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3- Nitropropionic acid로 유도된 신경손상에 대한 magnolol의 보호효과

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지도 교수 김 용 식

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의학과 약리학전공
이 가 희

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Protective effect of magnolol against 3- nitropropionic acid -induced neuronal damage

by

Ga Hee Lee

A Thesis Submitted to the Department of Pharmacology in partial Fulfillment of the Requirements for the Degree of Master of Philosophy in Medicine (Pharmacology) at Seoul National University College of Medicine

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Approved by Thesis Committee:

Professor ________________________ Chairman

Professor ________________________ Vice chairman

Professor ________________________

Abstract

3-Nitropropionic acid (3-NP), an irreversible complex II inhibitor of the electron transport chain, causes motor and cognitive deficits that are associated with excitotoxicity and excessive free radical generation. Recently, antioxidants have been implicated as a neuroprotection in the treatment of various neurological disorders. Magnolol, the major bioactive constituents of the bark of *Magnolia officinalis,* has been reported to possess various biological activities, such as anti-carcinogenic, anti-inflammatory and antioxidant activity. In this study, it was examined whether magnolol can protect neuronal damage induced by 3-NP in cultured cells and mouse brains.

When SK-N-SH cells were treated with 3-NP, decrease of cell viability, condensation and fragmentation in a view of nuclear morphology and increase of caspase-3 activity were shown. But pretreatment with magnolol protected cell death induced by 3-NP. And magnolol effectively reduced ROS generation, protein carbonylation induced by 3-NP. Change of HO-1 expression was also checked which is as an oxidative stress marker in this experiment. Magnolol inhibited 3-NP-induced increase in HO-1 expression. In *in vivo* study, lesion was induced in the right striatum of mouse by stereotaxic injection of 3-NP. Four days after the 3-NP injection, large lesions and extensive neuronal damages were produced in 3-NP-injected striata. The degree of neural damage was also confirmed by 3-NP-induced reduction of striatal GAD (glutamic acid decarboxylase) and TH (tyrosine hydroxylase) proteins. However, magnolol treatment significantly attenuated 3-NP-induced lesion volume and restored GAD and TH immunoreactivity in lesioned striatum. Also intrastriatal injection of 3-NP significantly increased level of lipid peroxidation, protein carbonylation and HO-1 expression in lesioned striatum 1 day after 3-NP injection. But all of these parameters were decreased in magnolol treated mice compared to 3-NP-injected mice.

These results suggest that magnolol protects 3-NP-induced neuronal damage in cultured cells and mouse brains by antioxidant action. Also these findings support that magnolol may be a potential candidate in preventing or reducing the metabolic impairment related disorders.

Keywords: 3-Nitropropionic acid (3-NP), Neuronal damage, Oxidative stress, Antioxidant, Magnolol

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ABBREVIATIONS

3- NP: 3-Nitropropionic acid

ROS: reactive oxygen species

HO-1: heme oxygenase-1

GAD: Glutamate decarboxylase

TH: Tyrosine hydroxylase

PI: propidium iodine

EDTA: ethylenediaminetetraacetic acid

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

TTC: 2, 3, 5-triphenyltetrazolium chloride

DNPH: 2, 4-Dinitrophenylhydrazine

DTT: dithiothreitol

Introduction

3-Nitropropionic acid (3-NP) is a naturally occurring fungal toxin that is an irreversible inhibitor of succinate dehydrogenase (SDH) in the tricarboxylic acid cycle and electron transport chain complex II [1]. Systemic administration of the mitochondrial toxin 3-NP to rodents results in selective striatal lesions and serves as an experimental model of Huntington's disease (HD), such as abnormal movements induced by degeneration of striatal GABAergic neurons [2-4]. The impairment of energy metabolism, excitotoxicity, and oxidative stress are recently proposed in the 3-NP-induced neurotoxicity [9-11]. Among these toxic mechanisms, there is growing evidence of a role of oxidative stress in 3-NP neurotoxicity [5]. Exposure of 3-NP to rats depleted glutathione pools [6], increased oxidative stress in the striatum and cortex region of the brain as observed by increased levels of lipid peroxidation [7]. Also there are many reports that several antioxidants are effective to protect 3-NP-induced neuronal damage *in vitro* and *in vivo* experimental conditions. For examples, endogenous and exogenous antioxidants such as vitamin C, vitamin E, taurine, melatonin effectively protected against 3-NP-induced neuronal damage in rats [8-10]. In case of curcumin, a potent antioxidant of dietary polyphenol, by its antioxidant activity showed neuroprotection against 3-NP-induced behavioral and biochemical alteration [17]. These findings supported that oxidative stress may be an important mediator of the neuronal damage induced by 3-NP.

Magnolol (5, 5'-di-2-propenyl-1, 1'-biphenyl-2, 2'-diol) is well-known as an anti-allergic, anti-asthmatic, antifungal and anti-inflammatory compound with a wide spectrum of pharmacological activities [11-13]. In addition, magnolol has been known to show high antioxidant activity in a variety of the oxidative stress, including the heart, intestine, liver and brain [14, 16]. And it has been reported that the antioxidant effect of magnolol is approximately 1,000 times more potent than α-tocopherol [11, 15]. A previous report has demonstrated that the antioxidant and free radical scavenging activity of magnolol contributes to the neuroprotective effect in the hippocampus of senescence-accelerated mice [12]. And it was reported that magnolol can be rapidly distributed in the brain after systemic administration [1] and protects those learning and memory impairment and cholinergic deficit in SAMP8 mice [19].

Based on previous reports [14-17] I can expect that magnolol effectively protects many kinds of neurotoxin-induced oxidative stress and neuronal damage. However, the effect of magnolol against 3-NP-induced neuronal damage has not reported yet. In the present study, I investigated whether magnolol can protect against 3-NP-induced oxidative stress and neuronal damage in cultured neuroblastoma cells and mouse brains.

Materials and methods

Cell culture

SK-N-SH human neuroblastoma cells were cultured in DMEM (GIBCO BRL, Grand Island, NY) with 10% Fetal Bovine Serum (FBS; GIBCO BRL), 100 U/mL penicillin, and 100 mg/mL streptomycin (P/S; GIBCO BRL) at 37 ℃ in a humidified 5% CO_2-controlled incubator.

