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Anti-inflammatory and Neuroprotective Effects of Magnolol in Chemical Hypoxia in Rat Cultured Cortical Cells in Hypoglycemic Media

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Abstract

Our previous studies demonstrated that magnolol protects neurons against chemical hypoxia by KCN in cortical neuron-astrocyte mixed cultures (14). In the present study, we examined whether the neuroprotective effect of magnolol involve modulating inflammatory mediators, prostaglandin E_2 (PGE_2) and nitric oxide (NO), induced by KCN (hypoxia) or KCN plus lipopolysaccharide (LPS). In glucose-absent (hypoglycemia) media, KCN or KCN plus LPS induced increases in lactate dehydrogenase (LDH) activity by 32% and 34%, and PGE_2 production by 12% and 32%, respectively. Both LDH and PGE_2 increases were suppressed by 100 μM magnolol. In addition, although KCN or LPS alone did not increase NO generation, KCN plus LPS increased NO generation. This increase was reduced by 100 μM magnolol or 10 μM L-NAME, but the LDH increase and PGE_2 production were not reduced by L-NAME. These findings suggest that the protective effects of magnolol against brain damage by KCN or KCN plus LPS in hypoglycemic media may involve inhibition of PGE_2 production, but inhibition of NO generation may not be important.

Key Words: hypoxia, magnolol, lactate dehydrogenase, cortical mixed culture, lipopolysaccharide, nitric oxide, prostaglandin E_2

Introduction

The mechanisms of neuronal degeneration following hypoxia/ischemia may involve calcium homeostasis (9), neurotransmitter release (10), oxidative stress (20), and inflammation (4). Models of chemical hypoxia are useful for exploring the molecular mechanisms of neurodegeneration (9). Potassium cyanide (KCN), involved in the disturbance of electron transport and the blockade of respiration (13), is known to produce irreversible changes in neurons (31). Its blockade of the respiratory electron transport chain is similar to that produced by hypoxia. Thus, cyanide intoxication has often been used as a model of chemical hypoxia which causes cell hypoxia and dysfunction leading to cell injury or death. Hence, this model becomes a convenient method for studying the action of drug effects in neuronal cultures (9, 10, 14).

Various products of the arachidonate cascade, most commonly PGE_2, have been implicated in inflammatory processes of the brain and neuronal injuries, including those induced by chemical hypoxia (2, 31), cerebral ischemia (1, 6, 32) or lipopolysaccharide (LPS) (22, 26). Furthermore, nitric oxide (NO) has also been involved in inflammatory processes (5, 21). Lipopolysaccharide (LPS), a bacterial endotoxin, has been used extensively to study the production of NO in glial cells (30) and prostaglandin (PG) production in rat astrocytes (24).

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Magnolol is an active component of *Magnolia officinalis*. In in vivo study, it can reduce the size of rat heart infarct caused by ischemia/reperfusion (11). In *in vitro* study, it can protect rat heart mitochondria against lipid peroxidation (16). It can reduce both prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4) levels in the pleural fluid of A23187-induced pleurisy (28). Our recent studies demonstrated that it effectively protected cells against chemical hypoxic injury or necrotic cell death in cortical neuron-astrocyte mixed cultures (14). The present experiments using the same cultures further investigated whether magnolol protects against neuronal injuries through modulation of the inflammatory mediators (PGE_2 and NO) evoked by either KCN, LPS, or KCN plus LPS.

Materials and Methods

Chemicals

Magnolol (Yoneyama Company, Osaka, Japan) was prepared as stock solutions in DMSO at a concentration of 100 mM and stored at -20°C. The final working concentration of magnolol was 100 μM dissolved in DMEM-Hepes with 0.1% DMSO. Chemicals were purchased from the indicated companies: DMSO, poly-D-lysine, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes), lipopolysaccharide (LPS) (*E. coli* 055:B5), D-(L)-glucose, boric acid, penicillin, streptomycin, microtubule associated protein-2 (MAP-2) and NG-nitro-L-arginine methyl ester dihydrochloride (L-NAME) from Sigma Chemical Co. (St. Louis, MO, USA); Dulbecco's modified Eagle's (DME) medium, neurobasal and B27 supplement from GibcoBRL (Grand Island, NY, USA); lactate dehydrogenase (LDH) standard and lactate dehydrogenase kits from Boehringer Mannheim (Germany); and potassium cyanide (KCN) from Merck Chemicals (Darmstadt, Germany).

