Neuroprotective effects of ethyl pyruvate by Nrf2-mediated anti-oxidation and Ca\textsuperscript{2+} chelation-mediated anti-inflammation
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Nrf2-mediated anti-oxidation and

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Neuroprotective effects of ethyl pyruvate by
Nrf2-mediated anti-oxidation and
Ca^{2+} chelation-mediated anti-inflammation

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ABSTRACT

Ethyl pyruvate (EP), a simple ester of pyruvic acid, has been shown to have protective effect under various pathological conditions, such as sepsis, endotoxemia, acute pancreatitis, and cerebral ischemia. During cerebral ischemia, oxidative stress and inflammation play critical roles in aggravating the delayed damaging process as well as the excitotoxicity-induced acute damaging process. Astrocytes are known to support the protection and survival of neurons against inflammation and oxidative damage. In order to verify the molecular mechanism underlying the anti-oxidative effect of EP in astrocyte, the activation of nuclear component E2-related factor 2 (Nrf2), a master transcriptional regulator of phase II antioxidative genes, was examined. EP was found to induce the Nrf2 translocation and upregulations of various genes downstream of Nrf2, such as hemeoxygenase 1 (HO-1), and these Nrf2 activation and HO-1 induction resulted in the amelioration of H$_2$O$_2$-induced oxidative damage in primary astrocyte cultures. EP dose-dependently induced HO-1 expression and suppressed H$_2$O$_2$-induced astrocyte cell death. siRNA-mediated HO-1 or Nrf2 knockdown and zinc protoporphyrin (ZnPP)-mediated inhibition of HO-1 activity showed that Nrf2 activation and HO-1 induction were responsible for the observed cytoprotective effect of EP, wherein ERK and Akt signaling pathways were involved. In addition to these protective effects in astrocytes, EP-conditioned
astrocyte culture media (ECM) conferred neuroprotective effects to primary neuronal cultures which were exposed to oxidative or excitotoxic stress. This seemed to be mediated by glial cell line-derived neurotrophic factor (GDNF) accumulated in ECM. In addition to HO-1, EP-induced Nrf2 activation increased the expressions of various anti-oxidant genes, including glutathione S-transferase (GST), NAD(P)H:quinone oxidoreductase 1 (NQO1), and glutamate-cysteine ligase modifier subunit (GCLM). For the anti-inflammatory effect of EP, a novel molecular mechanism via calcium chelation-mediated inhibition of high mobility group box 1 (HMGB1) secretion was examined. HMGB1 is a novel cytokine-like mediator which induces systemic inflammation and causes microglial activation during exitotoxicity-induced neuronal cell death. HMGB1 induces inflammatory pathways, when it is located in extracellular milieu. Since EP has been reported to inhibit HMGB1 secretion in models of endotoxemia/hemorrhagic shock, spinal cord ischemic injury, severe acute pancreatitis, and established lethal sepsis, the efficacy of EP for inhibiting the HMGB1 secretion in the postischemic brain and underlying molecular mechanism for this inhibition was examined. In the postischemic brain, HMGB1 was released extracellularly producing dual peaks around 1 day and 7 days after ischemic insult. Each of these peaks seemed to be generated from damaged neuronal cells and from inflammatory cells, respectively. EP administration reduced HMGB1 release independently with neuroprotective effects in the postischemic
brain. In addition, EP treatment suppressed A23187 (a calcium ionophore)-induced HMGB1 secretion both in BV2 cells and primary microglia cells, wherein activations of two calcium-mediated kinases, protein kinase C alpha (PKCα) and calcium/calmodulin-dependent protein kinase (CaMK) IV, were also inhibited. The nuclear translocation of two calcium-mediated kinases was inhibited by EP treatment, raising the possibility that EP might modulate an early step in the activation of PKC and CaMK IV, such as regulation of intracellular calcium level. Regarding this possibility, it was found that A23187-induced increase of intracellular calcium levels was significantly suppressed by EP treatment. In addition, A23187 induced serine phosphorylation and secretion of HMGB1 were markedly inhibited by EP treatment. The combined results suggested that EP functions as a novel Nrf2 activator that induces HO-1 expression in astrocytes and also confers inhibition of calcium-mediated HMGB1 release in microglia through its calcium chelation ability.
초 록

에틸파이루베이트 (ethyl pyruvate, EP)는 파이루베이트 (pyruvate)의 유도체로서 패혈증과 전신성 염증반응에서 염증을 억제하고 일시적 국소 허혈 동물 모델 (middle cerebral artery occlusion, MCAO)에서 신경계 보호 효과가 있음을 알려져 있다. 본 연구에서는 MCAO 모델, 1차 배양한 별아교세포, 신경세포, 미세아교세포, 그리고 미세아교 세포주인 BV2 세포를 사용하여 EP의 항산화, 항염증작용 관련 새로운 분자기전을 조사하였다. EP의 항산화 작용과 관련하여, H$_2$O$_2$로 산화적 스트레스를 유도한 별아교세포에서 EP는 nuclear factor E2-related factor 2 (Nrf2) 전사인자의 활성을 통한 보호효과를 보였다. 1차 배양한 별아교세포에 EP를 처리하면 항산화 효소로 알려진 hemeoxygenase 1 (HO-1)의 발현이 크게 증가하였고, 이는 HO-1 유전자에 위치하고 있는 antioxidant response element (ARE) 영역에 Nrf2가 결합하여 전사를 증가시키기 때문에이다. 이와 같은 EP에 의한 HO-1의 발현 증가는 H$_2$O$_2$ 처리에 의하여 유도되는 별아교세포의 손상을 현저히 억제할 뿐 아니라, EP를 처리한 1차 배양한 별아교세포의 세포 배양액은 1차 배양한 신경세포에 처리하였을 때, 별아교세포에서 유도된 신경교세포 유래 신경영양인자인 glial cell line-derived neurotrophic factor (GDNF)가 여러 산화적 스트레스로부터 신경세포를 보호하는 것을 관찰하였다. Nrf2 또는 HO-1 siRNA를 사용하여 해당 유전자를 억제하였을 때, GDNF의 유도와 배양액을 통한 신경세포 보호효과가 현저히 감소되며, 이는 Nrf2 활성과 HO-1 유도를 통해 GDNF가 발현되고, 이 과정이 신경세포 보호효과를 유도함을 시사하고 있다. 한편, high mobility group box 1 (HMGB1)
은 세포 안에서는 DNA와 결합하는 핵 구조단백질로서 전사의 핵심적인 역할을 하지만, 세포 밖으로 방출되었을 때는 면역계를 자극하여 염증을 유발하는 것이 보고된 바 있다. 신경계에서, EP가 HMGB1 분비를 억제함으로써, 이로 인해 수행되는 염증반응을 억제하는지 확인하기 위하여 MCAO 모델 1차 배양한 미세아교세포, BV2 세포에서 EP에 의한 HMGB1 분비 역제를 조사하였다. MCAO 동물 모델의 경우, 1일과 7일 후에 많은 양의 HMGB1이 뇌척수액(cerebral spinal fluid, CSF)으로 흘러나오는 것을 관찰하였고, EP를 허혈-재관류 4일 후부터 투여하였을 때 7일에 CSF에서 관찰되는 HMGB1 양이 감소되는 것을 관찰하였다. 이는 EP의 HMGB1 분비 억제작용이 뇌경색 형성억제와 독립적으로 진행된다는 것을 보여준다. BV2 세포에서 HMGB1은 lipopolysaccharides (LPS)에 의해 세포에서 인산화가 되어 세포질, 세포배양액으로 흘러나오며 HMGB1 인산화 과정에서 칼슘 의존적 인산화효소로 알려진 protein kinase C alpha (PKCα)와 calcium/calmodulin–dependent protein kinase (CaMK) IV가 역할을 한다는 것이 알려져있다. BV2 세포와 1차 배양한 미세아교세포에서 칼슘 이온투과담채(calcium ionophore, A23187)를 처리하면 세포 내 칼슘유입이 증가하고, 그 결과 HMGB1이 인산화되어 세포 밖으로 흘러나오는 것을 관찰하였다. 그러나 EP를 A23187과 함께 처리하면, 세포 내 칼슘의 양이 현저히 감소하는 동시에 HMGB1의 인산화도 현저히 억제되는 것을 확인하였다. 이 과정에서 PKCα와 CaMK IV의 핵으로의 유입도 현저히 억제되었다. 이와 같은 연구 결과들은, EP가 Nrf2를 활성화시키며 이를 통해 HO-1 유전자 발현과 GDNF 분비를 증가시켜 산화적 스트레스로 인한 세포 괴사로부터 범아교세포와 신경세포를 보호하고, 활성화된 미세아교세포에서 칼슘 의존적 HMGB1 분비를 억제하여 신경세포 손상을 억제시키는 것을 시사한다.
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INTRODUCTION

Protective effect of EP in CNS

Ethyl pyruvate (EP) has been reported to act as an anti-inflammatory and antioxidative molecule and to confer protective effects in various pathological conditions. In central nervous system (CNS), it has been reported that EP significantly reduced infarct volumes and alleviated neurological deficits in the postischemic rat brain (Yu et al., 2005). EP suppressed microglia activation and inflammatory cytokine induction in the postischemic brain and in primary microglia cultures (Yu et al., 2005; Kim et al., 2010). It was also shown that EP attenuated kainic acid-induced neuronal cell death in the CA1 and CA3 regions of the mouse hippocampus (Cho et al., 2006). It has been suggested that the neuroprotective effects of EP in the postischemic brain and postepileptic animal were shown to be related to the anti-inflammatory effects of EP and mediated by modulation of the nuclear factor-kappaB (NF-κB) and p38 MAPK signaling pathways (Yu et al., 2005; Cho et al., 2006; Kim et al., 2010). In an animal model of spinal cord injury, EP administration suppressed neuronal damage by reducing the numbers of apoptotic motor neurons and decreasing levels of high mobility group box 1 (HMGB1) in serum and injured tissue (Wang et al., 2009).
Anti-inflammatory and anti-oxidative effects of EP

