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Mitogen-Activated Protein Kinases (MAPKs) Mediate SIN-1/ Glucose Deprivation-Induced Death in Rat Primary Astrocytes

Byoung Kwon Yoo, Ji Woong Choi, Min Sik Choi, Mi Kyoung Ryu, Gyu Hwan Park, Mi Jin Jeon, and Kwang Ho Ko

Department of Pharmacology, College of Pharmacy, Seoul National University, Seoul 151-742, Korea

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Peroxynitrite is a potent neurotoxic molecule produced from a reaction between NO and superoxide and induces NO-mediated inflammation under neuropathological conditions. Previously, we reported that glucose deprivation induced ATP depletion and cell death in immunostimulated astrocytes, which was mainly due to peroxynitrite. In this study, the role of MAPKs (ERK1/2, p38MAPK, and JNK/SAPK) signal pathway in the SIN-1/glucose deprivation-induced death of astrocytes was examined. A combined treatment with glucose deprivation and 50 μM SIN-1, an endogenous peroxynitrite generator, rapidly and markedly increased the death in rat primary astrocytes. Also, SIN-1/glucose deprivation resulted in the activation of MAPKs, which was significantly blocked by the treatment with 20 μM MAPKs inhibitors (ERK1/2, PD98059; p38MAPK, SB203580; JNK/SAPK, SP600125). Interestingly, SIN-1/glucose deprivation caused the loss of intracellular ATP level, which was significantly reversed by MAPKs inhibitors. These results suggest that the activation of MAPKs plays an important role in SIN-1/glucose deprivation-induced cell death by regulating the intracellular ATP level.

Key words: MAPKs, Glucose deprivation, Peroxynitrite, SIN-1, ATP, Rat primary astrocytes

INTRODUCTION

After onset of cerebral ischemia, astrocytes become activated by proinflammatory cytokines from infiltrating blood-borne immune cells (Hallenbeck *et al.*, 1986; Lees, 1993). Activation of astrocytes is associated with ischemic injury through the marked generation of neurotoxic molecules such as nitric oxide (NO) and superoxide anion (O_2^-, Choi *et al.*, 2004; Murphy S., 2000; Barone and Feuerstein, 1999). Moreover, peroxynitrite, a product from the reaction between NO and O_2^-, has been implicated as a potent mediator in the pathogenesis of cerebral ischemia (Bal-price and Brown, 2001; Bolanos *et al.*, 1995).

The increased generation of peroxynitrite results in several deleterious effects including inactivation of enzymes, lipid peroxidation, and DNA damage (Virag *et al.*, 2003). In addition, various signaling events may also contribute to modulate peroxynitrite-induced cytotoxicity (Virag *et al.*, 2003; Cross *et al.*, 2000). Recently, it has been reported that mitogen-activated protein kinases (MAPKs; ERK1/2, p38MAPK, and JNK/SAPK) are involved in the intracellular signaling events induced by peroxynitrite in various cell types (Shrivastava *et al.*, 2004; Saeki *et al.*, 2000; Jope *et al.*, 2000).

In our previous studies, we demonstrated that immunostimulated astrocytes rapidly undergo cell death during glucose deprivation, which is due to the massive production of peroxynitrite (Yoo *et al.*, 2005b; Choi and Kim, 1998). We also reported that the 3-morpholinosydnomine (SIN-1)/glucose deprivation-induced death of astrocytes was correlated with ATP depletion and mitochondrial transmembrane potential (MTP) loss (Ju *et al.*, 2000; Shin *et al.*, 2002). However, the role of MAPKs signal pathway in the peroxynitrite-induced cytotoxicity is not fully understood. In the present study, we investigated whether the MAPKs signal pathways were involved in the SIN-1/glucose deprivation-induced cytotoxicity of astrocytes.