Cell Culture

Cultured cells were prepared from cerebral cortices of 1-day-old Sprague-Dawley rats as described previously (14). Cells were plated on poly-D-lysin-coated (3 μg/ml in 0.15 M borate buffer; pH 8.1) 24-well plates (Falcon) at a seeding density of 4×10^5/dish and onto 96-well plates (Falcon) at a seeding density of 1×10^5/well, and incubated at 37°C in a humidified incubator with 5% CO_2. After seeding for 2 hours, the culture media were replaced with neurobasal supplemented with B27 containing penicillin/streptomycin (PS), 0.5 mM glutamine, and 25 μM glutamate. On the fourth day *in vitro*, the media were changed and replaced with fresh neurobasal with B27. The cells were then further cultured for another 10 days prior to the experiments.

Immunostaining

On the day of the experiments, the cells were treated with vehicle (hypoglycemia) or 0.5 mM KCN (hypoxia) or KCN plus 100 μM magnolol in DMEM media containing 20 mM Hepes (glucose-free) for 2 hours. At the indicated time, the culture media were aspirated, the cells were washed twice with phosphate-buffered saline (PBS) (0.1 M Na_2HPO_4, 0.1 M NaH_2PO_4, 0.9% NaCl), fixed with 4% paraformaldehyde in phosphate buffer (PB) (0.1 M Na_2HPO_4 and 0.1 M NaH_2PO_4) for 10 minutes, and then the following procedures were performed as described in our previous report (14). The neuronal cells were characterized by immunostaining with the specific monoclonal antibody against MAP-2 (titer 1: 400).

Cytotoxicity Assessment

Cytotoxicities, as indicated by cell membrane integrities, were assessed by measuring the activity of LDH in the culture media by colorimetric detection of formazan, using a commercially available LDH diagnostic kit (Boehringer Mannheim), as a previous report (14). On the day of the experiments, the culture media were removed, and the cells were incubated with 0.5 mM KCN or KCN + 100 ng/ml LPS or 100 μM magnolol + KCN + LPS or 10 μM L-NAME + KCN + LPS in DMEM-free glucose with 20 mM Hepes media for the indicated time periods (30, 60, or 120 minutes). After incubation, the media were collected and centrifuged at 14000× g for 5 minutes. 50 μl of cell-free supernatants were transferred to a microtiter plate, then incubated with reaction mixture for 30 minutes at room temperature to develop color. The optical density was measured with a spectrophotometer (MRX, Dynatech Laboratories) at 490 nm, and the reference wavelength of 630 nm. An LDH standard curve was constructed and the activities of LDH were determined. The LDH activities were expressed as milliUnits per milliliter (mU/ml) of LDH.

Prostaglandin E2 Determination

Prostaglandin E_2 (PGE_2) was determined using a commercially available immunoassay kit (R&D Systems). Cultured cells were washed twice with fresh DMEM-Hepes media, and PGE_2 was conducted by adding 0.5mM KCN or KCN + 100 ng/ml LPS or 100 μM magnolol + KCN + LPS or 10 μM L-NAME

+ KCN + LPS in DMEM-Hepes media for the indicated time periods (30, 60 and 120 minutes). After incubation, the culture media were collected and centrifuged at 14000× g for 5 min at 4°C; 100 µl of supernatants were transferred to a microtiter plate and incubated with reaction reagents (including 50 µl of PGE_2 conjugate and 50 µl of PGE_2 antibody solution) for 2 hours at room temperature on a horizontal orbital microplate shaker (set at 500±50 rpm). Each well was then washed three times, and after the last wash any remaining wash fluid was removed and cells were incubated by adding developing color substrate to each well for 45 minutes at room temperature. The reaction was stopped by adding 50 µl of stop solution. A standard curve was constructed and the reaction color was read at 405 nm using a spectrophotometer (MRX, Dynatech Laboratories), with a reference wavelength of 630 nm. The PGE_2 in unknown samples was determined by comparison with the known standards curve. Data were expressed as picograms per milliliter (pg/ml) of PGE_2.

Nitric Oxide Measurement

Cayman's Nitrate/Nitrite Assay kits were used for the measurement of total Nitric oxide (NO) production (Cayman Chemical Company, Ann Arbor, MI, USA. Sensitivity : nitrite > 1.0 µM). After treatment, 80 µl of cell supernatants were transferred to a microtiter plate, and 10 µl of nitrate reductase reagent and enzyme co-factor were added. The plate was then incubated at room temperature for 2 hours. The Griess reagent was added, then the color was developed, and the optical density of each sample was read at 540 nm using a spectrophotometer (MRX, Dynatech Laboratories). The nitrite in unknown samples was determined by comparison with the known nitrate standard curve and data were expressed as microMolar (µM) of nitrite.