Anti-inflammatory effect of EP has been reported in various animal models of acute injury. The suppression of NF-κB activity has been proposed to be responsible for the anti-inflammatory effects of EP. EP down-regulates NF-κB activity, which results in suppression of proinflammatory cytokine production, such as iNOS, TNF-α, IL-1β, and COX-2, in mice subjected hemorrhagic shock and murine model of extrahepatic cholestasis (Yang et al., 2002; Yang et al., 2004). Similarly, EP decreased lipopolysaccharide (LPS, 20 mg/kg, IV)-induced plasma concentrations of TNF-α and IL-6, and simultaneously increased the level of IL-10 in plasma of endotoxic rats (Venkataraman et al., 2002). Anti-oxidative function has been reported as another important mechanism underlying protective effect of EP. EP has been shown to function as an effective ROS scavenger (Karabeyoglu et al., 2008; Zeng et al., 2007; Fedeli et al., 2007). It has been reported that EP directly scavenges H₂O₂ and other reactive intermediates, such as, superoxide radical and hydroxyl radical (Fedeli et al., 2007; Wang et al., 2005). EP treatment ameliorated redox stress in cultured hepatocytes (Mollen et al., 2007) and in rats subjected to hemorrhagic shock (Tawadrous et al., 2002). In addition to anti-inflammatory and anti-oxidative effects, anti-apoptotic and anti-coagulant effects of EP have also been reported (Wang et al., 2005; Zeng et al., 2007; Tsung et al., 2005a; Kung et al., 2011a). EP reduced neuronal apoptosis under various conditions, such as, in a
dopamine-induced neuronal cell death model (Wang et al., 2005) and in a rodent model of hypoxic-ischemic injury (Shen et al., 2010). In a severe disseminated intravascular coagulant model in rats, EP administration improved hepatic function, and attenuated tissue factor mRNA expression and cytokine release (Kung et al., 2011a).

**Activation of Nrf2**

Nuclear factor E2-related factor 2 (Nrf2) is a basic-region leucine zipper (bZIP) transcription factor that plays a crucial role in response to oxidative stress. Nrf2 binds to antioxidant responses elements (AREs) localized in the promoter regions of a battery of antioxidant and detoxifying genes, such as, hemeoxygenase 1 (HO-1) (Nguyen et al., 2003), NAD(P)H:quinone oxidoreductase 1 (NQO1) (Jaiswal, 2000), glutathione S-transferases (GST) (Itoh et al., 1999), and glutamate-cysteine ligase (comprising catalytic [GCLC] and modifier [GCLM] subunits) (Wild et al., 1999; Jeyapaul et al., 2000), and thus modulates their expressions. Nrf2 activity is tightly regulated by Kelch-like ECH-associated protein 1 (Keap1), an E3 ubiquitin ligase substrate adaptor, which targets Nrf2 for proteosomal degradation (Hayes and McMahon, 2009; Nguyen et al., 2009). During oxidative stress, Keap1 is inactivated by modification of its highly reactive cystein residues (Eggler et al., 2005; Zhang, 2006), and thus, the stability and nuclear accumulation of Nrf2 are
enhanced, which in turn induces genes downstream of Nrf2 (Kaspar et al., 2009).

**Protective effect of HO-1**

HO-1, formerly known as heat shock protein (HSP32), is the rate-limiting enzyme that catalyzes the degradation of heme to produce biliverdin, iron, and carbon monoxide (CO) (Maines, 1988). HO-1 expression is induced by the Nrf2-ARE pathway after exposure to various noxious stimuli, such as, hypoxia, proinflammatory cytokines, heavy metals, UV irradiation, and oxidative stress (Ryter and Choi, 2002). It has been shown that HO-1 upregulation suppresses cell death via antioxidative effects of its enzymatic products. Bilirubin is produced from biliverdin by biliverdin reductase and acts as a potent physiological antioxidant (Liu et al., 2003). In addition, CO has a strong vasodilatory effect that reduces vasoconstriction under pathological conditions, such as, ischemia (Dong et al., 2000), and Fe$^{2+}$ functions as a powerful pro-oxidant via the process that leads to the sequesteration of free cytosolic iron (Ward et al., 1994). Accumulating evidence indicates that HO-1 has a neuroprotective role, for example, HO-1-overexpressing neuroblastoma cell lines have been reported to be less prone to oxidative damage or to β-amyloid$_{1-40}$ than control cells (Le et al., 1999; Takeda et al., 2000). Similarly, cerebellar granule cells obtained from transgenic mice overexpressing HO-1 were found to be relatively resistant to glutamate- and to H$_2$O$_2$-induced oxidative damage.
(Maines et al., 1998; Chen et al., 2000), whereas astrocytes harvested from HO-1 knockout mice were vulnerable to hemin toxicity (Chen-Roelting et al., 2005). Furthermore, HO-1 has also been reported to have protective effects under various neuropathological conditions in vivo, such as, during ischemia (Panahian et al., 1999), traumatic brain injury (Beschorner et al., 2000), spinal cord injury (Lin et al., 2007), and also during neurodegenerative diseases, such as, Parkinson’s disease (PD) and Alzheimer’s disease (Schipper et al., 2009). Up-regulating HO-1 expression decreased infarct volumes in stroke and protected brain against exitotoxicity (Poss and Toneqawa, 1997; Satoh et al., 2006). Some bioactive ingredients such as oxymatrine and curcumin showed neuroprotection via upregulating HO-1 expression in rat focal brain ischemia-reperfusion model (Li et al., 2011; Yang et al., 2009a).

**HMGB1, a danger signal molecule, in pathology**

High mobility group box 1 (HMGB1) is a nonhistone DNA-binding protein. It is passively released from dying cells and also actively secreted from macrophagy and monocyte in response to exogenous and endogenous inflammatory stimuli such as, bacterial endotoxin, CpG DNA, TNF-α, IL-1, and IFN-γ (Chen et al., 2004; Lotze et al., 2005; Jiang et al., 2005; Rendon-Mitchell et al., 2003; Wang et al., 1999a; Abraham et al., 2000; Agnello et al., 2002). Extracellular HMGB1 aggravates
inflammatory processes by inducing various proinflammatory cytokines secretion (Scaffidi et al., 2002). In the postischemic brain, excessive release of HMGB1 after brain ischemic insult is a major cause of neuronal death and subsequent neurological and behavioral dysfunctions (Kim et al., 2006). In addition, extracellular HMGB1 aggravates pathological processes in various diseases such as lung inflammation, sepsis, arthritis, atherosclerosis, and cancer (Abraham et al., 2000; Sappington et al., 2002; Klune, et al., 2008; Rauvala and Rouhiainen, 2009; Sims et al., 2010). Increased plasma HMGB1 levels are observed in patients with inflammatory disease such as, sepsis and rheumatoid arthritis (Wang et al., 1999b; Taniguchi et al., 2003). For the secretory mechanism of HMGB1, it was shown that phosphorylation of serine residue of HMGB1 is an essential process for its translocation from nucleus to cytoplasm (Youn and Shin, 2006). Regarding this, activations of two calcium-mediated protein kinases, classical protein kinase C (cPKC) and calcium/calmodulin-dependent protein kinase (CaMK) IV, have been reported to be involved in HMGB1 phosphorylation (Oh et al., 2009; Zhang et al., 2008).

**Inhibition of HMGB1 signaling by EP**

Recently, it has been reported that EP is a pharmacological inhibitor of HMGB1 secretion (Ulloa et al., 2002; Dave et al., 2009; Chung et al., 2008; Liang et al.,
2009). EP administration significantly prevented lethality in mice with established endotoxemia or sepsis by reducing circulating levels of HMGB1, which resulted in the inhibition of the activation of p38 mitogen-activated protein kinase and NF-κB in macrophage cultures (Ulloa et al., 2002). In murine colitis, EP inhibited secretion of HMGB1 and cytokine production, and improved survival rates of the animal (Dave et al., 2009). EP also decreased expression of IL-12, p40 and nitric oxide production in LPS-activated murine macrophages (Dave et al., 2009). In addition to the HMGB1 secretion, suppression of HMGB1 expression by EP has also been reported in the traumatic brain injury (Su et al., 2011) and in myocardial ischemia/reperfusion injury (Hu et al., 2011). Therefore, EP suppresses HMGB1 secretion as well as its expression.

**Purpose of study**

The aim of the present study was to investigate novel molecular mechanisms underlying anti-oxidative and anti-inflammatory effects of EP in brain cells. For the anti-oxidative effect of EP, the molecular mechanism with respect to nuclear factor Nrf2 activation and HO-1 induction was examined in the primary astrocyte cultures and for anti-inflammatory effect, EP-mediated suppression of HMGB1 secretion in activated microglia cells was examined.
Materials and Methods

1. Primary astrocyte culture and H$_2$O$_2$ treatment

Primary astrocyte cultures were prepared as described previously (Gebicke-Haerter et al., 1989). The brains of newborn male Sprague-Dawley rats (< 1 d) were removed and cortex tissue was dissociated, chopped, and seeded into poly-l-lysine-coated culture flasks (Sigma, St Louis, MO, USA). They were cultured in plating media (Minimum Essential Medium (MEM; Sigma, St Louis, MO, USA) containing 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA), 2 mM glutamine, and 1% P/S for one week at 37°C in a 5% CO$_2$ incubator. Microglia were removed by tapping flasks and washing twice with PBS, and astrocytes were then incubated in growth media containing 5% FBS for one week. Media were changed every 2-3 days.

2. Cortical neuron-enriched cultures and treatment with H$_2$O$_2$, Zn$^{2+}$ ion, and Fe$^{2+}$ ion

Cortical neuron-enriched cultures were prepared from the cerebral cortices of E17 Sprague-Dawley rats. Briefly, cortex tissue were dissociated and cells were seeded in Dulbecco’s modified Eagle’s medium (DMEM; Hyclone, Logan, UT, USA) containing 10% FBS on 24 well plates. After 24 hrs, the medium was replaced with
DMEM containing 2% B27 (Gibco, Carlsbad, CA, USA). These neuron-enriched cultures were maintained for 7 to 10 days with medium changes every 3 days. Cells (1 x 10^5/well) were then treated with 70 μM H_2O_2 (sigma, St Louis, MO, USA) for 1 hr, 400 μM Zn^{2+} ion (sigma, St Louis, MO, USA) for 30 min, or 400 μM Fe^{2+} ion (Sigma, St Louis, MO, USA) for 30 min.