MATERIALS AND METHODS

Materials

SIN-1 was purchased from Sigma (St. Louis, MO). Glucose-free Dulbecco's modified Eagle's medium

Correspondence to: Kwang Ho Ko, Department of Pharmacology, College of Pharmacy, Seoul National University, San 56-1, Shinlim-Dong, Kwanak-Gu, Seoul 151-742, Korea
Tel: 82-2-880-7848, Fax: 82-2-885-8211
E-mail: khk123@snu.ac.kr

(DMEM), DMEM/F12, and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY). Rabbit monoclonal antibodies against phospho-Tyr-ERK1/2 and polyclonal antibodies against ERK2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). PD98059, SB203580, and SP600125 were purchased from Calbiochem (La Jolla, CA). All other reagents were from Sigma (St. Louis, MO).

Culture of rat primary astrocytes and treatment of SIN-1/glucose deprivation

Rat primary astrocytes were cultured from prefrontal cortices of 2- to 4-day-old Sprague-Dawley rat pups as previously described (Choi *et al.*, 2004; Shin *et al.*, 2002). In brief, cells were dissociated by mild trypsinization (10 min at 37°C DMEM containing 0.1% trypsin) and passed through sterile nylon sieves (pore size, 80 μm). Cells were plated onto poly-L-lysine-coated 75 cm^2 culture flasks and maintained for 7 days in DMEM supplemented with 10% heat-inactivated FBS. Cells were replated onto poly-D-lysine-coated 24 or 6 well plates. After 5-7 days, cells were treated with 50 mM SIN-1 in glucose-free media or 5 μM glucose-containing media.

Measurement of lactate dehydrogenase (LDH)

Astrocytic cell death was monitored by measuring activity of LDH released into the medium, as described previously (Yoo *et al.*, 2005a; Shin *et al.*, 2001). Briefly, the media were mixed with NADH and pyruvate (0.1% wt/vol) and incubated at 37°C. Thirty minutes later, coloring reagent (Sigma, St. Louis, LO) was added and incubated for 20 min at room temperature. The reaction was stopped by adding 0.4 N NaOH, and the absorbance was measured at 450 nm. The LDH activity was calculated from the standard curve. The LDH amounts corresponding to the complete death were measured from the culture media treated with 0.1% Triton X-100 for 30 min at 37°C. Cell death was determined as a percent of LDH release from control.

Western blot analysis

Cells were washed twice with PBS and lyzed with 2 X SDS-PAGE sample buffers. An aliquot containing 20 μg of total protein was separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat dried milk in PBS including 0.2% tween-20 (BLOTTO) for 1 h. The membranes were incubated with antibodies against phospho-ERK1/2 (for the determination of activated ERK1/2) or ERK2 (for the determination of total level of ERK) overnight at 4°C and then with peroxidase-conjugated secondary antibodies for 2 h at room temperature. Specific bands were detected using the ECL system (Amersham, Buckinghamshire, U.K.).

Measurement of p38 MAPK and JNK/SAPK activity

p38MAPK and JNK/SAPK activity were measured using immunoprecipitation-based MAPK assay kits (Cell signaling, Beverly, MA). Briefly, cells were rinsed once with ice-cold PBS and lyzed by adding 1X ice-cold lysis buffer. After sonication, the supernatant which contains 400 μg of protein was incubated with 20 μL of immobilized phospho-p38MAPK antibody or phospho-JNK/SAPK antibody overnight at 4°C. After centrifugation, the pellet was supplemented with 200 μM ATP and 2 μM ATF-2 and c-JUN a substrate of p38MAPK and JNK/SAPK, respectively. The samples were loaded on SDS-PAGE gel and the activity of p38MAPK and JNK/SAPK were analyzed by Western blotting using a phospho-ATF-2 antibody and a phospho-c-Jun antibody, respectively.

Measurement of the intracellular ATP levels

The level of intracellular ATP was determined using luminescence-based method, as described previously (Choi *et al.*, 2004). In brief, cells were harvested with 10% trichloroacetic acid (TCA) and sonicated for 15 sec on ice. The lysates were supplemented with 2 mM EDTA and 2 mg/mL BSA. After centrifugation, the supernatants were adjusted to neutrality with 4 M KOH and the ATP content was determined using commercial assay kit (ATP luminescence detection kit, Molecular Probe, Eugene, OR).