Statistical Analysis

Data were reported as mean±s.e.m. values from the indicated number of cultures. For single comparisons, the statistical significance between means was determined by Students *t*-test. For multiple comparisons, data were analyzed by one-way analysis of variance (ANOVA). A level of $P<0.05$ was considered statistically significant.

Results

Immunochemical characterization of the culture 14 days after seeding revealed a composition of 30% astroglial cells and 70% neuronal cells, as described previously (14). Cortical cultured cells were treated with chemical hypoxia by KCN (0.5 mM) for 2 hr and the neuronal cells using immunochemical staining against neuronal marker (MAP-2) were examined. The results, as shown in Fig. 1, revealed that the neuronal loss and impairment of neuronal network

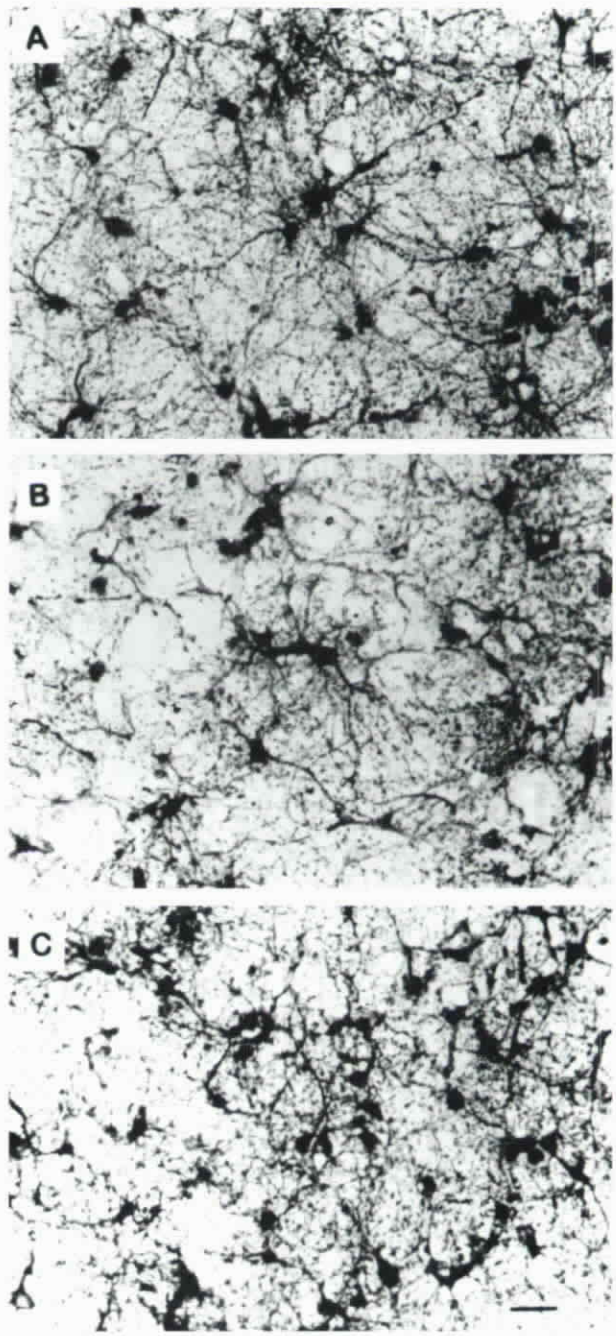

Fig. 1. Cell morphology of mixed cortical cultures after 2-hr treatment with vehicle (A), KCN (B), and KCN plus magnolol (C) in glucose-free media. Primary mixed culture cells were grown in neurobasal containing B27 and PS for 14 days. The seeding density was 2.0×10^5 cells/well at chamber slide. Fixed 14-day-old cells were examined for MAP-2-positive neurons. Bar = 50 µm.