Cell viability assay

Cell viability was determined by propidium iodide (PI) staining of nuclei. Approximately $5x10^3$ cells per well into 96 well plates were seeded and then changed in DMEM supplemented containing 1% FBS. Cells were pretreated 5 μM magnolol for 1 h before being exposed to 5 mM 3-NP and incubated for 24 h without changing the medium. And then, PI (5 μg/ml) was added to the culture medium, and PI uptake into damaged neuronal cells was observed with a fluorescence microscope. To determine the apoptotic morphology nuclei were stained with Hoechst 33258. The Hoechst staining is sensitive to DNA and used to assess the changes in nuclear morphology. Cells were plated onto coverslip and exposed to 5 mM 3-NP for 12 h. Cells were rinsed with Phosphate-buffered saline (PBS; pH 7.4), and then fixed 4% paraformaldehyde (PFA) for 30 min. After washing twice with PBS, cells were stained with Hoechst 33258 (20 μg/ml) in PBS containing 0.5% DMSO, and observed with a fluorescence microscope.

Measurement of caspase-3-like protease activity

Cells were washed three times with PBS and were harvested in 50 mM Tris–HCl (pH 7.4), 1 mM EDTA, and 10 mM EGTA. Lysates were homogenized and centrifuged at 1,000 rpm for 10 min. Protein lysate (150 μg of protein) was mixed with assay buffer (20 mM HEPES (pH 7.4), 2 mM EDTA, 0.1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic acid, and 5 mM DTT) and caspase-specific substrates (Ac-DEVD-pNA). After 4 h incubation at 37℃, the absorbance of pNA released as a result of caspase-3 activity was measured at 405 nm in a microplate reader. The reaction mixture without enzyme or substrate was used as a control.

Measurement of ROS generation

Intracellular ROS produced by 3-NP treatment in cells was measured by using a DCF-DA method. In brief, cells ($1x10^5$/35 mm dish) were cultured with 3-NP for 9 h in the presence or absence of magnolol. After incubation, cells were washed and loaded with 10 μM 2′,7′-dichlorofluorescein diacetate (DCF-DA; Sigma) for 15 min at 37℃. Subsequently, cells were washed twice with PBS. Photomicrographs were taken with a fluorescence microscope equipped with UV supply system (Olympus IX70, Japan). To quantitate the production of ROS, cells were loaded with 10 μM DCF-DA for 15 min and collected, lysed with 0.1% Tween 20 with PBS on ice. After centrifugation at 10,000 rpm for 5 min, the supernatants were saved. The DCF fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 525 nm with spectrofluorometer (Kontron instruments S.P.A, TI/SFM25).

Animals

Seven week-old male ICR mice (28-30 g) were used for this study. All procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. 3-NP was dissolved in saline and pH was adjusted to pH 7.4 with 1 N NaOH. The mouse were anesthetized with equithesin and placed in a stereotaxic device so that the bregma and lambda landmarks were in the same horizontal plane. Injections were made in a volume of 2 μl over 5 min with 3-NP (200 nmol) into the right striatum at the following coordinates: AP + 0.5 mm, ML - 2.0 mm, DV - 3.3 mm from bregma and dura mater. And the needle was left in place for 5 min before being slowly withdrawn. The sham-injected animals made by injecting 2 μl of saline adjusted at pH 7.4, instead of 3-NP. Magnolol was dissolved in 10% ethanol/phosphate-buffered saline. Magnolol (10, 30 mg/kg) was intraperitoneally administered to mouse 1 day, 2 day, 1 hour before and 2 hours, 1 day after the unilateral striatal injection of 3-NP (total five injections). Mice were sacrificed at day 1 or day 4 after 3-NP injection.

2, 3, 5-triphenyltetrazolium chloride (TTC) staining

Four days after 3-NP injection, animals were sacrificed by rapid decapitation. And brains were removed, placed on brain slicer, and cut into 1-mm coronal sections. Slices were stained with 2% TTC at room temperature in the dark for 30 min and then fixed in cold 4% paraformaldehyde. Unstained areas were defined as striatal neuronal damage induced by 3-NP.

Areas of lesions as noted by pale staining were measured on the posterior surface of each section with an image analysis system (MCID; Imaging Research Inc., Canada), and the volume (mm^3) of each lesion was calculated by summing the results of multiplying each lesion area by 1 mm.

Tissue preparation

Mice were killed with equithesin and then decapitated followed by rapid removal of striatum. Striatal protein extracts were prepared by homogenization in a lysis buffer to which protease inhibitors had been freshly added. Homogenates were centrifuged at 10,000 rpm for 10 min at 4℃ and supernatants were then used for Western blot analysis.

Measurement of lipid peroxidation

MDA as an index of lipid peroxidation was measured as described by Ohkawa et al (14). Briefly, brain tissues were homogenized with sodium phosphate buffer. The reagents (1.5 ml of 20% acetic acid, 1.5 ml of 0.8% thiobarbituric acid and 0.5 ml of 8.1% sodium dodecyl sulfate buffer mixture) were added to 1 ml of processed tissue sample. The mixture was then heated 100℃ for 60 minutes. The mixture was cooled on ice. After centrifugation at 4,000 rpm for 10 minutes, Supernatants were used to calculate the level of MDA by measuring absorbance at the 532 nm wavelength. The results were expressed as the percentage of control (sham-injected mice).

Measurement of protein carbonylation

The appearance of carbonyl groups in proteins was analyzed by immunodetection of protein-bound 2, 4-dinitrophenylhydrazone after derivatization with the corresponding hydrazine. The homogenate fractions were harvested by centrifugation and washed once in TE-buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Samples were mixed with SDS to a final concentration of 6% (w/v) and were derivatized with an equal volume of DNPH derivatization solution [Fluka; 20 mM DNPH in 10% trifluoracetic acid (v/v)] and incubated at room temperature for 15 minutes. The derivatization reaction was stopped by adding neutralization solution [2 M Tris, 30% glycerol (v/v), bromophenol blue]. Then Western blot for DNP was determined.