3. Primary microglia culture and cell culture

Primary microglia cultures were prepared from Sprague-Dawley rats (< 1 d). Microglial cultures were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA), 2 mM glutamine, and 1% P/S for two week at 37°C in a 5% CO_2 incubator. Briefly, the brains of newborn male SD rats (< 1 d) were removed and cortex tissue was dissociated, chopped, and seeded into poly-l-lysine-coated culture flasks (Sigma, St Louis, MO, USA). Floating microglia were collected from 10- to 14-day-old astrocyte-microglia-mixed primary cultures by filtering through a 40-μm-pore-size cell strainer (BD Falcon, Franklin Lakes, NJ, USA). Microglial cultures were re-seeded into 24 well plates and subjected to various treatments after 24 hr. BV2 cells were grown in DMEM supplemented with 5% FBS and 1% P/S.
4. Ethyl pyruvate and sodium pyruvate treatment

EP (Sigma, St. Louis, MO, USA) was added to Ringer’s solution containing sodium (130 mM), potassium (4 mM), calcium (2.7 mM) and chloride (139 mM) (pH 7.0). Primary astrocyte cultures were then treated with Ringer’s EP solution in MEM containing 5% FBS. Cells were also treated with sodium pyruvate (Sigma, St. Louis, MO, USA) in MEM containing 5% FBS.

5. Immunoblotting and co-immunoprecipitation analysis

Cells were washed twice with cold PBS and lysed with RIPA buffer (50 mM Tris-HCl (pH 7.4), 1% NP40, 0.25% sodium-deoxycholate, 150 mM NaCL, 1 mM Na3VO4, and 1 Mini protease inhibitor cocktail tablet (Roche Diagnostics, Basel, Switzerland). Lysates were centrifuged at 12000 rpm for 15 min at 4°C and supernatants were loaded onto 12% SDS-PAGE gels. Primary antibodies, which were diluted at 1:1000, were as follows: anti-HO-1 (Stressgene, Victoria, Canada), anti-Nrf2 (Santa Cruz biotechnology, Santa Cruz, CA, USA), anti-p-ERK (Cell Signaling, Beverly, MA, USA), anti-p-AKT (Cell Signaling, Beverly, MA, USA), anti-GST (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-NQO1 (Invitrogen, Carlsbad, CA, USA), anti-GCLM (Abcam, Cambridge, MA, USA), anti-SOD2 (Stressgene, Victoria, Canada), anti-HMGB1(Abcam, Cambridge, MA, USA for media; Santa Cruz Biotechnology, Santa Cruz, CA, USA for lysate), anti-
PKCα (BD Biosciences, San Jose, CA, USA), anti-CaMK IV (BD Biosciences, San Jose, CA, USA), anti-p62 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Lamin B (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-α-Tubulin (Calbiochem, Darmstadt, Germany); and were detected by BM Chemiluminescence Blotting Substrate (Roche, Mannheim, Germany) using goat anti-rabbit, anti-mouse or donkey anti-goat horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Total lysates containing 500 µg of protein were immunoprecipitated with 2 µl of anti-HMGB1 antibody overnight at 4°C. Pre-equilibrated protein G PLUS-Agarose (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was then added and incubated for 2 hrs at 4°C on a rotating wheel. Beads were then washed three times with RIPA buffer and separated by SDS-PAGE. Primary antibody was diluted at 1: 1000 for anti-phosphoserine (Millipore Bioscience Research Reagents, Temecula, CA, USA).

6. Measurement of cell viability and cytotoxicity assay

Cell viabilities after treatments were analyzed using the MTT (3-(4,5-dimethylthiazol-3-yl) 2,5-diphenyl tetrazolium bromide) method. Briefly, primary astrocytes were cultured in 24 well tissue culture plates and treated with serum-free DMEM containing 100 µM H₂O₂ for 1 hr. Cells were stained with 500 µg/ml MTT (Sigma, St. Louis, MO, USA) at 24 hrs later. The medium was then
carefully aspirated and 200 µl of DMSO was added to solubilize the colored formazen product. The optical density was read at 550 nm. Cell viabilities after A23187 treatment were also analyzed using the MTT (3-(4,5-dimethylthiaziazol-3-yl) 2,5-diphenyl tetrazolium bromide) method. Lactate dehydrogenase release (LDH) activity was measured with a colorimetric assay kit (Roche Diagnostics, Basel, Switzerland). LDH activity released from cytosol of damaged cells into the supernatant was quantified with a coupled enzymatic assay.

7. siRNA transfection

Transient transfections were carried out using Dharmafect 3 transfection reagent (Dharmacon, Lafayette, CO, USA), according to the manufacturer’s instructions. Briefly, siRNA and lipid complexes were added to wells (to final concentration of 100 nM siRNA and 1 µl/well of Dharmafect 3). HO-1 siRNA oligonucleotide sequences were chosen using Invitrogen’s Stealth RNAi™ siRNA duplex oligonucleotides (Invitrogen, Carlsbad, CA, USA). Rat HO-1-specific siRNA (5’-AUG GCA UAA AUU CCC ACU GCC ACG G-3’ and 5’-CCG UGG CAG UGG GAA UUU AUG CCA U-3’) and a nonspecific siRNA (5’-AUG CAC GAU AUA ACC UCA CCG UCG G-3’ and 5’-CCG ACG GUG AGG UUA UAU CGU GCA U-3’) were provided by Invitrogen (Carlsbad, CA, USA). Rat Nrf2-specific siRNA oligonucleotides (SMARTpool) were purchased from Dharmacon (Lafayette, CO,
8. Treatments of ZnPP, PD98059, and wortmannin

Zinc protoporphyrin (ZnPP) was provided by Sigma (St Louis, MO, USA), and PD98059 and wortmannin by Calbiochem (Calbiochem, San Diego, CA, USA). Primary astrocytes were incubated with 5 mM EP for 12 hrs with or without 1, 5, and 20 μM ZnPP. Cells were then treated with 100 μM H₂O₂. PD98059 and wortmannin were preincubated in primary astrocytes with indicated amounts for 15 or 30 min. After pre-incubation, 5 mM EP was treated for 12 hrs and EP-induced HO-1 expression was examined by immunoblot analysis.

9. Plasmid transfection and luciferase assays

Wild type (E1 and E2) and ARE-mutated (E1-M739 and E2-M45) HO-1 promoter/luciferase fusion constructs (Alam et al., 2003) were kindly donated by Dr. Alam from Ochsner Clinic Foundation (New Orleans, LA, USA). HO-1 promoters in the E1 and E2 constructs harbor three ARE sequences. The E1-M749 and E2-M45 constructs contain mutations in three ARE core sequences. Dharmafect 3 (Dharmacon, Lafayette, CO, USA) was used as a plasmid transfection reagent. Plasmid constructs/Dharmafect 3 complexes (1: 3.5 ratio) were added to each well and cells were incubated for 4 hrs at 37°C in a 5% CO₂ incubator. Media were
then replaced with fresh media containing 5% FBS and cells were incubated for an additional 24 hrs. Luciferase assays were performed using β-Galactosidase Enzyme Assay System with Reporter Lysis Buffer according to the manufacturer’s instructions (Promega, Madison, WI, USA).

10. Electrophoretic mobility shift assays

Primary astrocytes (1 x 10^6 cells) were harvested and suspended in 400 µl of ice-cold buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM DTT, 0.2 mM PMSF) on ice for 15 min. Suspensions were then centrifuged at 12000 rpm for 10 min at 4°C, and pellets (nuclear fractions) were saved. Pellets were resuspended in buffer C (20 mM HEPES, pH 7.9, 20% glycerol, 0.4 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF) and incubated on ice for 40 min with occasional gentle shaking, and centrifuged at 12000 rpm for 15 min. ³²P-labeled double strand promoter fragments harboring the Nrf2 binding domain (ARE) were used as probes. For supershift assays, nuclear extracts were preincubated with 1 µg of anti-Nrf2 antibody for 2 hrs.

11. Immunocytochemistry

Primary astrocyte cultures were treated with 5 mM EP for 1, 3, 6, 12, or 24 hrs. Cells were then fixed in PBS containing 4% PFA. Anti-Nrf2 antibodies were diluted
at 1: 100 and FITC-labeled anti rabbit IgG (Jackson ImmunoRes, West Grove, PA, USA) was used as a secondary antibody. Cells were double-stained with DAPI (100 ng/ml).

12. Ethyl pyruvate conditioned medium and GDNF production assay

To evaluate effects of GDNF accumulated in the EP-treated primary astrocyte cultures, culture media were collected at indicated times after 12 hr EP (5 mM) incubation. EP-conditioned astrocyte culture media was then added to the neuron-enriched cultures with and without co-treatment of 2.5 μg/ml of GDNF antibody (Abcam, Cambridge, MA, USA). GDNF secretion in the ethyl pyruvate conditioned media was detected using GDNF Emax Immunoassay System according to the manufacturer’s instructions (Promega, Madison, WI, USA).

13. Treatment of recombinant GDNF

Recombinant rat GDNF (rrGDNF) (Sigma, St. Louis, MO, USA) at various dosages was added to the culture medium of primary neuronal cultures after H₂O₂, Zn²⁺, or Fe²⁺ treatment. After 24 hrs, cell supernatants were harvested and used in LDH assay to evaluate the effects of rrGDNF at 10, 50, and 100 ng/ml doses, respectively.
14. Surgical procedures for MCA occlusion and CSF sampling

All experiments were carried out in accordance with “The Guidelines for Animal Research at Inha University School of Medicine”. Middle cerebral artery (MCA) occlusion was carried out as described (Kim et al., 2006). In brief, Male Sprague-Dawley rats (250-300g) were anesthetized with 5% isoflurane in a gas mixture of 30% oxygen and 70% nitrous oxide and maintained using 0.5% isoflurane in the same gas mixture during the operation. Occlusion of right common carotid artery was maintained by a suture method for 1 hr and this was followed by reperfusion for 1 or 7 d. A thermoregulated heating pad and a heating lamp were used to maintain the rectal temperature at $37 \pm 0.5 ^\circ C$. For sampling CSF, rats were anesthetized and the head was horizontal to the table. The skin was incised and the occipital bone was cleared of muscle to expose atlanto-occipital membrane. The needle of 1 ml disposable plastic syringe was passed through to the dura-mater into the cisterna magna of the rats and 50 to 100 $\mu$l CSF like fluid was obtained into the syringe. To avoid contaminating CSF with blood, supernatant was achieved for pure CSF with centrifugation of CSF like fluid.