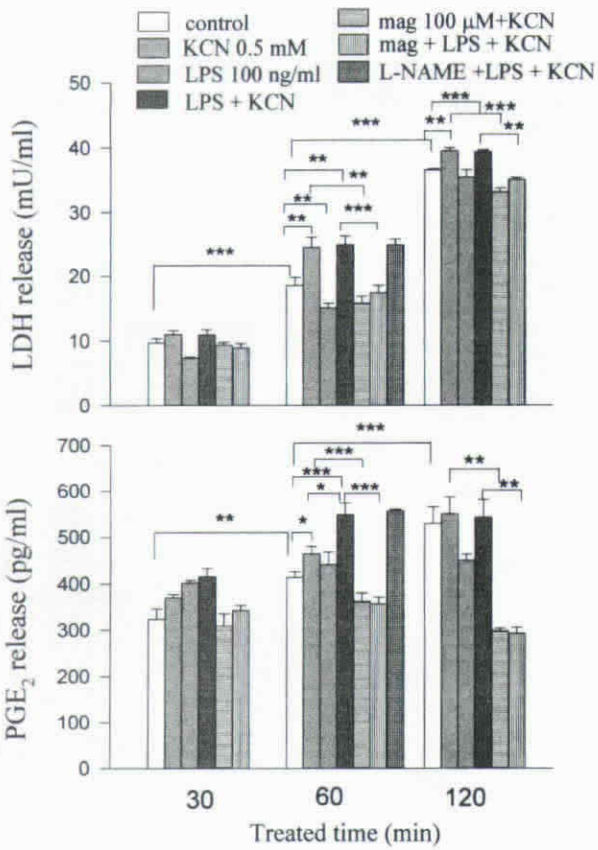

Fig. 2. Effects of magnolol (mag) on LDH and PGE_2 release induced by KCN, and LPS + KCN in glucose-free culture media. Cells were seeded on 24-well plates. Bars indicate LDH and PGE2 values (mean±SEM, n=8) in the media after 30, 60 and 120 min without treatment (control), with KCN, LPS, LPS + KCN, mag + KCN, mag + LPS + KCN. *P<0.05, **P<0.01, ***P<0.001 by Students t-test following one-way ANOVA.

after KCN treatment (1B), and the neurotoxicity was attenuated by the presence of magnolol (1C).

Cortical cells were treated with KCN (0.5 mM), LPS (100 ng/ml), magnolol (100 μM), L-NAME (10 μM) or their combination for various time periods, and the cell toxicity and prostaglandin cascade were evaluated by LDH release and PGE_2 production, respectively. The results, as shown in Fig. 2, were that LDH and PGE_2 releases were time-dependently increased in the control (hypoglycemic media). Treatments with drugs for 30 minutes caused no significant LDH or PGE_2 changes as compared with the control. Treatment with KCN 0.5 mM alone for 60 and 120 minutes induced significant increases in LDH release (32%, $p<0.01$ and 8%, $p<0.01$, respectively), and PGE_2 release (12%, $p<0.05$ and 4%, $p>0.05$, respectively). In contrast, treatment with LPS 100 ng/ml alone for 60 and 120 minutes caused a reduction in LDH release (19%, $p<0.01$ and 3%, $p>0.05$, respectively), but no significant change in PGE_2 release. Despite the opposite effects of KCN and LPS on LDH release, treatment with KCN plus LPS for 60 and 120 minutes still increased LDH release to a level similar to KCN treatment. Nevertheless, treatment with KCN plus LPS for 60 minutes potentiated PGE_2 increase (21% vs. 12% and 19% vs. 6% as compared with KCN or LPS alone, respectively), but treatment for 120 minutes did not enhance PGE_2 increase. When magnolol was added, the LDH increase induced by KCN treatment for 60 and 120 minutes was reduced by 35% and 16%, respectively. On the other hand, the PGE_2 increases induced by KCN treatment for 60 minutes and 120 minutes were reduced by 26% and 57%, respectively. Since PGE_2 release was not significantly affected by LPS treatment alone for 60 or 120 minutes, the effects of magnolol on LPS were not tested. Certainly, LDH and PGE_2 increases by KCN plus LPS for 60 and 120 minutes were also reduced by 30% and 11%, and 40% and 58%, respectively. Note that at 120 minutes, PGE_2 increases appeared to be not significantly different among the control (hypoglycemic media without drug treatment), KCN, and KCN plus LPS groups. This indicated that PGE_2 increases in the KCN and the KCN plus LPS groups were mainly attributable to the 120-minute hypoglycemic effect. Therefore, the results that magnolol significantly blocked PGE_2 production induced by KCN or KCN plus LPS treatment for 120 minutes may indicate magnolol was also able to block PGE_2 production induced by hypoglycemia. Since the above results revealed that an important time occurred at 60 minutes, L-NAME was added. LDH and PGE_2 increases by KCN plus LPS for 60 minutes were not significantly changed by L-NAME. These results may suggest that NO generation did not contribute to LDH release or PGE_2 production induced by KCN plus LPS.