Statistical analysis

Data were expressed as the mean ± standard error of mean (S.E.M) and analyzed for statistical significance by using one way analysis of variance (ANOVA) followed by Newman-Keuls test as a post-hoc test and a P value < 0.05 was considered significant.

RESULTS

The death of astrocytes was determined by measuring the amount of LDH released into the medium. In agreement with our previous report (Shin *et al.*, 2002), the combined treatment with glucose deprivation and 50 μM SIN-1, an endogenous peroxynitrite generator, rapidly and markedly enhanced cell death of rat primary astrocytes (Fig. 1). However, neither glucose deprivation nor the treatment with 50 μM SIN-1 alone induced death of astrocytes, which was similar to our previous report (Shin *et al.*, 2002).

To investigate whether MAPKs signal pathways are activated by SIN-1/glucose deprivation in astrocytes, cell extracts were analyzed for MAPKs activation. The activation of ERK1/2 was determined by Western blotting using an anti-phospho-ERK1/2 antibody detecting the activated form of ERK1/2 and the activity of p38MAPK and JNK/SAPK was monitored by immunoprecipitation-

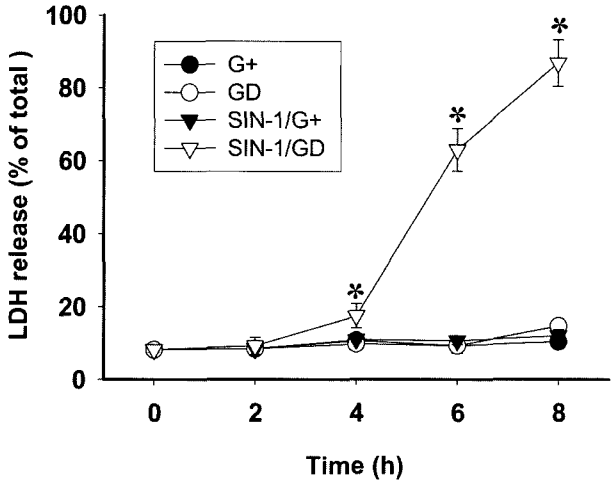

Fig. 1. Time course of LDH release in SIN-1/glucose deprivation-treated astrocytes. Cells were incubated in the absence (glucose deprivation, GD) and presence (G+) of 5 mM glucose in the presence or absence of SIN-1 (50 μM) for the indicated times. The amounts of LDH released into the medium from cells were measured as described in Materials and Methods. Data represent means ± S.E.M. (n=4). *Significant difference from control (unstimulated or time zero, $p<0.05$).