15. Infarct volume assessment

Rats were decapitated after 1 or 7 d of reperfusion and whole brains were dissected coronally into 2-mm brain slices using a metallic brain matrix (RBM-40000, ASI,
Springville, UT, USA) and stained with 1% 2,3,5-triphenyl tetrazolium chloride (TTC) at 37°C for 15 min. Brain slices were then in 4% paraformaldehyde for preservation. The areas of infarct tissue were measured using Scion Image program (Frederick, MD, USA). To account for the cerebral edema and differential shrinkage resulting from tissue processing, the area of ischemic lesion was measured in each section by subtracting the area in the ipsilateral hemispheres from that of the contralateral hemispheres. The infarct volumes were calculated (in mm³) by integrating the infarct sizes for each infarct-containing tissue section.

16. Ethyl pyruvate and A23187 treatment

EP (Sigma, St. Louis, MO, USA) was added to Ringer’s solution containing sodium (130 mM), potassium (4 mM), calcium (2.7 mM) and chloride (139 mM) (pH 7.0). EP was administered intravenously (5 mg/kg) 30 min before or at various time after MCA occlusion. Primary microglial cultures and BV2 cells were treated with 1, 2.5, and 5 mM of EP with or without A23187 (Sigma, St Louis, MO, USA) for 30 min. A23187 was diluted in tyroid buffer (137 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl₂, 1.1 mM MgCl₂, 11.9 mM NaHCO₃, 0.4 mM NaH₂PO₄, and 5.6 mM glucose, pH 7.2) and incubated in cells. After 30 min, A23187-contained tyroid buffer was changed to fresh culture media containing 5% FBS.
17. Preparation of cell extracts and protein contents in media

Nuclear extracts and cytosolic extracts were prepared essentially using NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific, Rockford, IL, USA), according to the manufacturer’s instruction. Briefly, cells were pelleted by centrifugation at 500 x g for 5 min and the pellet was harvested by removing the supernatant. The pellet was resuspended in 100 µl of cytoplasmic extraction reagent (CER1) and vortexed vigorously to lyse the cells incubating on ice for 10 min. 5.5 µl of CER2 was added to the pellet containing soluble fraction and vortexed. After 1 min, cytosolic extracts were collected from supernatant after centrifugation at 16000 x g. The insoluble (pellet) fraction was suspended in 50 µl of nuclear extraction reagent (NER), which contains nuclei and vortexed. After 40 min, nuclear extracts were collected from supernatant after centrifugation at 16000 x g. For preparation of protein contents in media, 500 µl of media from treated cells were harvested and centrifuged at 500 x g for 5 min to remove cellular debris. Media were then concentrated using Nanosep centrifugal devices (Pall Life Sciences, Ann Arbor, MI, USA) according to the manufacturer’s instruction, and protein contents in media were collected.

18. Calcium assay and staining

BV2 cells and primary microglia cultures were treated with 2.5 µM of A23187 for
30 min. Cells were then fixed in PBS containing 4% PFA at indicated time after A23187 treatment. BV2 cells were then incubated with 4 μM of Flou-4 (Invitrogen, Carlsbad, CA, USA) for 30 min and rinsed twice in PBS. Increased fluorescence excitation at 488nm was screened by confocal microscopy and microplate reader.

19. Statistical Analysis

Statistical analysis was performed by analysis of variance (ANOVA) followed by the Newman-Keuls test. All data were presented as means ± SEMs and statistical difference was accepted at the 5% level.
Results

1. Protective effect of EP-mediated Nrf2 activation and HO-1 induction in astrocytes via autocrine and paracrine mechanisms

1-1. EP pretreatment suppressed H₂O₂-induced cell death in primary astrocyte cultures

To investigate whether the neuroprotective effects of EP shown in the postischemic brain and in a KA-induced seizure animal model (Yu et al., 2005; Cho et al., 2006) are related with its antioxidative effect in astrocytes, protective effect of EP in primary astrocyte cultures was examined after H₂O₂ treatment. Pretreatment of primary astrocyte cultures with 1, 2.5, 5, or 10 mM of EP for 12 hrs protected astrocytes from H₂O₂-induced cell death (100 μM, for 1 hr) (Fig. 1A). In particular, 2.5 and 5 mM of EP increased cell survival from 32.6 ± 0.7% to 62.2 ± 2.4 % and 63 ± 1.8 %, respectively (Fig. 1A). When cells were treated with 2.5 or 5 mM of EP for 3 hrs, cell survival was also significantly increased, and it increased further to peak at a preincubation time of 6 hrs (Fig. 1B). In contrast to EP, treatment with pyruvate (a primary EP metabolite) showed no protective effect under the same conditions (2.5 or 5 mM for 12 hrs) (Fig. 1C), suggesting that the cytoprotective effect of EP is not derived from pyruvate. These results indicate that EP has a
marked protective effect against H$_2$O$_2$-induced cell death in primary astrocyte cultures.

1-2. EP pretreatment induced HO-1 in primary astrocyte cultures

Because HO-1 is an important antioxidant defense enzyme, it was examined whether HO-1 levels are increased in astrocytes after EP treatment. HO-1 was significantly induced in primary astrocyte cultures preincubated with EP (Fig. 2A). HO-1 induction peaked after 12 hrs of incubation with 2.5 mM of EP at 3.3-fold and with 5 mM of EP at 4.9-fold (Fig. 2A). HO-1 expression in primary astrocyte cultures was also increased by H$_2$O$_2$ (100 μM); it began to increase at 1 hr and continued to increase until 12 hrs (Fig. 2B).

1-3. EP-induced HO-1 up-regulation is responsible for the cytoprotective effect of EP in H$_2$O$_2$-treated primary astrocyte cultures

To determine whether HO-1 induction is responsible for the cytoprotective effects of EP in H$_2$O$_2$-treated primary astrocyte cultures, siRNA-mediated HO-1 or Nrf2 knockdown was carried out. Nrf2 has been known to bind ARE in the promoter region and induce the expression of HO-1 (Nguyen et al., 2003). At 24 hrs after HO-1 siRNA transfection (after 12 hrs of EP treatment), EP-induced HO-1 level was only 12.3 ± 1.3% of that of HO-1 siRNA non-transfected control cells (Fig.
Figure 1. Cytoprotective effect of EP pretreatment in H$_2$O$_2$-treated primary astrocyte cultures

(A) Primary astrocyte cultures were treated with 1, 2.5, 5, or 10 mM of EP for 12 hrs and then treated with H$_2$O$_2$ (100 μM) for 1 hr. (B) Cells were pre-treated with EP (2.5 or 5 mM) for 1, 3, 6, 12, or 24 hrs and then treated with H$_2$O$_2$ (100 μM) for 1 hr. (C) Cells were pre-treated with 1, 2.5, 5, or 10 mM of pyruvate for 12 hrs and then treated with H$_2$O$_2$ (100 μM) for 1 hr. In all experiments, MTT assays were carried out 24 hrs after H$_2$O$_2$ treatment. Changes in cell survival observed in three independent experiments are presented as averages±SEMs. *p<0.05 and **p<0.01 versus the untreated control.
Figure 2. HO-1 induction by EP in primary astrocyte cultures

(A) Cells were treated with 2.5 or 5 mM of EP for 6, 12, or 24 hrs and HO-1 levels were determined by immunoblotting. Protein levels determined in three independent experiments are presented as averages±SEMs. (B) HO-1 expression levels in primary astrocyte cultures were determined at 1, 3, 6, 12, or 24 hrs after H_{2}O_{2} treatment (100 μM, 1 hr) by immunoblotting. Protein levels determined in three independent experiments are presented as averages±SEMs. *p<0.05 and **p<0.01 versus the untreated control.
Figure 3. Inhibition of EP-mediated cell survival by suppressing Nrf2 or HO-1 level and HO-1 activity

(A) Primary astrocyte cultures were transfected with HO-1 siRNA, Nrf2 siRNA, or nonspecific siRNA. Twelve hours after transfection, cells were treated with 5 mM EP for 12 hrs and HO-1 or Nrf2 levels were examined (24 hrs after siRNA transfection) by immunoblotting (A). (B) Cell viabilities were determined by MTT assay at 24 hrs after 1 hr of H$_2$O$_2$ treatment (36 hrs after siRNA transfection). (C) Primary astrocyte cultures were co-incubated with 1, 5, or 20 μM of ZnPP and 5 mM EP for 12 hrs, and then treated with 100 μM H$_2$O$_2$ for 1 hr. MTT assays were carried out 24 hrs after H$_2$O$_2$ treatment. Changes in cell survivals and protein levels in three independent experiments are presented as averages±SEMs. *p<0.05 and **p<0.01 versus the control.
and similarly, after Nrf2 siRNA transfection, Nrf2 level was 19.2 ± 4.2% that of Nrf2 siRNA non-transfected control cells (Fig. 3A). In HO-1-siRNA-transfected cells, the cell viability of EP (5 mM)-pretreated/H_2O_2-treated cells reverted to 30.1 ± 2.9% of normal cells, which was comparable to that observed in EP-untreated/H_2O_2-treated primary astrocyte cultures (36.5 ± 2.2%) (Fig. 3B). Similarly, when the primary astrocyte cultures were transfected with Nrf2 siRNA, cell viability was 34.9 ± 3.8% of normal cells (Fig. 3B). In contrast, cell viabilities in non-specific siRNA-transfected primary astrocyte cultures were similar to those of EP-treated cells (Fig. 3B). The results indicate that Nrf2-mediated HO-1 induction is responsible for the protective effect of EP. To confirm the importance of HO-1, effect of ZnPP (a HO-1 inhibitor) in EP/H_2O_2-treated astrocyte cultures was examined. Co-treatment with ZnPP (1, 5, or 20 μM) and EP (5 mM) for 12 hrs before H_2O_2 treatment attenuated EP-mediated cytoprotection in a ZnPP-concentration dependent manner (5 μM ZnPP treatment reduced cell viability to 26.6 ± 1.9%) (Fig. 3C). These results indicate that HO-1 plays a crucial role in cytoprotection afforded by EP in astrocytes.