To answer whether the NO pathway was involved in the inflammatory response by KCN or KCN plus LPS and in the neuroprotective effect of magnolol, we measured total NO production (indicated as nitrite content) to examine NO generation after the cells were treated with KCN, LPS, magnolol, 10 μM L-NAME (NOS nonselective inhibitor) or their combinations in hypoglycemic media for 30, 60, and 120 minutes. As shown in Fig. 3, KCN or LPS treatment alone for various time periods had no effect on NO production. However, KCN plus LPS treatments for 30, 60, and 120 minutes induced NO increase by 99%, 70%, and 87%, respectively, when compared with KCN treatment, and by 62%, 63%, and 77%, respectively, when compared with LPS treatment. These results indicated that interactions of KCN with LPS may have played a role in potentiation of NO generation. Furthermore, the NO increases induced by KCN plus LPS were attenuated by

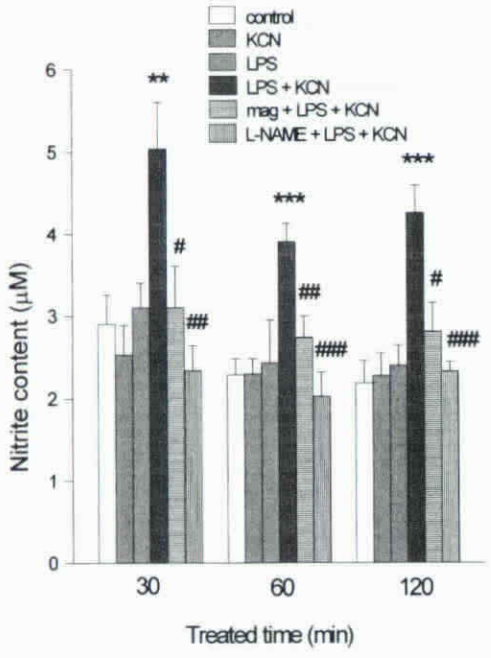

Fig. 3. Effects of magnolol (mag) and L-NAME treatment on KCN+LPS induced nitric oxide generation in glucose-free culture media. Cells were seeded on 24-well plates. Nitric oxide generation was indicated by extracellular nitrite (NO_2^-) content. Bars indicate mean±SEM (n=8). Asterisks indicate statistically significant. $^{**}P<0.01$, $^{***}P<0.001$ vs. KCN; $^{\#}P<0.05$, $^{\#\#}P<0.001$, $^{\#\#\#}P<0.001$ vs. LPS+KCN by Students t-test following one-way ANOVA.

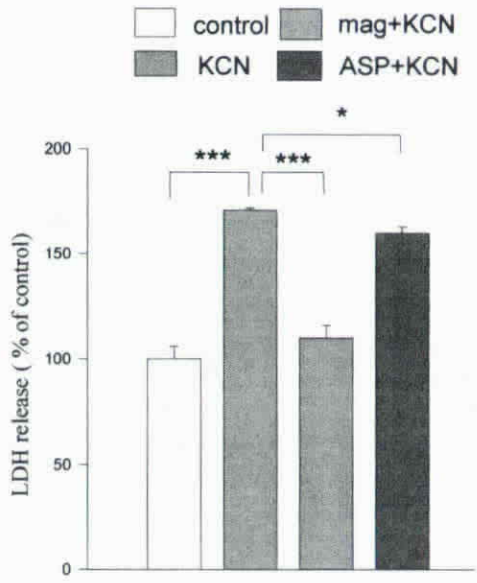

Fig. 4. Effects of 100 μM of magnolol (mag) and aspirin (ASP) on 0.5 mM KCN induced LDH release in glucose-free culture media for 120 min. Cells were seeded onto 96-well plates. LDH values are expressed as mean±SEM (n=5) in the media without treatment (control), with KCN, mag + KCN, ASP + KCN. Data are expressed as a percentage of control (% of control). $^{*}P<0.05$, $^{***}P<0.001$ by Students t-test following one-way ANOVA.

magnolol 100 μM and L-NAME 10 μM. Interestingly, although L-NAME attenuated the NO increase (Fig. 3), it did not attenuate the LDH and PGE_2 releases which were significantly attenuated by magnolol (Fig. 2). These results indicated that reductions in LDH and PGE_2 releases by magnolol may not be attributable to its ability to attenuate NO production.