Western blot analysis

Cells were harvested or brain tissue were homogenized in lysis buffer supplemented with 1:1,000 protease inhibitor cocktail (Roche) on ice. Lysates were homogenized and centrifuged at 1,000 rpm for 10 min. After protein concentration was determined by the Bradford assay (Bio-Rad Lab), samples were denatured in sample buffer containing for 10 min at 100°C. Protein samples are run in polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose (NC) membrane. Blotted NC membrane incubated for 1 h with Tris-buffered saline containing 0.1% Tween (TBST), 5% skim milk and 2% BSA powder. Membranes were incubated overnight at 4℃ with the primary antibody solution in TBST. Blots were analyzed with goat anti-GAD-65/67 (C-20, Santa Cruz; 1:1,000), anti-TH (sc-14007, Santa Cruz; 1:500), rabbit anti-DNP (D9656, Sigma; 1:10,000), rabbit anti-HO-1 (SPA-895, Stressgen; 1:2,000). The membranes were then washed three times (10 min each) with TBST, the membranes were further incubated with the secondary antibody (1:2,000), and the proteins were visualized by using an enhanced chemiluminescence reagent (Amersham Biosciences, Buckinghamshire, UK). The antibody directed against ß-actin (MAB1501R, Chemicon; 1:10,000) was used to normalize the amount of protein per lane.

Statistical analysis

All data are presented as mean ± standard error of the mean (S.E.M.). Statistical comparison between different treatments was done by one-way analysis of variance (ANOVA) with Duncan's multiple comparison. Results with $p < 0.05$ were considered as statistically significant.

Results

The effect of magnolol in 3-NP-treated cells

Magnolol protects 3-NP-induced cytotoxicity

To evaluate protective effect of magnolol against 3-NP-induced cytotoxicity, SK-N-SH cells were treated with 5 mM 3-NP for 24 h and then cytotoxicity was assessed by PI staining. Treatment with magnolol at 2.5-10 μM for 48 h did not induce cell death (data not shown). Treatment with 3-NP increased PI fluorescence. PI-positive cells were detected from 6 h and the number of PI-positive cells was increased over 24 h after 3-NP treatment. Percentage of PI-positive cells in 3-NP treatment for 24 h was increased to 48.0 ± 3.0% of total cells (vs. 13 ± 1.6% in control group). But pretreatment with magnolol significantly reduced PI-positive cells (27.5 ± 4.4% of total cells), compared to '3-NP-treated group (Fig. 1). These results showed that magnolol effectively protected SK-N-SH cells from 3-NP-induced cytotoxicity.

Magnolol inhibits morphological changes and apoptosis-related proteins expression after 3-NP treatment

To evaluate the effect of magnolol on 3-NP-induced apoptosis, cells were stained with Hoechst 33258 12 h after 3-NP treatment. Apoptotic features such as nuclear shrinkage, chromatin condensation or fragmentation were shown after 3-NP treatment for 12 h. But, pretreatment with 5 μM magnolol significantly reduced 3-NP-induced chromatin condensation and nuclear fragmentation (Fig. 2A).

To further investigate the suppressive effect of magnolol on 3-NP-induced cell death, I examined whether magnolol can suppress the 3-NP-stimulated caspase-3 activity. As expected, exposure of cells to 3-NP led to enhance caspase-3 activity in the cell lysate (Fig. 2B). However, treatment of magnolol effectively reduced caspase-3 activation induced by 3-NP.

Magnolol protects cells from oxidative stress induced by 3-NP

To determine the effect of magnolol on 3-NP-induced oxidative stress in cultured cells, ROS generation was measured using fluorescent dye, DCF-DA. DCF fluorescence was started to increase 6 h after 3-NP treatment, and significantly elevated to more than 20-fold 9 h after 3-NP treatment compared to control. Pretreatment with magnolol for 1 h before 3-NP treatment

significantly decreased 3-NP-induced ROS generation (Fig 3A, B). Treatment with 3-NP for 9 h also increased levels of protein carbonylation, compared to control group. But, pretreatment with magnolol protected 3-NP-induced increase in protein carbonylation in cells (Fig 3C).

Magnolol reduces 3-NP-induced HO-1 expression

It is generally accepted that induction of HO-1 mediates a cellular adaptive response against the various type of stress *in vitro* and *in vivo* study [20]. And it is well-known that several polyphenol compounds, such as curcumin, baicalein, resveratrol, etc are able to induce HO-1 expression and the HO-1 inducing activity is related to the additional protective mechanisms of these chemicals as well as their direct antioxidant action [19-21]. So, we evaluated whether the protective effect of magnolol, a natural polyphenolic compound, may be mediated by HO-1 induction. Cells were treated with various concentrations of magnolol (5~20 μM), but magnolol did not induce HO-1 expression in these concentrations (data not shown). To the contrary, 3-NP treatment induced the expression of HO-1 protein in cultured cells in a time-dependent manner. Treatment with 3-NP slightly increase HO-1 expression at 1-3 h with a maximum level at 6 h exposure. Induction of HO-1 expression was showed at least for 12 h after 3-NP treatment (Fig. 4A). However, pretreatment with magnolol inhibited 3-NP-induced expression of HO-1 protein (Fig. 4B).

The effect of magnolol in unilateral injection of 3-NP to mouse striatum.

Magnolol reduces 3-NP-induced neuronal damage

Neuronal damage was examined by TTC staining. 3-NP was injected into the right striata of male mice as described previously [9]. Brain was removed 4 days later, and prepared for TTC staining. Injection of 3-NP was produced neuronal damage in lesioned side of striatum 4 days after intrastriatal 3-NP injection. Lesion volume was 15.9 ± 2.1 mm^3 in 3-NP-injected mice (n = 5), compared with sham-injected mice. However, magnolol treatment effectively reduced the 3-NP-induced striatal neuronal damage to 4.2 ± 0.3 mm^3 (10 mg/kg magnolol treatment, n = 5) and 1.1 ± 1.0 mm^3 (30 mg/kg magnolol treatment, n = 5) (Fig. 5).

Magnolol protects the reduction of GAD and TH contents in the striatum after 3-NP injection

To support the protective effect of magnolol on 3-NP-induced striatal neuronal damage, specific striatal neuronal markers (GAD, TH) were also determined by immunoblotting after treatment with 30 mg/kg magnolol. Striatal GAD contents were markedly decreased 4 days after 3-NP injection to 43.0 ± 4.7% of sham-injected mice (Fig. 6). However, the treatment with magnolol at 30 mg/kg significantly restored striatal GAD contents to 61.3 ± 3.7% of sham-injected mice. TH contents were also reduced to 66.8 ± 2.2% of sham-injected mice after 3-NP injection. Treatment of magnolol at 30 mg/kg was also increased TH contents in striatum to 78.6 ± 5.7% of sham-injected mice, respectively.