1-4. EP induced the nuclear translocation of Nrf2

Ablation of EP-induced protective effect in Nrf2-knockdowned cell prompted to examine whether EP induces the cytoplasm to nuclear translocation of Nrf2 in
primary astrocyte cultures. In a double labeling experiment with anti-Nrf2 antibody and DAPI, Nrf2 was found to be localized in the cytoplasm in control cells (Fig. 4A). However, within 3 hrs of EP treatment (5 mM), Nrf2 translocation to nuclei was observed (Fig. 4B), and Nrf2 remained in nuclei after 6 hrs of EP treatment (Fig. 4C). Nrf2 continued to be detected in nuclei until 12 hrs after EP treatment, but subsequently was also detected in cytoplasm (Fig. 4D). In contrast, Nrf2 was observed in the cytoplasm in Ringer’s solution-treated control cells at all time points (Fig. 4E-H). These results suggest that EP induces Nrf2 translocation in primary astrocyte cultures.

1-5. EP increased Nrf2 binding to ARE in HO-1 promoter

Next, Nrf2-ARE interaction in EP-treated astrocytes was examined using an ARE-reporter assay and EMSA. When primary astrocyte cultures were transfected with HO-1 promoter-luciferase fusion constructs (E1 and E2; Alam et al., 2003), luciferase activities were higher in EP-treated cells than those observed in mock-transfected control cells (Fig. 5A,B). In contrast, luciferase activities in primary astrocyte cultures transfected with ARE mutants of both reporter constructs (E1 M739 and E2 M45) (Alam et al., 2003) were significantly lower than in wild type E1- or E2-transfected cells, respectively (Fig. 5A,B). EP-induced Nrf2-ARE binding was further confirmed by EMSA using ARE-containing oligonucleotides as
Figure 4. Nrf2 translocation from cytoplasm to nucleus by EP

Primary astrocyte cultures were treated with 5 mM EP (A-D) or Ringer’s solution (E-H) for 3, 6, or 12 hrs. Double fluorescence staining was performed using anti-Nrf2 antibody and DAPI. Nrf2-positive cells were identified using a FITC-conjugated secondary antibody. Arrows indicate Nrf2 translocation from cytoplasm to nucleus and the arrowheads indicate the cytoplasmic localization of Nrf2. The scale bar represents 20 μm.
Figure 5. Activation of Nrf2-ARE pathway by EP in primary astrocytes

(A, B) Promoter activities were assayed using the HO-1 promoter/luciferase fusion constructs, E1, E2, and the ARE-mutation constructs, E1-M739 and E2-M45. Twelve hours after transfecting reporter constructs, cells were treated with 5 mM EP for 6 or 12 hrs. Luciferase activities were assayed at 24 hrs after transfection. Changes in luciferase activities were determined using four independent experiments and are presented as averages±SEMs. *p<0.05 and **p<0.01 versus the control.
Figure 6. Nrf2-ARE interaction by EP in primary astrocytes

(A, B) The EP-induced interaction between Nrf2 and ARE was examined by EMSA. Nuclear extracts were prepared from primary astrocyte cultures at 3 and 6 hrs after treatment with 5 mM EP. Cold, 100 fold molar excess of unlabelled probe; SS, supershift assay, which were conducted by pre-incubating with antibody against Nrf2.
probes. Incubation of nuclear extracts prepared from EP-treated primary astrocyte cultures with $\gamma^{32}$p-labeled HO-1 specific ARE oligonucleotides enhanced binding activity between HO-1-ARE and Nrf2 (Fig. 6A) and the preincubation of reaction mixtures with anti-Nrf2 antibody notably decreased shifted Nrf2 band intensities (Fig. 6B), suggesting that EP triggers HO-1 expression via Nrf2 binding to ARE in HO-1 promoter.

1-6. ERK and AKT signaling pathways were involved in the EP-mediated enhancement of HO-1 expression

To identify the signal transduction pathways mediating EP-induced HO-1 expression in primary astrocyte cultures, activations of various kinases known to be involved in HO-1 induction were examined. A transient increase in phosphorylated ERK after 3 hrs and a sustained increase in phosphorylated AKT after 1 hr of EP treatment were observed (Fig. 7A), respectively, suggesting that ERK and AKT participate in EP-mediated HO-1 induction in astrocytes. When the primary astrocyte cultures were treated with PD98059 or wortmannin (pharmacological inhibitors of ERK and AKT, respectively), both significantly suppressed EP-mediated HO-1 induction (Fig. 7B). These results further support the notion that the ERK and AKT signaling pathways are involved in the EP-mediated induction of HO-1 in primary astrocyte cultures.
1-7. EP-mediated HO-1 induction enhanced GDNF expression in primary astrocyte cultures

Recent report showing neurotrophic factor induction by HO-1 (Hung et al., 2008) prompted to investigate the effect of EP-mediated HO-1 induction on GDNF expression in primary astrocyte cultures. Accumulation of secreted GDNF protein in EP-conditioned (12 hrs incubation) primary astrocyte culture media was detected after 6 hrs of EP treatment and was further increased at 12 hrs and then increased level was maintained until 48 hrs (Fig. 8A). To determine whether HO-1 up-regulation by EP is responsible for GDNF induction, effects of HO-1 knockdown on GDNF accumulation were examined. In HO-1 siRNA-transfected cells (Fig. 8B), protein levels of GDNF in culture media were reduced to 63.3 ± 9.7% of the non-transfected cell levels (Fig. 8C). In contrast, secreted GDNF protein levels in non-specific siRNA-transfected primary astrocyte cultures were similar to those of EP-treated cells (Fig. 8C). These results suggest that EP-mediated HO-1 induction is responsible, at least in part, for GDNF induction.

1-8. EP-HO-1-mediated GDNF induction protected neuronal cells damaged from \( \text{H}_2\text{O}_2, \text{Zn}^{2+}, \text{and Fe}^{2+} \) ion

Next, the neuroprotective effect of GDNF accumulated in EP-conditioned media
Figure 7. Activations of ERK and Akt by EP-mediated HO-1 induction in primary astrocyte cultures

(A) Primary astrocyte cultures were incubated with 5 mM EP for 1, 3, or 6 hrs. Phosphorylated ERK and Akt levels were determined by immunoblotting with anti-pERK and pAkt antibodies. (B) Cells were preincubated with the indicated amounts of PD98059 or wortmannin, for 15 and 30 min, respectively, and treated with 5 mM EP for 12 hrs. EP-induced HO-1 expressions were examined by immunoblotting.
Figure 8. Accumulation of GDNF in EP-conditioned astrocyte culture media

(A) Primary astrocyte cultures were incubated with 5 mM EP for 12 hrs and GDNF levels in media of primary astrocyte culture were determined at 6, 12, 24, and 48 hrs after EP treatment. (B,C) Astrocytes were transfected with HO-1 siRNA or nonspecific siRNA. After 12 hrs of transfection, cells were treated with 5 mM EP for 12 hrs and then HO-1 levels in total cell lysate were examined by immunoblotting (B). Twelve hours after EP treatment, media were replaced with fresh one and after another 12 hrs, secreted GDNF protein levels were analyzed by GDNF production assay kit (C). **p<0.01 versus the untreated control.
(ECM) was examined. When primary neuronal cultures were treated with ECM which was collected after 12 hrs of incubation with 5 mM EP in primary astrocyte cultures, H$_2$O$_2$ (50-70 µM, 1 hr), Zn$^{2+}$ ion (200-400 µM, 30 min), and Fe$^{2+}$ ion (200-400 µM, 30 min)-induced neuronal cell deaths were significantly suppressed (Fig. 9A-C). Mean neuronal death induced in 70 µM H$_2$O$_2$-treated neuron-enriched primary cultures was reduced to 65.6±2.3% by ECM (Fig. 9A). Similarly, mean neuronal deaths observed in 400 µM Zn$^{2+}$ ion- or 400 µM Fe$^{2+}$ ion-treated neuronal cultures were reduced to 66.5±3.8% and 58±9.8%, respectively (Fig. 9B,C). In addition, these ECM-mediated neuroprotective effects were not observed when ECM collected from Nrf2 siRNA-transfected cells was used or when ECM was incubated with GDNF antibody (Fig. 10A-C), but not by control IgG or by non-specific siRNA. These results indicate that the protective effects were due to the GDNF present in ECM. Moreover, recombinant GDNF treatment significantly reduced H$_2$O$_2$-, Zn$^{2+}$, and Fe$^{2+}$-induced cell death in primary neuronal cultures (Fig. 11), further corroborating the notion that EP-induced GDNF secretion from astrocyte confers neuroprotection.

1-9. EP-induced Nrf2 activation increased the expressions of various antioxidant genes

EP-induced Nrf2 translocation to the nucleus and the interaction between Nrf2 and
(A-C) Primary astrocyte cultures were treated with 5 mM of EP for 12 hrs and then EP-conditioned astrocyte culture media (ECM) were collected. Primary neuronal cultures were treated with indicated amounts of H_2O_2 for 1 hr (A) or with Zn^{2+} ion for 30 min (B) or with Fe^{2+} ion for 30 min (C). Media were then replaced with ECM and LDH assays were carried out 24 hrs after H_2O_2, Zn^{2+} ion, or Fe^{2+} ion treatment. Changes in cell death observed in three independent experiments are presented as averages±SEMs. *p<0.05 and **p<0.01 versus the control.
Figure 10. Neuroprotective effect of GDNF accumulated in EP-conditioned astrocyte culture media

(A-C) Primary neuronal cultures were treated with 70 μM of H2O2 for 1 hr (A) or with 400 μM Zn2+ ion for 30 min (B) or with 400 μM Fe2+ ion for 30 min (C) and media were then replaced with ECM in the presence or absence of 2.5 μg/ml GDNF antibody or with ECM prepared from Nrf2 siRNA-transfected astrocyte cultures. Non-specific IgG or non-specific siRNA was used as a negative control, respectively. LDH assays were carried out 24 hrs after H2O2, Zn2+ ion, or Fe2+ ion treatment. Changes in cell death observed in three independent experiments are presented as averages±SEMs. *p<0.05 and **p<0.01 versus the control.
Figure 11. Neuroprotective effect of recombinant GDNF treatment in culture media