To characterized whether the neuroprotective effect of magnolol was mediated by PGE_2 production, we further used an anti-inflammatory drug, aspirin, which involves inhibition of PGE_2 production through nonselective cyclooxygenase inhibition. 100 μM of aspirin reduced 6% of the LDH release induced by KCN ($p<0.05$ vs. KCN), but 100 (M magnolol reduced 35% of the LDH release ($p<0.001$ vs. KCN) in mixed cultured cells and DMEM-glucose free media (Fig. 4). These results indicated that the neuroprotective effect of magnolol can be attributed to its inhibition of PGE_2 production through inhibition of arachidonate cascade.

Discussion

The present findings clearly demonstrated that in hypoglycemic media, magnolol 100 μM significantly reduced LDH and PGE_2 increases induced by either KCN (hypoxic stress) or KCN plus LPS (inflammatory stimulation), and also significantly reduced NO generation by KCN plus LPS. These findings suggested that magnolol may protect against neuronal injuries (reduced LDH release) induced by hypoxic stress and/or inflammatory stimulation and this protective effect may involve inhibition of the production of PGE_2, but not NO, by KCN and LPS.

Chemical hypoxia mediated by cyanide results in a serious injury to neuronal cells (14, 20), including inflammatory mediator release (4, 15) and free radical generation (3, 17). Products of the arachidonate cascade, particularly PGE_2, have been indicated to be involved in the genesis of brain inflammation (8). These findings suggest that the increase in PGE_2 by hypoxia is an important element resulting in cell death. This notion is supported by our present findings that KCN (hypoxic stress) or KCN plus LPS (inflammatory stimulation) induced an increase in LDH release concomitant with an increase in PGE_2 release. However, the results that LPS treatment for 60 minutes significantly reduced LDH release (Fig. 2) as compared with the control (hypoglycemic media with vehicle) suggested that LPS may protect cells from hypoglycemic injury, although LPS did not prevent the LDH increase by KCN (Fig. 2, KCN + LPS vs. KCN). Our present results agree with the results of Dawson et al. (23) that LPS may protect against ischemic injury induced by middle cerebral artery occlusion. Their findings inferred that the

inflammatory response induced by LPS may play a role in protecting cells. However, our results were different from other studies in which LPS was capable of stimulating human monocytes or rat liver cells to release LDH (7, 12, 18).

In the present experiments, the NO generation was not induced simply by KCN or LPS alone, but was significantly enhanced by LPS plus KCN (Fig. 3). The results suggested that hypoxic stress or an inflammatory stimulus alone may not be able to induce NO generation, but combinations of hypoxic stress and inflammatory stimuli may interact with each other to enhance NO synthesis. Alternatively, chemical hypoxia may provide a novel signal to promote NO synthesis during the state of inflammation induced by LPS stimulation in rat mix cortical cultured cells.

The NO pathway is involved in the hypoxic inflammatory response (19). Reaction of NO with superoxide free radicals may form peroxynitrite (ONOO−) to cause cell injury (25). Thus, inhibition of NO production by magnolol in the present findings may explain the neuroprotective effect of magnolol. Other studies implied that magnolol may inhibit cytotoxicity, as indicated by the inhibition of LDH release (14), via inhibition of hydroxyl radical production (27, 29). However, it should be noted that combination of KCN and LPS significantly increased NO production and reached a plateau as early as 30 minutes after treatment (Fig. 3), while LDH release was increased with time (Fig. 2). Furthermore, NO was essentially not increased by treatments with vehicle in the hypoglycemic media (control) for 30, 60, and 120 minutes (Fig. 3), while LDH release (cell injuries) was time-dependently increased by the same treatments (Fig. 2). In other words, the severity of cell injury was not correlated with NO generation.

It was reported that magnolol protected the cell against lipid peroxidation in rat heart mitochondria (16) and reduced infarct size. It suppressed ventricular arrhythmia in rats subjected to coronary ligation (11). Magnolol was shown to exert anti-inflammatory effects on mouse hindpaw edema, and could reduce PGE_2 and LTB4 levels stimulated by A23187 in the pleural fluid (29). Our recent study demonstrated that magnolol, in addition, was able to protect cortical neuronal cells from chemical (KCN) hypoxic injuries (14). The present study further demonstrated that its neuroprotective effect may involve inhibition of PGE_2 production induced by hypoxic stress (KCN) or/and inflammatory stimulation (LPS), but inhibition of NO production may not be important. Nevertheless, the cellular mechanism by which magnolol protects the cells from hypoxic injuries remains further investigation.

Acknowlegdgments

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