Magnolol significantly reduces 3-NP-induced oxidative stress

The amount of MDA and protein carbonylation as the parameters of oxidative stress in the striatum was measured 1 day after 3-NP injection. There was a significant increase in MDA levels in 3-NP-injected mice compared to sham-injected mice (9.6 ± 1.4 vs 2.7 ± 1.4 µmole/mg protein). Magnolol significantly attenuates 3-NP-induced increase in the levels of MDA (3.0 ± 1.1 µmole/mg protein) (Fig. 7A). Protein carbonylation in the lesioned striatum also increased in 3-NP-injected group, compared to control group (over 940% of control, Fig. 7B). But magnolol treatment prevented 3-NP-induced increase in protein carbonyl content near to control level (116% of control). These results indicated that magnolol effectively protected the striatum from 3-NP-induced oxidative stress.

Magnolol reduces 3-NP-induced increase in HO-1 expression in lesioned striatum

In this experiment I observed that 3-NP significantly increased HO-1 expression and magnolol effectively inhibited 3-NP-induced HO-1 expression in cultured cells. So, I tried to evaluate that magnolol can inhibit HO-1 induction by 3-NP in lesioned brain, too. One day after 3-NP injection, the expression of HO-1 protein in the striatum in 3-NP-injected group was up-regulated compared to sham-injected group (194 % of control). However, treatment with magnolol significantly decreased the expression of HO-1 induced by 3-NP (133 % of control). Magnolol alone did not affect HO-1 expression (Fig. 8).

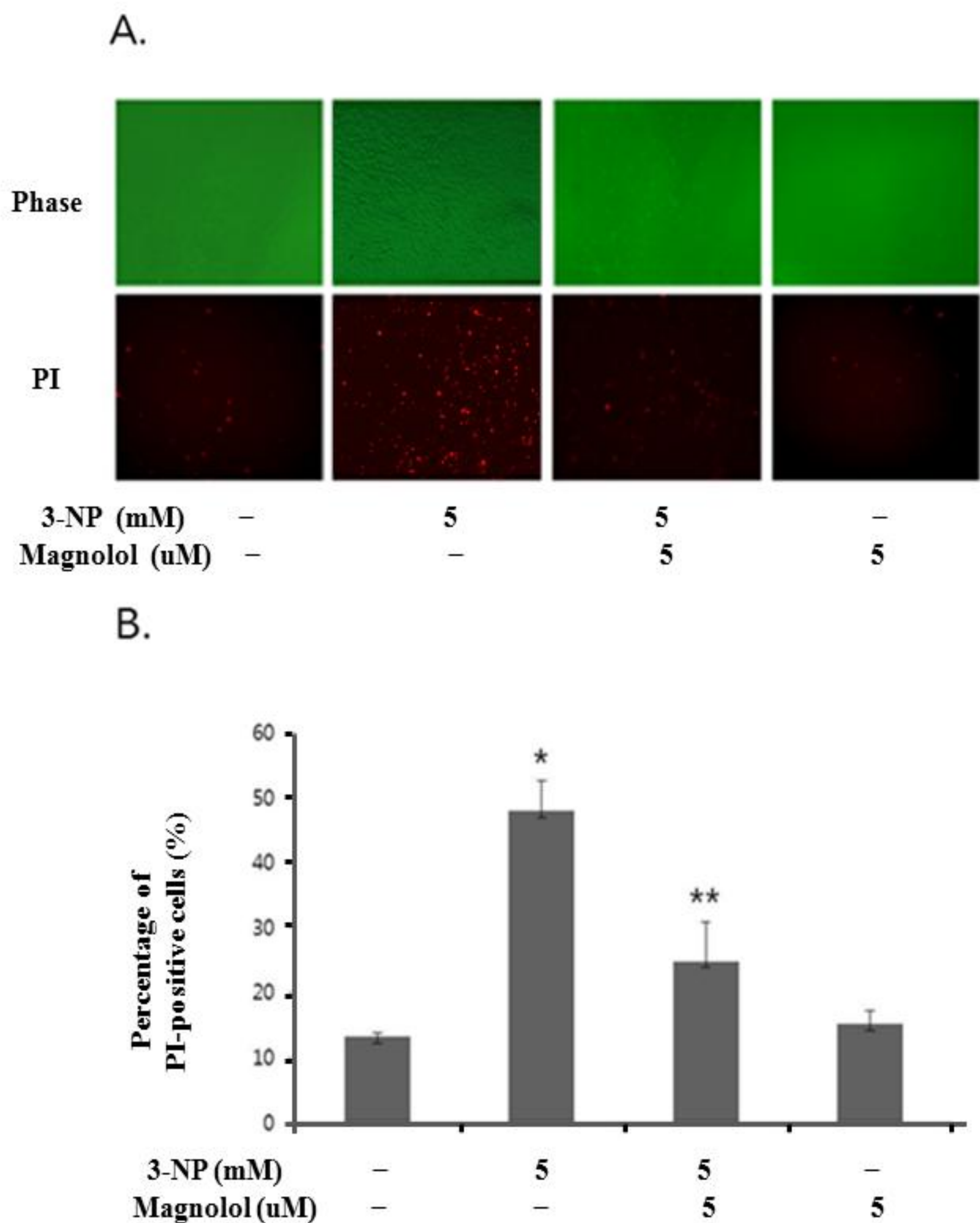
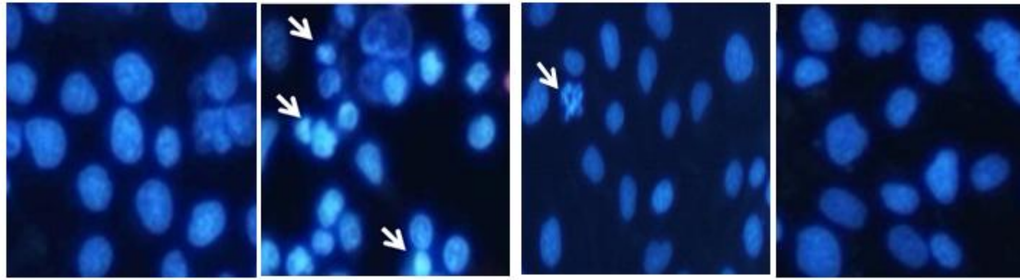
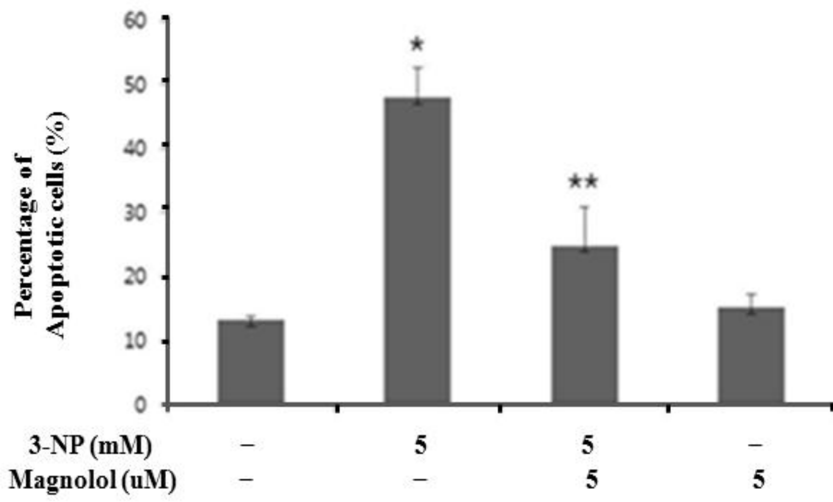