Recombinant rat GDNF (10, 50, 100 ng/ml) was added to culture medium of primary neuronal cultures after \( \text{H}_2\text{O}_2 \) (70 \( \mu \text{M} \) of \( \text{H}_2\text{O}_2 \) for 1 hr), \( \text{Zn}^{2+} \) ion (400 \( \mu \text{M} \) \( \text{Zn}^{2+} \) ion for 30 min), or \( \text{Fe}^{2+} \) ion (400 \( \mu \text{M} \) \( \text{Fe}^{2+} \) ion for 30 min) treatment. LDH assays were carried out 24 hrs after \( \text{H}_2\text{O}_2 \), \( \text{Zn}^{2+} \) ion, or \( \text{Fe}^{2+} \) ion treatment. Changes in cell death observed in three independent experiments are presented as averages±SEMs. *\( p<0.05 \) and **\( p<0.01 \) versus the control.
Figure 12. Induction of genes downstream of Nrf2 by EP in primary astrocyte cultures

Primary astrocyte cultures were treated with 2.5 or 5 mM of EP (A,B) or pyruvate (C) for 6, 12, or 24 hrs and levels of HO-1, GST, NQO1, GCLM, SOD2, and α-tubulin were determined by immunoblotting. (B) Primary astrocyte cultures were transfected with Nrf2 siRNA or non-specific siRNA. Twelve hours after transfection, cells were treated with 5 mM EP for 12 hrs and HO-1, GST, NQO1, GCLM, SOD2, and α-tubulin levels were examined (24 hrs after siRNA transfection) by immunoblotting.
ARE prompted to examine whether various antioxidant genes modulated by Nrf2 (other than HO-1) are also induced by EP. Levels of GST, NQO1, and GCLM were markedly increased by EP treatment (2.5 mM, 6 hrs) and peak induction occurred after 12 hrs of EP treatment (Fig. 12A), indicating that EP increases the expressions of the genes modulated by Nrf2. These genes were not induced after Nrf2 knockdown by Nrf2 siRNA transfection, but not after transfection with non-specific siRNA (Fig. 12B), which further confirmed the Nrf2-dependendent natures of these inductions. These genes were also induced after pyruvate treatment but at markedly lower levels (Fig. 12C), suggesting that pyruvate may need much higher concentration or employ different molecular mechanisms for Nrf2-downstream gene induction.

2. Inhibition of HMGB1 release by EP via chelating calcium ion

2-1. EP treatment reduces infarct volume and HMGB1 release in rat MCAO model

Pretreatment with 5 mg/kg of EP at 30 min before the ischemic insult (MCAO, 60 min) reduced the infarct volume to 18.2 ± 4.0% of that of the control animals 1 day after MCAO (Fig. 13A,B). To investigate the possibility that neuroprotective effect of EP is related with the suppression of HMGB1 release in the postischemic brain,
extracellular HMGB1 levels were examined after EP administration. HMGB1 was accumulated in the CSF after MCAO, producing dual peaks around 1 day and 7 days after MCAO (Fig. 14A), similarly to the previous report (Kim et al, 2006; Kim et al., submitted). The administration of EP (5 mg/kg/day, I.V.) for 4 days at 4, 5, 6, and 7 d after MCAO reduced CSF HMGB1 level at 7 d after MCAO by 53.8±4.2% compared to the Ringer solution-treated MCAO control (Fig. 14B). However, in EP administered rats, the infarct volume measured 7 days after MCAO was similar to Ringer solution-treated MCAO group (Fig. 14C), suggesting that the reduction of HMGB1 release is not an indirect outcome of neuroprotective effects of EP.

2-2. EP inhibits HMGB1 secretion from activated microglia, in particular, at the level of translocation from nucleus to cytoplasm.

Since it has been known that extracellular HMGB1 functions as a cytokine, it was examined whether EP inhibits HMGB1 secretion and this inhibition suppresses delayed microglia activation. As shown in a previous report (Kim et al., submitted), in LPS (0.2 μg/ml)-treated BV2 cells, HMGB1 was secreted in a remarkably delayed manner (Fig. 15A), beginning 18 hrs after LPS treatment, and HMGB1 level in culture media was sustained at 24 hrs (Fig. 15A). Immunoblot analysis using protein preparations from nuclear and cytoplasmic fractions revealed that
Figure 13. Amelioration of ischemic infarct volume by pre-administration of EP in the postischemic brain

(A) 5mg/kg of EP was administered intravenously 30 min before 1h MCAO. Infarction volumes were assessed at 1d post-MCAO by TTC staining and quantification data of infarction volumes were presented as means±SEM (B). **p<0.01 versus the control.
Figure 14. Reduced HMGB1 release by EP treatment in rat MCAO model

(A) The CSF levels of HMGB1 after MCAO were determined by immunoblotting at the indicated times after 1h of MCAO. (B,C) 5mg/kg of EP was administered intravenously at 4d, 5d, 6d, and 6hr before 7d after 1h MCAO. Infarction volumes were assessed at 7d post-MCAO by TTC staining and quantification data of infarction volumes were presented as means ± SEM (C). Western blot analysis of HMGB1 in CSF from EP-administered or untreated MCAO groups was determined at 1d or 7d after 1h MCAO (A,B). *p<0.05 versus the control.
HMGB1 was translocated to cytoplasm 6 hrs after LPS treatment and further accumulated in the cytoplasm at 12 hrs after LPS treatment (Fig. 15B). Nuclear to cytoplasmic translocation of HMGB1 was markedly inhibited in EP-treated cells (Fig. 15B), indicating that EP inhibits nuclear to cytoplasmic translocation of HMGB1. However, total HMGB1 levels combining cell homogenates and media were comparable in all conditions (Fig. 15A,B), suggesting that the amounts of HMGB1 were not changed by LPS and/or EP treatments.

2-3. EP inhibits HMGB1 phosphorylation

It has been reported that HMGB1 is phosphorylated and then translocated from nucleus to the cytoplasm in LPS-treated macrophages (Zhang et al., 2008; Oh et al., 2009) and colon cancer cells (Kang et al., 2009). Thus, it was examined whether EP inhibits HMGB1 secretion by blocking its phosphorylation. In LPS-treated BV2 cells, HMGB1 was phosphorylated at its serine residues, and this phosphorylation began at 1 hr after LPS treatment and maintained at 4 hrs after treatment (Fig. 16A). Co-treatment of EP (5 mM) with LPS blocked HMGB1 phosphorylation almost completely (Fig. 16B), indicating that EP suppressed LPS-induced HMGB1 phosphorylation.
Figure 15. HMGB1 secretion induced by LPS treatment in BV2 cells

(A,B) BV2 cells were treated with 200 ng/ml of LPS. Indicated times after LPS treatment, HMGB1 levels in media, cytoplasmic, and nucleus of BV2 cells or 5 mM EP co-treated BV2 cells were examined by immunoblotting.
2-4. EP inhibits A23187-induced HMGB1 secretion in BV2 cells

To further demonstrate that inhibition of HMGB1 phosphorylation by EP can be attributed not only to an indirect outcome of anti-inflammatory effect but to a direct effect, effect of EP on HMGB1 secretion in calcium ionophore (A23187)-treated BV2 cells was examined. It has been previously shown that increase in intracellular $\text{Ca}^{2+}$ concentration induces HMGB1 secretion via the activation of two kinases, PKC and CaMK IV (Zhang et al., 2008; Oh et al., 2009). BV2 cells were treated with A23187 (1, 2.5, or 5 μM) for 30 min and cell survivals were examined at 24 hrs after the A23187 treatment (Fig. 17A). To avoid necrotic cell death, after A23187 treatment for 30 min, cells were incubated with culture media containing FBS (1, 2.5, or 5%) for 24 hr. When BV2 cells were treated with 1 or 2.5 μM of A23187 together with 5% FBS, no cell death was detected (Fig. 17A). In both conditions, HMGB1 secretion occurred as early as 3 hr and peaked at 24 hr, although more HMGB1 was secreted at 2.5 μM of A23187 (Fig. 17B). In contrast, HMGB1 level in cell lysate fractions was decreased time dependently, especially in 2.5 μM A23187-treated cells, indicating that $\text{Ca}^{2+}$-induced HMGB1 secretion in BV2 cells without concomitant cell death (Fig. 17B). Treatment of EP blocked A23187-induced HMGB1 secretion in BV2 cells, wherein 2.5 and 5 mM EP completely blocked HMGB1 secretion at 24 hr after A23187 treatment (Fig. 18A). In particular, 5 mM of EP completely blocked nuclear to cytoplasmic translocation
Figure 16. Inhibition of LPS induced HMGB1 phosphorylation by EP treatment

(A, B) BV2 cells were treated with 200 ng/ml of LPS either in the presence or absence of 5 mM EP for indicated times and total cell lysate was collected, immunoprecipitated for HMGB1, and subjected to immunoblotting with anti-pSer antibody.
of HMGB1 observed at 6 hr after A23187 treatment (Fig. 18B). Together these results indicate that EP inhibits HMGB1 secretion by blocking its translocation from nucleus to cytoplasm, and it seems to be a direct effect independent of cell death.

2-5. EP blocked A23187-induced HMGB1 phosphorylation

In A23187-treated cells, HMGB1 was phosphorylated at its serine residues, and this phosphorylation began 1 hr after A23187 treatment and peaked at 4 hrs after treatment (Fig. 19A). Co-treatment of 5 mM EP blocked HMGB1 phosphorylation almost completely (Fig. 19B). When the nuclear translocation of two kinases, cPKC and CaMK IV known to be involved in HMGB1 phosphorylation, was investigated, levels of nuclear PKCα were increased in BV2 cells immediately after A23187 treatment and maintained in the nucleus till 1 hr (Fig. 19C). Similarly, nuclear CaMK IV levels were increased from 30 min after A23187 treatment (Fig. 19C). Co-treatment of EP with A23187 significantly suppressed nuclear translocations of PKCα and CaMK IV, suggesting that EP inhibits activation of the two kinases and both of them are activated by Ca\(^{2+}\).