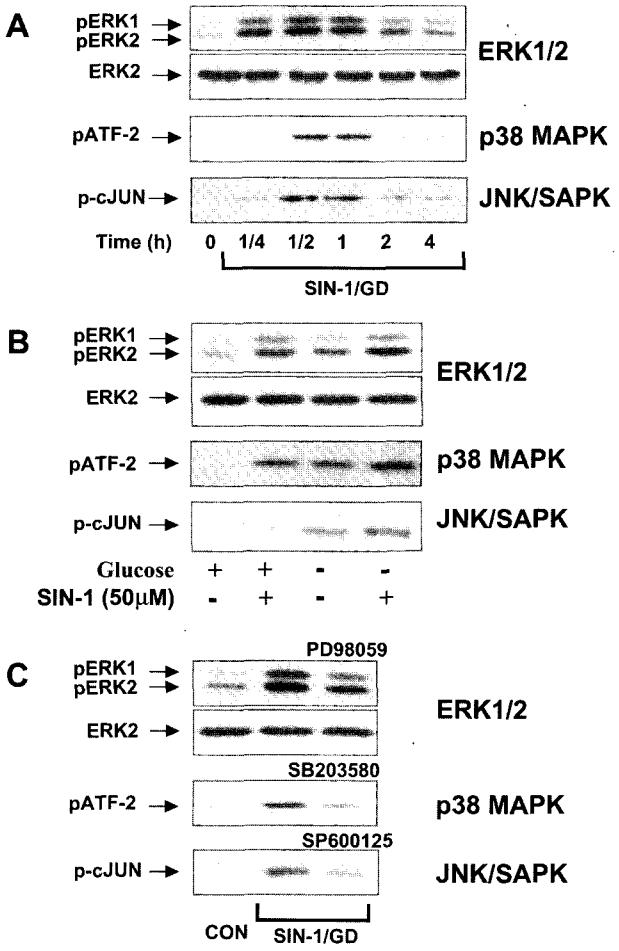

Fig. 2. Activation of MAPKs signal pathway (ERK1/2, p38 MAPK, and JNK/SAPK) in SIN-1/glucose deprivation-treated astrocytes. (A) Cells were exposed to the combined treatment with SIN-1 (50 μM) and glucose deprivation (GD) for the indicated times. (B) Cells were incubated with glucose deprivation (GD), SIN-1 (50 μM), or a combination of glucose deprivation (GD) and SIN-1 (50 μM) for 1 h. (C) Cells were exposed to the combined treatment with SIN-1 (50 μM) and glucose deprivation (GD) in the presence or absence of inhibitors, i.e., ERK inhibitor PD98059 (20 μM), p38 inhibitor SB203580 (20 μM), and JNK inhibitor SP600125 (20 μM) for 1 h. Cell extracts were subjected to Western blot analysis by using a specific antibody against active form of ERK1/2, and immunoprecipitation-linked kinase assay for the p38MAPK and JNK/SAPK activity using activity assay kits as described in Materials and Methods. Data are representative of three independent experiments.

based kinase assay (Fig. 2). After treatment with SIN-1/glucose deprivation, the phosphorylated forms of ERK1/2 were detected within 15 min and the maximum activity was observed between 30 min and 1 h and sustained up to 6 h (Fig. 2A). SIN-1/glucose deprivation-induced ERK1/2 activation in astrocytes was inhibited by PD98059, a specific inhibitor of ERK1/2 upstream kinase (Fig. 2C). The activity of p38MAPK and JNK/SAPK was reached a maximum between 30 min and 1 h and thereafter declined (Fig. 2A). The treatment with specific inhibitors for p38MAPK (SB203580) or JNK/SAPK (SP600125) blocked these activations (Fig. 2C). To examine whether the SIN-1/glucose deprivation-induced death of astrocytes is caused by the increased activation of MAPKs signal pathway, we examined the ability of SIN-1/glucose deprivation to activate MAPKs signal pathway in astrocytes compared with SIN-1-treatment or glucose deprivation alone. As shown in Fig. 2B, the combined treatment with SIN-1 and glucose deprivation more increased the activity of MAPKs signal pathway compared with SIN-1 treatment or glucose deprivation alone.

To determine whether MAPKs signal pathway is involved in the SIN-1/glucose deprivation-enhanced death, astrocytes were exposed to SIN-1/glucose deprivation in the presence of PD98059, SB203580 or SP600125. As shown in Fig. 3, SIN-1/glucose deprivation-enhanced cell death was partially but significantly blocked by MAPK inhibitors.

In our previous report, glucose deprivation caused ATP depletion occurred ahead of cell death in LPS/IFN-γ-treated primary astrocytes *via* the production of peroxynitrite (Yoo *et al.*, 2005b). We also reported that ATP depletion and cell death occurred only in SIN-1/glucose deprivation-treated astrocytes (Shin *et al.*, 2002). The combined treatment with SIN-1 and glucose deprivation significantly decreased intracellular ATP levels in astrocytes (Fig. 4). However, similar to our previous report (Shin *et al.*, 2002), neither glucose deprivation nor SIN-1 treatment alone significantly altered ATP levels of astrocytes in all time