Fig. 1. Effect of magnolol on cytotoxicity induced by 3-NP in cultured cells

Cell death was evaluated by PI staining. Cells were pretreated with magnolol (5 μM) for 1 h followed by exposure to 3-NP (5 mM). Magnolol significantly prevented the cytotoxicity induced by 3-NP at 24 h incubation. Cell death is expressed as a percentage of the total number of cells (approximately 100 cells per field were counted). Values are expressed as mean ± SEM. * indicates $p < 0.05$ compared with the control and ** indicates $p < 0.05$ compared with the 3-NP treated group.

A.





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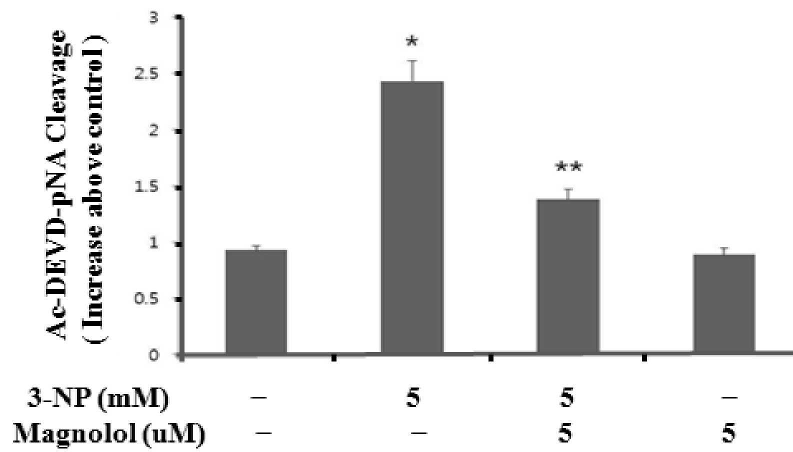
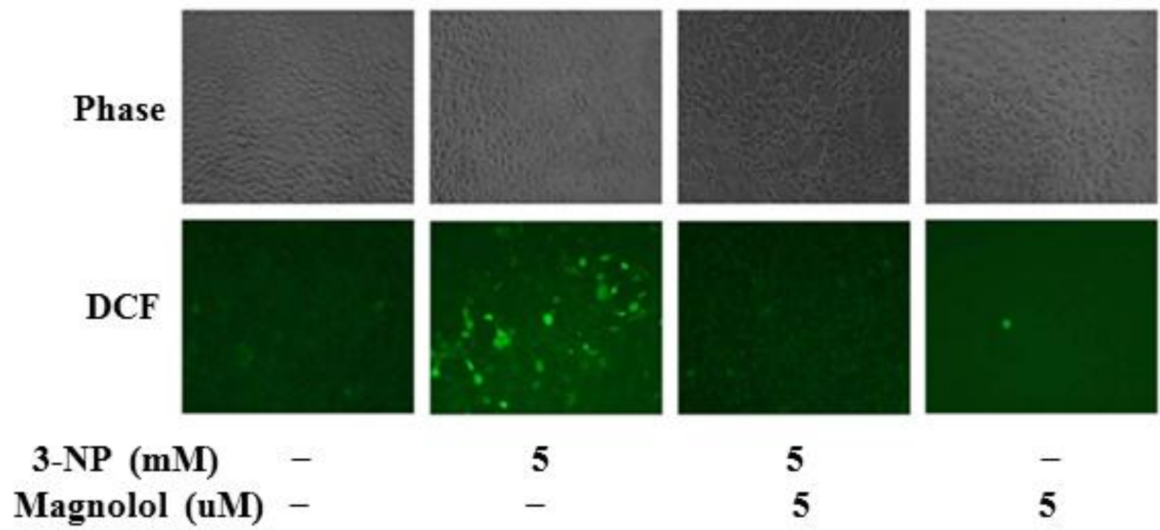


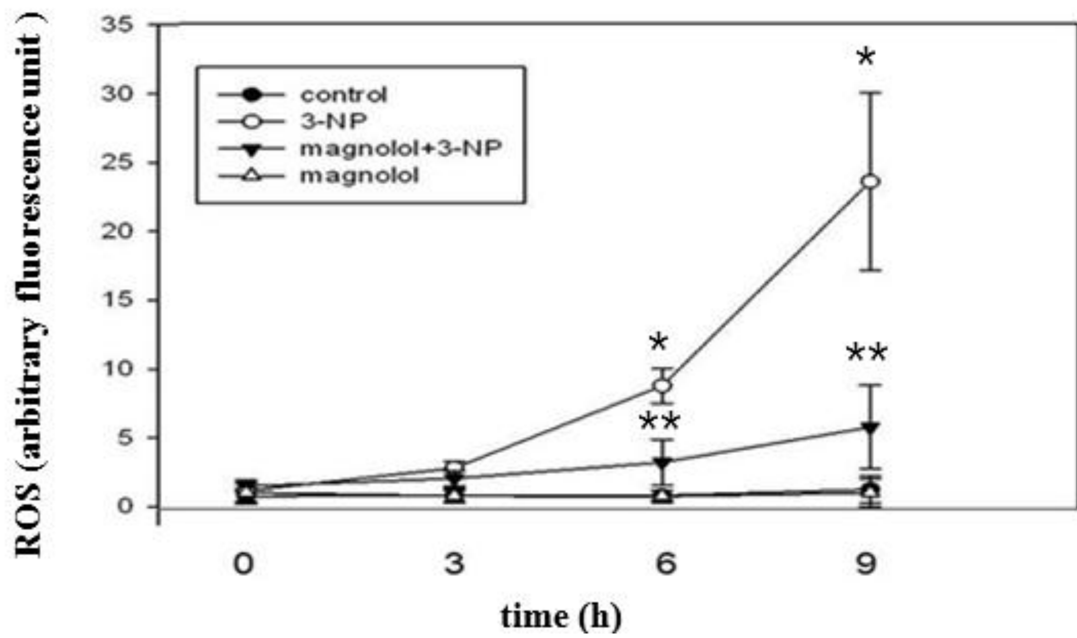
Fig. 2. Effect of magnolol on morphological changes and caspase-3 activity induced by 3-NP treatment in cultured cells

