions of mitochondrial K_{ATP} channels in the regulation of mitochondrial matrix volume, ATP production, and Ca^{2+} homeostasis in mitochondria, which determines the outcome of ischemic stress on cellular function and survival. Hence, to examine whether K^+ channel opening activity was involved in the protective mechanism of KR-31378 against oxidative neuronal injury, its neuroprotective effect was compared with other known K^+ channel openers and the antioxidant trolox. We observed that the neurotoxic action of $FeSO_4$ was blocked by KR-31378 and trolox in a dose-dependent manner, but not by other K^+ channel openers such as BMS-180448, BMS-191095, and diazoxide.

To address this issue further, we have tested whether the protective effect of KR-31378 was reversed in the presence of K_{ATP} channel blockers such as glibenclamide or 5-hydroxydecanoic acid. The experimental results demonstrated that the level of protection obtained with KR-31378 was well preserved even in the presence of K_{ATP} blockers, which suggested that K_{ATP} channel opening activity was not directly related to the protective mechanism of KR-31378 against oxidative stress-induced neuronal death. Our result somewhat disagrees with the previous report (Goodman and Mattson, 1996) in which K^+ channel openers, including diazoxide, protected hippocampal neurons against $FeSO_4$ toxicity. This discrepancy might be due to the differences in pure hippocampal cultures and cortical mixed culture system. However, it was also reported that their protective mechanism was independent of K^+ channel opening activities, which is in good agreement with our result.

Then, we have focused on the antioxidant activity of KR-31378 in this neuronal injury model. Previously, we have demonstrated that KR-31378 has a direct radical-absorbing capacity devoid of iron-chelating activity, and it effectively protects H_2O_2-induced A7r5 cell death (Kim et al., 2001). Formation of reactive oxygen species and the ensuring oxidation of biological molecules are well-recognized mechanisms of tissue damage in many diseases such as myocardial ischemia–reperfusion, stroke, traumatic head injury, and spinal cord injury (Bains and Shaw, 1997). Our results confirmed the antioxidative mechanism of KR-31378, showing that it suppressed both intracellular accumulation of reactive oxygen species and lipid peroxidation in cortical cell cultures exposed to $FeSO_4$, which were measured by DCF fluorescence and malondialdehyde formation, respectively.

Typically, equilibrium exists between the generation of reactive oxygen species and the antioxidant defenses, which maintains homeostatic control over the cell's oxidative state. The enzymatic defenses include superoxide dismutase, catalase, and glutathione peroxidase. Superoxide dismutase converts the superoxide radical to H_2O_2, which is removed by catalase and glutathione peroxidase. Neurons may be particularly susceptible to oxidative stress because of the high rate of oxidative metabolic activity and low level of antioxidant enzymes, such as catalase and glutathione peroxidase. In addition to the direct radical-scavenging activity of KR-31378, we have checked whether its antioxidant activity is due to its ability to change activities of the endogenous antioxidant enzymes. We found that $FeSO_4$ decreased the activities of glutathione peroxidase and catalase greatly, and KR-31378 restored these enzyme activities significantly. Catalase and glutathione peroxidase are enzymes of major importance in the detoxification of hydrogen peroxides—the peroxides generated in the highest quantity in the brain. Certain enzymes of the antioxidant network appear to be controlled by gene activation via redox-sensitive transcription factors, allowing the antioxidant system to respond to fluctuations in the production of oxidizing species (Rahman, 2000; Poulsen et al., 2000). In fact, recent studies demonstrated that overexpression of catalase in neural cells provides protection against the H_2O_2-induced toxicity (Mann et al., 1997) and erythropoietin treatment restores glutathione peroxidase activity decreased by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in the substantia nigra (Genc et al., 2002). However, the exact mechanism of the effect of KR-31378 on glutathione peroxidase and catalase activities remains to be determined.

To monitor the extent of glutathione depletion induced by $FeSO_4$ and to investigate whether KR-31378 affects the glutathione level, we assayed glutathione content in the cortical neurons following treatment with $FeSO_4$ in the absence or presence of KR-31378. A significant reduction of total glutathione content was evident in the cultures treated with $FeSO_4$ alone, which would further stimulate mitochondria to produce more reactive oxygen species, resulting in cell death. Glutathione is considered to be the most important intracellular nonprotein thiol compound in mammalian cells and plays a crucial role as a free radical scavenger, particularly effective against the hydroxyradical, for which there is no known enzymatic defense system. Therefore, the ability of glutathione to nonenzymatically scavenge both singlet oxygen and hydroxyradical provides a first line of antioxidant defense (Coyle and Puttfarcken, 1993). There has been strong evidence that glutathione depletion causes nerve cell death in culture (Li et al., 1997). The loss of glutathione may cause mitochondrial damage and the impairment of mitochondrial function may lead to a decrease in cytosolic glutathione. The depletion of glutathione contents induced by $FeSO_4$ in the present study is probably due to the changes in the cellular turnover of glutathione from a deficiency of ATP supplied by mitochondria (Mithofer et al., 1992) and the active transport of oxidized glutathione out of the cultured cells (Akerboom and Sies, 1989; Kondo et al., 1995). In addition to its critical role as a free radical scavenger, glutathione may act as a redox modulator of ionotropic receptors and serve as a neuroprotectant against glutamate excitotoxicity, which together suggest that alterations in glutathione status may be deleterious to normal neuronal function (Bains and Shaw, 1997). Furthermore, KR-31378 attenuated the loss of total glutathione caused by $FeSO_4$, which provided evidence that KR-31378 treatment decreases oxidative stress by restoring reduced level of the natural antioxidant, glutathione. This may be achieved via its modulatory action on the altered glutathione metabolism.

In conclusion, we found that KR-31378 protected neurons from ischemia–reperfusion brain injury through its modulatory action on the antioxidative defense mechanisms toward reactive oxygen species. In addition, KR-31378 has K^+ channel opening activity and anti-inflammatory actions by suppressing nuclear factor-κB-mediated proinflammatory gene expression and ensuring neutrophil migration into the ischemic brain (data not shown). Although K_{ATP} channel opening activity was not directly related to the protective mechanism of KR-31378 against oxidative stress-induced neuronal death, K_{ATP} channels can contribute to the hypoxic hyperpolarization in the ischemic brain. There is also compelling evidence that endothelial inflammatory cell interactions play a prominent role in the progression of cerebral infarction (Kuroda et al., 1999). Thus, the protection of neurons by KR-31378 seen after reperfusion may provide clinically beneficial outcomes alone or in combination with thrombolytic therapy.

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