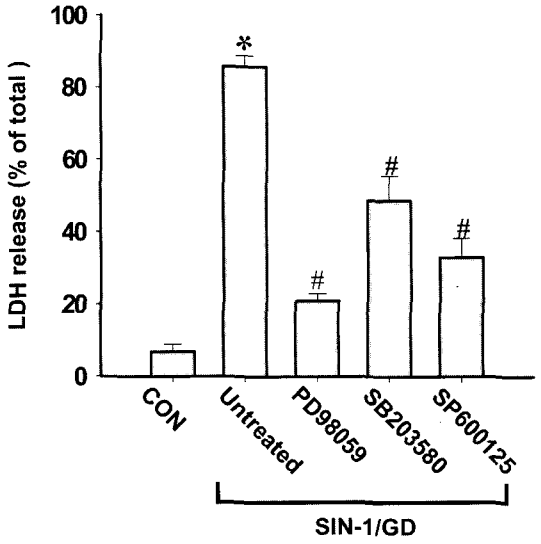

Fig. 3. Effect of MAPK inhibitors on the SIN-1/glucose deprivation-induced death of astrocytes. Cells were exposed to the combined treatment of SIN-1 (50 μM) and glucose deprivation (GD) in the absence or presence of PD98059 (20 μM), SB203580 (20 μM), or SP600125 (20 μM). Eight hours later, cell death was determined by measuring the amounts of LDH released into the medium from cells. *Significant difference from untreated cells (CON, $p<0.05$). #Significant difference from the SIN-1/glucose deprivation-treated cells (SIN-1/GD, $p<0.05$).

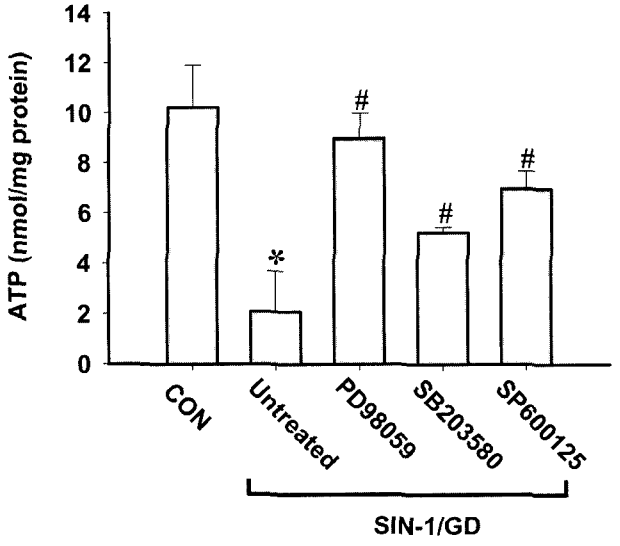

Fig. 4. Effects of MAPK inhibitors on the SIN-1/glucose deprivation-induced ATP depletion of astrocytes. Cells were exposed to the combined treatment of SIN-1 (50 μM) and glucose deprivation (GD) in the absence or presence of PD98059 (20 μM), SB203580 (20 μM), or SP600125 (20 μM). Five hours later, cellular ATP levels were determined using a chemiluminescence detection kit. *Significant difference from untreated cells (CON, $p<0.05$). #Significant difference from the SIN-1/glucose deprivation-treated cells (SIN-1/GD, $p<0.05$).

points examined (data not shown). Thus, we addressed that MAPKs activation by the combined treatment with SIN-1 and glucose deprivation mediated intracellular ATP depletion. The treatment with specific inhibitors of MAPKs (20 μM) reversed the SIN-1/glucose deprivation-induced ATP depletion (Fig. 4).

DISCUSSION

The present study was aimed to investigate the involvement of MAPKs signal pathway in the SIN-1/glucose deprivation-induced death of astrocytes. Our data suggest that the combined treatment with SIN-1 and glucose deprivation accelerates the death of astrocytes, which is mediated by MAPKs signal pathways (i.e., ERK1/2, p38MAPK, and JNK/SAPK). First, three major MAPKs were activated in glucose deprivation/SIN-1-treated astrocytes. Second, the augmented cell death was blocked by specific inhibitors of these MAPKs. Third, the inhibitory effects of MAPK inhibitors on cytotoxicity for astrocytes were dependent on restoration from the depletion of intracellular ATP level.