Cells were pretreated with magnolol (5 μM) for 1 h and further incubated in the presence or absence of 5 mM 3-NP for 12 h. Cells were stained with Hoechst 33258 and assayed for nuclear morphology and determined caspase-3 like activity. (A) The extent of apoptotic cell death was assessed by counting condensed or fragmented cells using a fluorescence microscopy after Hoechst staining. (B) Enzymatic activity of Ac-DEVD-pNA (caspase-3-like enzyme activity) in 3-NP-treated cells in the presence or absence of magnolol. * indicates $p < 0.05$ compared with the control and **indicates $p < 0.05$ compared with the 3-NP treated group.

A.

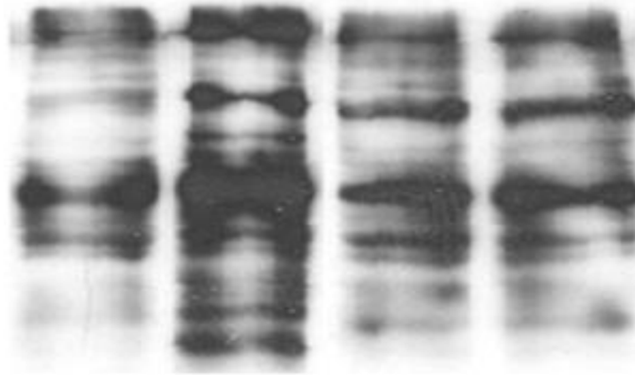


B.



C.

Protein carbonylation



3-NP (mM) – 5 5 –
Magnolol (uM) – – 5 5

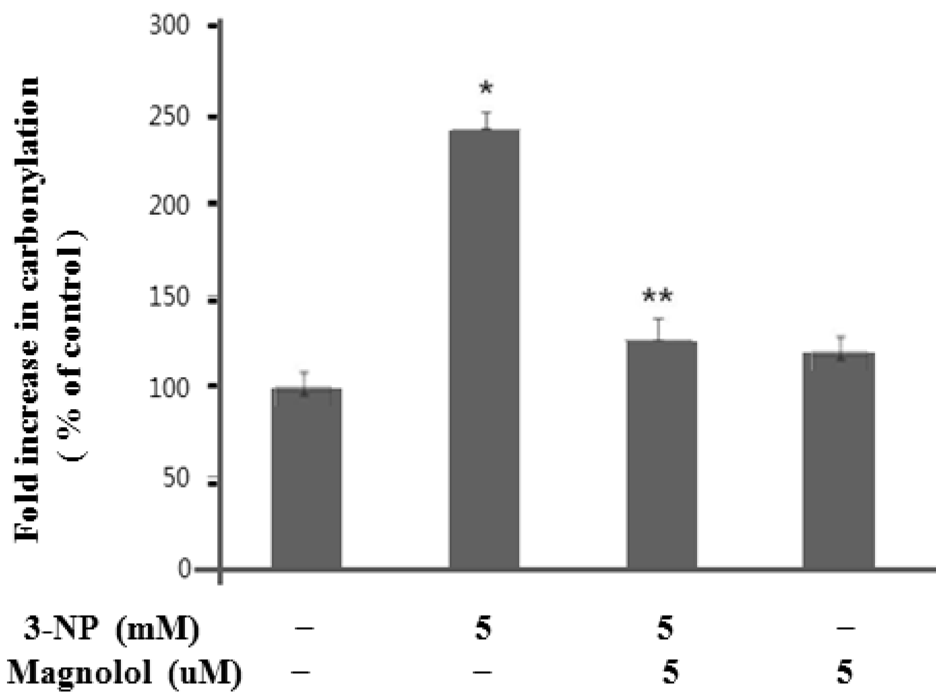
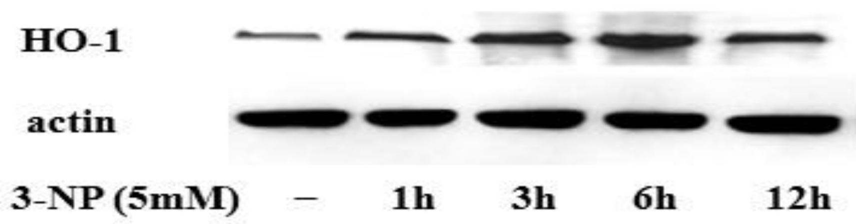
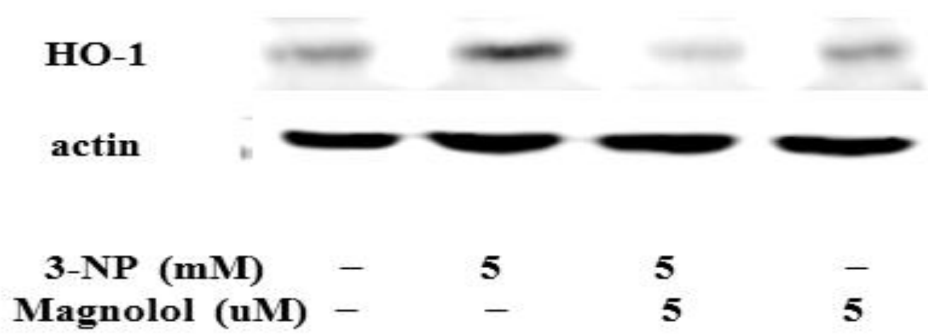


Fig. 3. Protective effect of magnolol on oxidative stress induced by 3-NP in cultured cells
(A) Representative imaging of DCF fluorescence 9 h after 3-NP (5mM) exposure in cells. Increased DCF fluorescence by 3-NP treatment was effectively reduced by magnolol. (B) Time-dependent change of ROS generation. DCF fluorescence was started to increase 6 h after 3-NP treatment and significantly elevated 9 h after 3-NP treatment compared to control. Pretreatment with magnolol for 1 h before 3-NP treatment significantly decreased 3-NP-induced ROS generation. (C) Protein carbonylation induced by 3-NP. Treatment with 3-NP significantly increased protein carbonylation but magnolol inhibited 3-NP-induced protein carbonyls in these cells.