2-6. EP functions as an intracellular calcium chelator

To investigate the possibility that EP directly chelates intracellular Ca\(^{2+}\),
Figure 17. HMGB1 secretion induced by A23187 in BV2 cells

(A) Cell viabilities were determined by MTT assay at 24 hr after 30 min of A23187 treatment with or without further 1, 2.5, and 5% of FBS addition. (B) BV2 cells were treated with 1 and 2.5 μM of A23187. 3, 6, 12, and 24 hr after A23187 treatment, HMGB1 levels of media and lysate of BV2 cells were examined by immunoblotting.
Figure 18. Inhibition of A23187 induced HMGB1 release by EP treatment

(A,B) A23187 was pre-incubated with 2.5 or 5 mM of EP in BV2 cells for 30 min. HMGB1 levels in media, cytoplasm, and nucleus of BV2 cells were harvested, and subjected to immunoblot analysis with anti-HMGB1 antibodies 24 hr after A23187 treatment and anti-α-tubulin and p62 antibodies for control as each fraction (A). 6 hr after A23187 treatment with or without 5 mM of EP, HMGB1 levels in media, cytoplasm, and nucleus of BV2 cells were subjected to immunoblotting with each antibody (B).
intracellular Ca\(^{2+}\) levels were examined by staining with Fluo-4, fluorescent Ca\(^{2+}\) indicator. Fluo-4 has been known to reveal free Ca\(^{2+}\) in cell cytoplasm, which are probably localized in Ca\(^{2+}\) storing organells, such as, secretory granules and endoplasmic reticulum (ER) (Fig. 20A, 1\(^{st}\) line) (Gee et al., 2000; Yoo, 2010). After A23187 treatment, increased intracellular Flou-4 positive fluorescence was detected as early as 30 min and continued to be detected until 24 hr (Fig. 20A, 2\(^{nd}\) line). The Ca\(^{2+}\) influx induced by A23187 was notably reduced by co-treatment with EP, since significantly lower fluorescence compared to untreated control was detected at all time points (Fig. 20A, 3\(^{rd}\) line). To confirm the capacity of EP for intracellular Ca\(^{2+}\) chelation, 1, 2.5 and 5 mM of EP was introduced for 1 hr after A23187 treatment. As expected, intracellular Ca\(^{2+}\) levels after A23187 treatment was also notably reduced by post-treatment with EP (Fig. 20A, 4\(^{th}\) line). Fluorescence excitation measured by microplate reader at 24 hr after A23187 treatment showed that EP treatment suppressed intracellular Ca\(^{2+}\) levels in a dose dependent manner (Fig. 20B).

2-7. EP inhibits A23187-induced HMGB1 release in primary microglia cultures

The suppression of HMGB1 secretion by EP was further examined in primary microglia cultures. Primary microglia cultures were treated with A23187 (1 \(\mu\)M) for 30 min and cells were cultured with 5% FBS for 24 hr after A23187 treatment to
Figure 19. Inhibition of phosphorylation of HMGB1 by EP treatment

(A,B) BV2 cells were treated with 2.5 μM A23187 either in the presence or absence of 5 mM EP for 30 min. 1, 2, or 4 hr later, total cell lysate was collected, immunoprecipitated for HMGB1, and subjected to immunoblotting with anti-pSer antibody (A). To compare with observation of the phosphorylation of HMGB1 in LPS and A23187 treated cells, 0.2 μg/ml of LPS was treated for 4 hr and followed immunoprecipitated (B). After A23187 treatment, nuclear fractions were harvested after 0, 30 min, and 1 hr and nuclear fraction of 5 mM of EP co-treated with A23187 in BV2 cells was also collected after A23187 treatment (C). Representative blot of two individual experiments was performed by immunoblotting using anti-PKCα and CaMK IV antibodies. Lamin B antibody was used for control as nuclear fraction (C).
avoid necrotic condition (Fig. 21A). When primary microglia cultures were treated with 0.1, 0.25, 0.5 or 1 μM of A23187, HMGB1 secretion was detected at 24 hr after A23187 treatment (Fig. 21B). In terms of the temporal profile, HMGB1 release was detected from 9 to 24 hr in 1 μM of A23187-treated primary microglia cultures (Fig. 21C). At 12 hrs after A23187 treatment, HMGB1 translocation from nuclei to cytoplasm was observed in A23187 treated primary microglia cultures, however, 5 mM of EP co-treatment with A23187 significantly inhibited HMGB1 translocation (Fig. 21D). These results indicate that EP inhibits calcium-mediated HMGB1 release both in BV2 cells and in primary microglia cultures.
Figure 20. Reduced intracellular calcium levels by EP treatment in BV2 cells and chelation of EP and pyruvic acid with calcium ion

(A,B) Intracellular Ca\textsuperscript{2+} levels were measured at 0.5, 1, 6, or 24 hr after A23187 treatment in the presence or absence of various concentrations of EP. Ca\textsuperscript{2+} levels were measured with Flou-4 staining and positive fluorescence excitation in three individual experiments was screened with microplate reader (B). Images of Flou-4-stained Ca\textsuperscript{2+} influx by A23187 was identified by confocal microscopy (A).
Figure 21. Inhibition of A23187 induced HMGB1 release by EP treatment in primary microglia cultures

(A) Cell viabilities were determined by MTT assay at 24hr after 30 min of A23187 (1 μM) treatment with or without further 5% of FBS addition. (B) Primary microglia cultures were treated with 0.1, 0.25, 0.5, and 1 μM of A23187 and 24 hr after A23187 treatment, HMGB1 release in media was examined by immunoblotting. (C) 1 μM of A23187 was incubated in primary microglia cultures for 30 min. HMGB1 levels in media were harvested, and subjected to immunoblot analysis with anti-HMGB1 antibodies 3, 6, 9, 12, 15, 18, and 24 hr after A23187 treatment (C). 12 hr after A23187 treatment with or without 5 mM of EP, HMGB1 levels in media, cytoplasm, and nucleus of primary microglia cultures were subjected to immunoblotting with anti-HMGB1 antibodies and and anti-α-tubulin and p62 antibodies for control as each fraction (D).
Discussion

Nrf2-mediated anti-oxidative effect by EP

The present study showed that EP-mediated Nrf2 activation and subsequent HO-1 induction enhances the viability of H$_2$O$_2$-treated primary astrocyte cultures. It also showed that EP-conditioned astrocyte culture media exert a protective effect on neurons exposed to excitotoxic- or oxidative-insults, at least in part, via GDNF accumulated in the media. Regarding anti-oxidative effects of EP, various molecular and cellular mechanisms have been proposed, for examples, the inhibition of ROS-dependent STAT signaling, direct ROS scavenging, and the suppression of NF-κB signaling (Han et al., 2005; Kim et al., 2008; Johansson et al., 2008; Liu et al., 2009). Here, the results showed that EP exerted protective effect in autocrine and paracrine manner by causing marked activation of Nrf2, inductions of its downstream genes, and subsequent GDNF induction. EP-mediated HO-1 induction has been previously reported in bone marrow-derived macrophages, lung, and joints (Dave et al., 2009; Di Paola, et al., 2010; Kung et al., 2011b). This appears to be a molecular mechanism specific for EP since pyruvate was markedly less potent for inducing Nrf2-downstream genes (Fig. 12C) and for protecting the H$_2$O$_2$-treated cells (Fig. 1C) in a comparable concentration range. In view of the fact that most of
the protective functions of EP and pyruvate are common to both, the EP-mediated inductions of various Nrf2-downstream genes, such as HO-1, may explain the markedly better neuroprotective potency of EP than pyruvate under neuropathological conditions during ischemia in the brain (Yu et al., 2005).

**Nrf2- mediated neuroprotective effect**

Nrf2-mediated neuroprotection has been reported in various pathological conditions, especially, in neurodegenerative diseases. Nuclear Nrf2 levels have been reported to be reduced in Alzheimer’s disease (Ramsey et al., 2007) and intrahippocampal injection of a Nrf2-expressing lentiviral vector improved spacial learning in a mouse model of Alzheimer’s disease (Kanninen et al., 2009). Nrf2-mediated neuroprotection has also been reported in a MPTP mouse model of Parkinson’s disease, generated using Nrf2\(^{-/-}\) mice (Chen et al., 2009). Furthermore, DJ-1, a Parkinson’s disease associated protein (Singh 151-153), has been reported to stabilize Nrf2 and dysfunction of DJ-1 has been reported to result in decreased Nrf2 activity (Clements et al., 2006). In addition, the therapeutic potency of Nrf2-ARE pathway activation has been reported in Huntington’s disease, cerebral ischemia, and traumatic brain injury (Calkins et al. 2009). Nrf2 expression restricted to astrocytes has been found to confer neuroprotection in a MPTP-mediated Parkinson’s disease mouse model (Chen et al., 2009). Similarly, grafting of Nrf2-
overexpressing astrocyte has been reported to protect animals from neurotoxic legions (Calkins et al., 2005), which suggests that astrocytic Nrf2 activation is crucial for neuroprotection. The neuroprotection afforded by EP-conditioned astrocyte culture media but not by Nrf2 siRNA-transfected astrocyte culture media (Fig. 10) further supports this notion.

**Neuroprotective effect of GDNF accumulated in EP-conditioned media**

Of the various Nrf2 downstream genes, HO-1 has been reported to protect tissues by restoring redox homeostasis and reducing inflammation due to its anti-oxidant, antiapoptotic, and anti-inflammatory effects (Cuadrado et al., 2008; Lee et al., 1996; Willis et al, 1996). The present study also shows that HO-1 induced by EP enhanced GDNF levels in astrocytes, and that it is responsible, at least in part, for the protective effects of EP-conditioned astrocyte culture media (Figs. 8-11). The protective effects of GDNF have been demonstrated under various neurodegenerative pathogenic conditions (Akerud et al., 2001; Cunningham and Su, 2002; Capowski et al., 2007). In particular, astrocytes are potent GDNF providers, wherein it exerts many functions critical for neuronal survival (Wilson, 1997). In a recent report by Hung et al. (2008), the downstream products of HO-1 (bilirubin and CO) were shown to modulate the expressions of BDNF and GDNF in neurons and astrocytes, respectively, which agrees with our findings. Regarding the
mechanisms involved, it has been reported that astrocyte-secreted GDNF protects dopaminergic neurons against 6OHDA cytotoxicity in synergy with the GSH antioxidant system (Sandhu et al., 2009). Furthermore, in a previous study, astrocytes and neurons were found to express separate components of GDNF signaling complex, which suggests that they might utilize different pathways to mediate the autocrine and paracrine effects of GDNF (Sandhu et al., 2009). Therefore, it appears that GDNF exerts its neuroprotective effects in various ways. Furthermore, in addition to its cytoprotective effects on neurons, it was observed during our study that EP-conditioned media suppress LPS-induced microglia activation (unpublished data), which may contribute to the robust neuroprotective effect of EP in vivo (Yu et al., 2005).