In various cell types including neurons, it has been reported that peroxynitrite could lead to cell death *via* the activation of MAPKs signal cascade (Shrivastava *et al.*, 2004; Saeki *et al.*, 2000; Jope *et al.*, 2000). In general, astrocytes are more resistant to peroxynitrite than neurons because astrocytes have a superior ability for glycolysis and antioxidant system than neurons (Bolanos *et al.*, 1995; Lisasoain *et al.*, 1996). In some cases, astrocytes were also damaged by the treatment with SIN-1 (above 200 μM), a peroxynitrite releasing reagent (Muyderman *et al.*, 2004; Ying *et al.*, 2005). However, in this study, 50 μM SIN-1 did not induce any cell death, but the combind treatment of SIN-1 and glucose deprivation induced a marked cell death (Fig. 1). Similarly, 8 h glucose deprivation alone did not induce cell death, which is coincident with our previous reports (Shin *et al.*, 2002; Yoo *et al.*, 2005b). Interestingly, the treatment with 50 μM SIN-1 or glucose deprivation alone activated MAPKs pathway. Furthermore, the combined treatment of astrocytes with SIN-1 and glucose deprivation more activated MAPKs pathway than the treatment with SIN-1 or glucose deprivation alone, and an inhibition of MAPKs by their inhibitors significantly reduced the combined treatment of SIN-1 and glucose deprivation-induced death of astrocytes. Nebeyrat *et al.* (2003) reported that MAPKs signal pathway (ERK1/2, p38 MAPK, and JNK/SAPK) in hepatocytes were activated by SIN-1 in a concentration-dependent manner, which correlated with cell death. Only when MAPKs signal pathway were potently activated, hepatotoxicity occurred. The results from our study also indicate that the combined treatment with SIN-1 and glucose deprivation-induced cell death is mediated by MAPKs signal pathway and may be dependent on the level of MAPKs activation in astrocytes, too.

Maintenance of cellular ATP levels is very important in

cell survival, and the decrease in cellular ATP levels is one of main factors for the induction of cell death (Erecinska and Silver, 1989; Waterhouse *et al.*, 2001). In this study, exposure of astrocytes to the combined treatment with SIN-1 and glucose deprivation caused the decrease of ATP levels in astrocytes. In our recent studies, ATP loss occurred ahead of cell death, and the restoration of cellular ATP level is enough to block cell death (Shin *et al.*, 2002; Yoo *et al.*, 2005b). However, it is not clear whether the changes of cellular ATP levels are associated with the activity of MAPKs. In this study, the combined treatment with SIN-1 and glucose deprivation-induced ATP loss was prevented by MAPKs inhibitors (PD98059, SB203580, and SP600125), which indicates that the activation of MAPKs signal pathway induced cell death by depleting cellular ATP contents. Only when MAPKs signal pathway are potently activated, ATP depletion occurred. These findings indicate that similar to the case of cell death, ATP loss may also be dependent on the level of MAPKs activation. Serveral reports indicated that MAPKs signal pathway is involved in the regulation of mitochondrial function. Loss of mitochondrial potential under various conditions inducing cell death was prevented by inhibitors of MAPKs signal pathway (Kim *et al.*, 2004; Lee *et al.*, 2005; Myatt *et al.*, 2005). Since the normal function of mitochondria is essential to keep the intracellular ATP level, the restoration of cellular ATP levels by the inhibition of MAPKs signal pathway is most likely due to the blockade of mitochondrial dysfunction in astrocytes.

In conclusion, the present study demonstrated that an overactivation of three major MAPKs signal pathway by the combined treatment with SIN-1 and glucose deprivation induces ATP depletion and thereby cell death of astrocytes. Stress such as SIN-1/glucose deprivation can occur under the circumstances of ischemic brain. In ischemic brain, the activation of MAPKs signal pathway may play a role not only in the production of inflammatory mediators but also the subsequent progression of brain injury. The findings from the present study may furnish the evidences that targeting of MAPKs is useful for a neuroprotective therapy against ischemic brain injury.

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