A.
HO-1
actin
3-NP (5mM)
–
1h
3h
6h
12h


B.
HO-1
actin
3-NP (mM) – 5 5 –
Magnolol (uM) – – 5 5

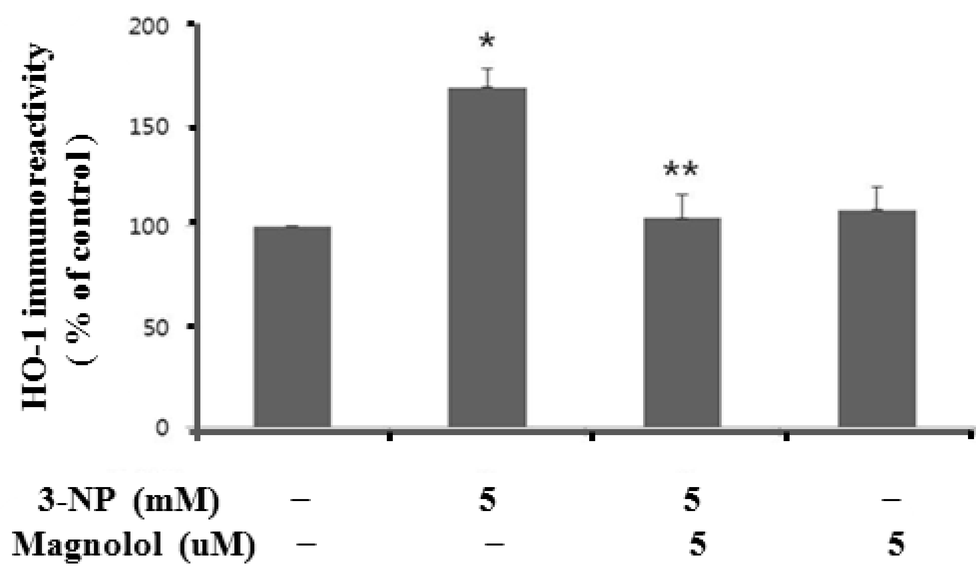
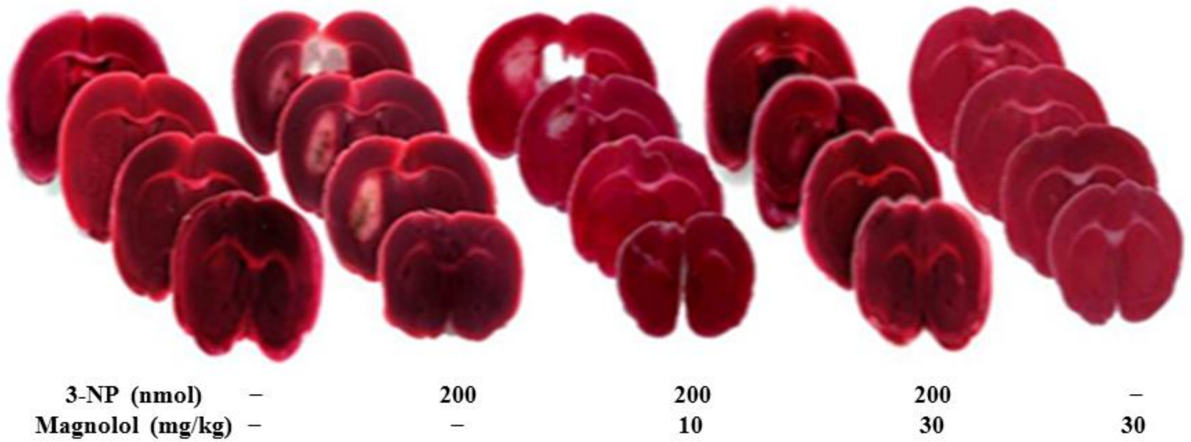

HO-1 immunoreactivity
(% of control)
200
150
100
50
0
*
**
3-NP (mM) – 5 5 –
Magnolol (uM) – – 5 5

Fig. 4. Effect of magnolol on heme oxygenase (HO)-1 expression induced by 3-NP in cultured cells

(A) Time-dependent increase in HO-1 expression by 3-NP (5mM). Cell lysates were electrophoresed and expression of HO-1 protein was detected at the indicated times. (B) Expression of HO-1 protein induced by 3-NP with and without magnolol treatment. Cells were pretreated with magnolol for 1 h prior to the addition of 3-NP at 6 h incubation. ß-actin was used as a loading control. Densitometric analysis (HO-1/ß-actin) for each band for independent experiments, n = 7. * indicates $p < 0.05$ compared with the control and ** indicates $p < 0.05$ compared with the 3-NP treated group.