**Keap1/Nrf2 signaling pathway**

Nrf2 activity is tightly regulated by Keap1, which binds to Nrf2 in the cytoplasm and modulates the ubiquitin-mediated proteosomal degradation of Nrf2 (Motohashi and Yamamoto, 2004). Keap1 is a cystein-rich protein, and in particular, its two cystein residues (C273, and C288) are crucial for its association with Nrf2 (Zhang and Hannink, 2003). Furthermore, it has been reported that many ARE inducers, for example, t-butyl hydroquinone (tBHQ) and sulforaphane, activate Nrf2 by covalently modifying the reactive cysteine residues of Keap1 (Li et al. 2006). It is
likely that EP is capable of covalently modifying the reactive cysteine residues of Keap1 and a previous report that showed EP inhibits the DNA binding activity of p65 by covalently modifying C38 of p65 (Han et al., 2005) supports this possibility. However, further study is required to determine the detailed mechanism involved.

**Inflammation-promoting activity of HMGB1**

Extracellular HMGB1 aggravates damaging process via exaggerating inflammation. Regarding the cytokine-like function of HMGB1, it has been reported that extracellular HMGB1 upregulates inflammatory cytokines, such as, IL-1, IL-6, and TNF-α and causes activation of macrophagy/monocytes and maturation of dendritic cell (DC) (Andersson et al., 2000; Rovere-Querini et al., 2004; Messmer et al., 2004; Dumitriu et al., 2005). In the brain, HMGB1 is released after cytokine treatment and then participates in inflammatory processes (Wang et al., 1999a; Agnello et al., 2002). In particular, in the postischemic brain, HMGB1 is massively released during excitotoxicity-induced acute damaging process and then aggravates neuronal damage triggering inflammatory processes (Kim et al., 2006; Kim et al., 2011). In addition, HMGB1 was markedly induced in activated microglia, astrocytes, and microvascular structures in the postischemic brain, and these gradual inductions were sustained for several days (Kim et al., 2008).
Extracellular HMGB1 in pathogenesis

High plasma levels of HMGB1 have been reported under various pathological conditions. In sepsis patients, serum HMGB1 levels are markedly elevated (Wang et al., 1999b) and HMGB1 levels in non-surviving patients (83.7 ng/ml) are significantly higher than in patients that survive (25.2 ng/ml) (Gibot et al., 2007). In another study, HMGB1 levels were found to be significantly higher in patients with severe acute pancreatitis (13.33±2.11 ng/ml) than in healthy controls (0.16±0.03 ng/ml) or than in patients with mild pancreatitis (2.64±0.19 ng/ml) (Kocsis et al., 2009). Goldstein et al. (2006) also found that plasma levels of HMGB1 are elevated by up to 10-fold in stroke patients, which adds to the pathological relevance of HMGB1 in stroke. Since extracellular HMGB1 from various cellular origins plays a role in aggravating brain damage in the postischemic brain (Kim et al., 2006; Muhammad et al., 2008; Qiu et al., 2008; Yang et al., 2010), inhibiting HMGB1 functions or blocking certain step along the HMGB1 secretory pathway could provide important tools for alleviating postischemic brain damage.

HMGB1 as a therapeutic target

Various approaches to modulate HMGB1 function have been attempted and many of them have conferred protective effect. In the postischemic brain, short hairpin (sh)RNA-mediated HMGB1 downregulation suppressed infarct size, microglia
activation, and expression of proinflammatory cytokines (Kim et al., 2006). Recently, administration of HMGB1 A box, which is known to be a functional peptide antagonist of full-length HMGB1 protein, remarkably reduced ischemic brain infarct volumes, functional neurological deficits, and proinflammatory cytokine inductions (Jin et al., 2011). Treatment with anti-HMGB1 antibody also conferred inhibitory effect to angiogenesis in chorioallantonic membrane of the chick embryo (van Beijnum et al., 2006). Furthermore, Toll-like receptor 4 (TLR4, a receptor of HMGB1)-defective mice exhibited less damage in hepatic injury after liver ischemia-reperfusion (Tsung, et al., 2005b). These observations are in line with the previous report that HMGB1−/− necrotic cells have a significantly reduced ability to induce proinflammatory cytokines (Scaffidi et al., 2002).

**Inhibition of HMGB1 signaling by EP**

Regarding the suppression of HMGB1 secretion, many studies have reported that EP improves survival and attenuates damage in various animal disease models via inhibition of HMGB1 (Su et al., 2011; Hu et al., 2011; Dave et al., 2009; Wang et al., 2009; Yang et al., 2009b; Yang et al., 2008; Fink, 2007; Ulloa et al., 2002). Wang et al. (2009) reported that administration of EP at 30 min before ischemia or 6 hr after reperfusion significantly attenuated motor functional deficit and reduced the number of apoptotic neurons through inhibiting HMGB1 release. In experimental
severe acute pancreatitis (SAP) model in rats, delayed EP treatment protected animals against organ injury and prolonged survival time via reducing serum levels of HMGB1 (Yang et al., 2008). Similarly, EP administration inhibited HMGB1 release from hepatic tissue of mice with SAP and decreased hepatic local inflammation and liver injury (Yang et al, 2009b). In mice with LPS-induced acute lung injury, EP treatment reduced the lung permeability index and improved survival through inhibiting the release of HMGB1 (Shang et al., 2009). These capacities of EP, i.e., inhibiting HMGB1 release or secretion, serve as an effective therapeutic means against inflammatory response, but the mechanisms are still not well known.

**EP-mediated Ca^{2+}-chelation inhibits HMGB1 release from activated microglia**

Increase of the intracellular calcium level is a major initiative factor of ischemic cell death (Dirnagl et al., 1999) and has been known to be closely associated with HMGB1 secretion (Tsung et al., 2007). Recently, two calcium dependent kinase, cPKC and CaMK IV, have been shown to be involved in nucleocytoplasmic shuttling of HMGB1 (Oh et al., 2009; Zhang et al., 2008). Surge of cPKC in the nuclei began to be detected as early as 5 min after LPS treatment and peaked at 20 min in RAW264.7 cells (Oh et al., 2009) and CaMK IV activation was observed at 15 min after LPS treatment in RAW264.7 cells (Zhang et al., 2008). In this study, it
was shown that EP-mediated Ca\textsuperscript{2+}-chelation inhibits HMGB1 release from activated microglia. Recent report showing reduced HMGB1 release by calcium chelation with BAPTA treatment agrees with the present result and further supports the notion that calcium chelation might be an important means to inhibit HMGB1 secretion (Tsung et al., 2007; Oh et al., 2009).

**Ca\textsuperscript{2+}-chelation mediated therapeutic approaches**

Since metal ions act as controllers for enzymes, co-factor, and cellular transporters (Green, 2008), chelation of metal ions has been investigated as an important tool to modulate cell death and survival in various pathological conditions (Angel et al., 2002). Calcium overload-induced mitochondrial dysfunction has been shown to trigger the cell death following ischemic, traumatic brain injury and several neurodegenerative diseases (Friberg and Wieloch, 2002; Starkov et al., 2004; Norenberg and Rao, 2007; Bezprozvanny, 2009; Gibson et al., 2010). It has been reported that 3-aminopyridine-2-carboxaldehyde thiosemicarbazone (PAN-811), which has been known to modulate calcium homeostasis, reduced infarct size by a modest 59% in a rat MCAO model and inhibited free radical production (Jiang et al., 2006a,b), suggesting that metal ion chelation might be an important therapeutic target for neuroprotection.
**Therapeutic potential of EP**

Together, these results reveal that EP may have therapeutic potentials for treating the disease related with oxidative and inflammatory pathways. EP appears to reduce oxidative stress-induced damages in various diseases, such as cancer, stroke, myocardial infarction, diabetes, and major disorders by inducing Nrf2 and up-regulating its down-stream genes. In addition, EP effectively inhibits HMGB1 release in microglia cells and alleviates inflammation. Actually, EP has been used as a nontoxic food additive. Although a recent clinical study failed to prove a therapeutic potential of EP in cardiopulmonary bypass (Fink, 2008), it is still believed that modifications of EP and/or combination treatment with other drugs may be able to improve therapeutic efficacy of EP. Finding paracrine function of EP for protecting neurons and other cell types will provide more beneficial effects of EP.
REFERENCES


Chung, K.Y., Park, J.J., Kim, Y.S., 2008, The role of high-mobility group box-1 in


group box 1 levels in patients with cerebral and myocardial ischemia. Shock 25, 571-574.


Kocsis, A.K., Szabolcs, A., Hofner, P., Takács, T., Farkas, G., Boda, K., Mándi, Y.,


Li, W., Yu, S.W., Kong, A.N., 2006. Nrf2 possesses a redox-sensitive nuclear exporting signal in the Neh5 transactivation domain. J. Biol. Chem. 15, 27251-27263.


Panahian, N., Yoshiura, M., Maines, M.D., 1999. Overexpression of heme
oxygenase-1 is neuroprotective in a model of permanent middle cerebral artery occlusion in transgenic mice. J. Neurochem. 72, 1187-1203.


IFN-γ induces high mobility group box 1 protein release partly through a TNF-dependent mechanism. J. Immunol. 170, 3890-3897.


Taniguchi, N., Kawahara, K., Yone, K., Hashiguchi, T., Yamakuchi, M., Goto, M.,
Inoue K., Yamada, S., Ijiri, K., Matsunaga, S., Nakajima, T., Komiya, S.,


Tsung, A., Kaizu, T., Nakao, A., Shao, L., Bucher, B., Fink, M.P., Murase, N.,


Tsung, A., Klue, J.R., Zhang, X., Jeyabalan, G., Cao, Z., Peng, X., Stolz, D.B.,
ischemia involves Toll-like receptor 4 dependent reactive oxygen species production an calcium-mediated signaling. J. Exp. Med. 204, 2913-2923.


Ethyl pyruvate attenuates spinal cord ischemic injury with a wide therapeutic window through inhibiting high-mobility group box 1 release in rabbits. Anesthesiology 110, 1279-1286.


Youn, J.H., Shin, J.S., 2006. Nucleocytoplasmic shuttling of HMGB1 is regulated
by phosphorylation that redirects it toward secretion. J. Immunol. 177, 7889-7897.