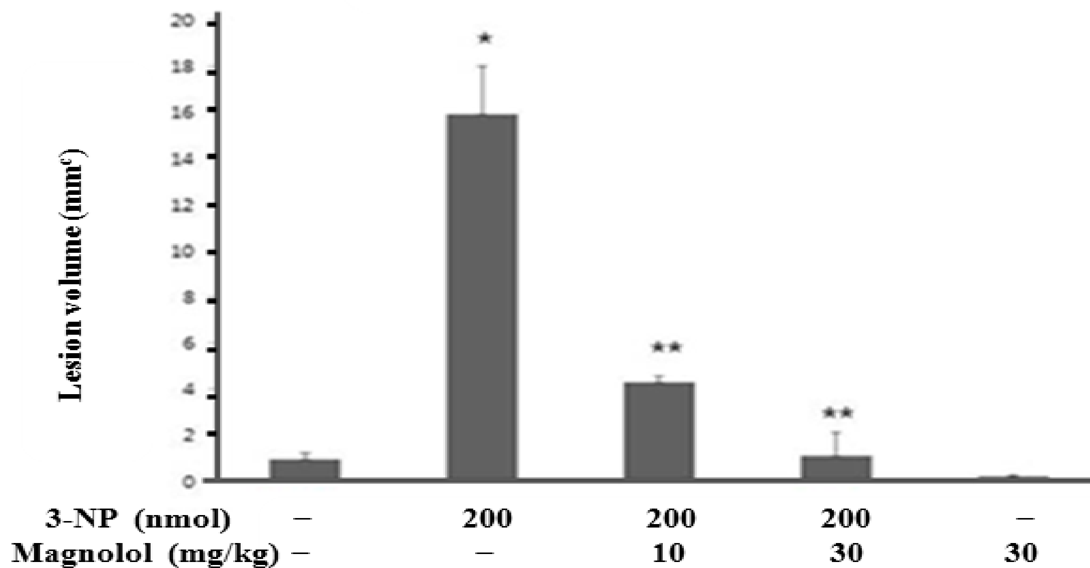


Fig. 5. **Effect of magnolol on striatal damage induced by intrastriatal injection of 3-NP (A)** Representative brain section photographs, and (B) quantitative analysis of the striatal lesion size in the TTC stained sections 4 days after 3-NP injection. Injection of 3-NP in the striatum induced extensive neuronal damage while few or no lesion was observed in the sham-operated mice. However, treatment with magnolol markedly reduced the lesion volume induced by 3-NP. Values are expressed as mean ± SEM (n = 5). * indicates $p < 0.05$ compared with the control and ** indicates $p < 0.05$ compared with the 3-NP treated group.

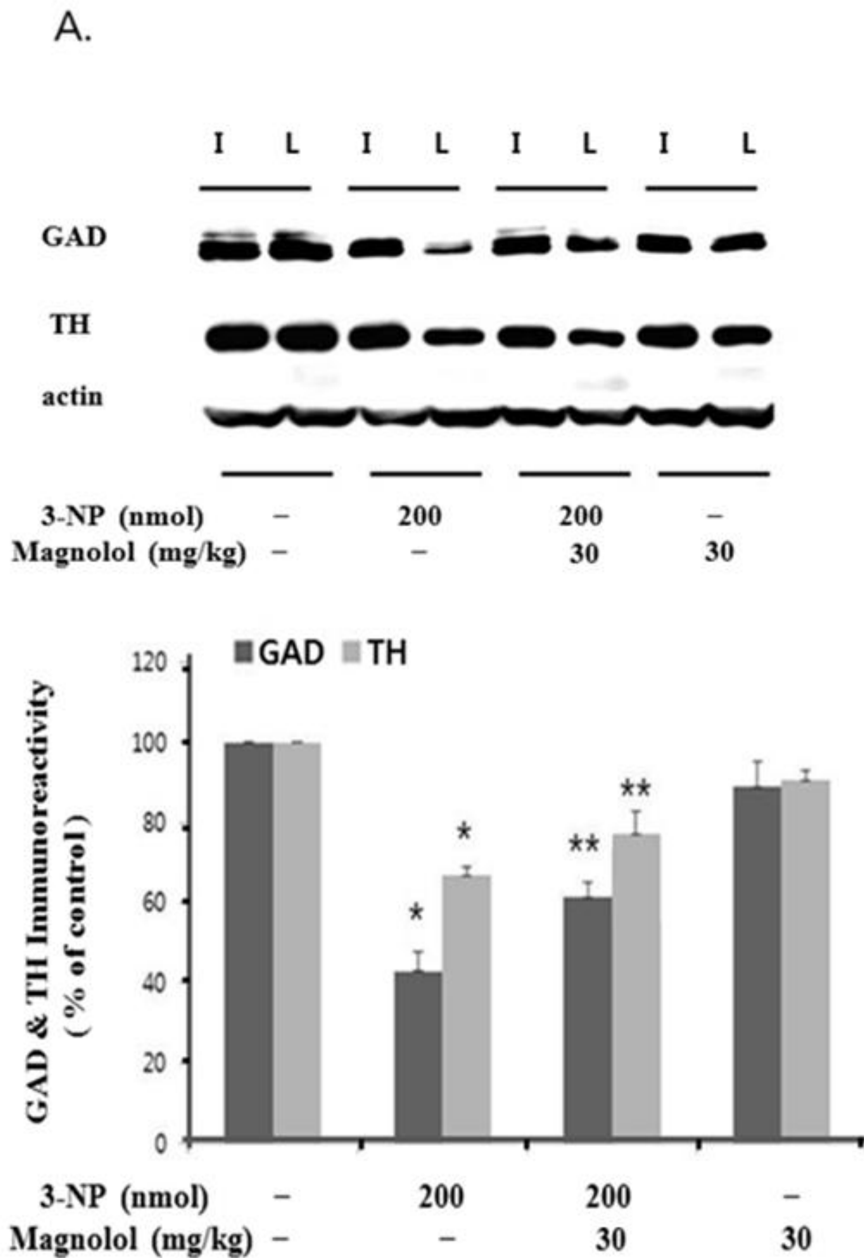
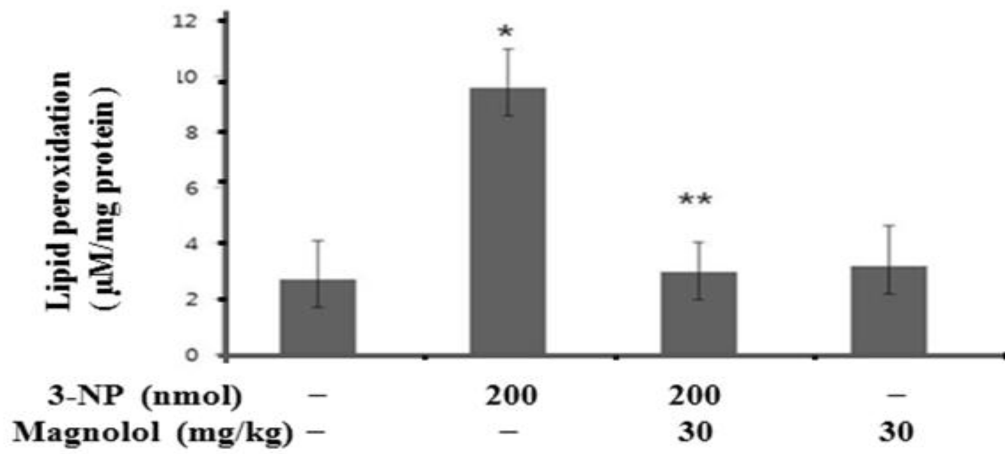


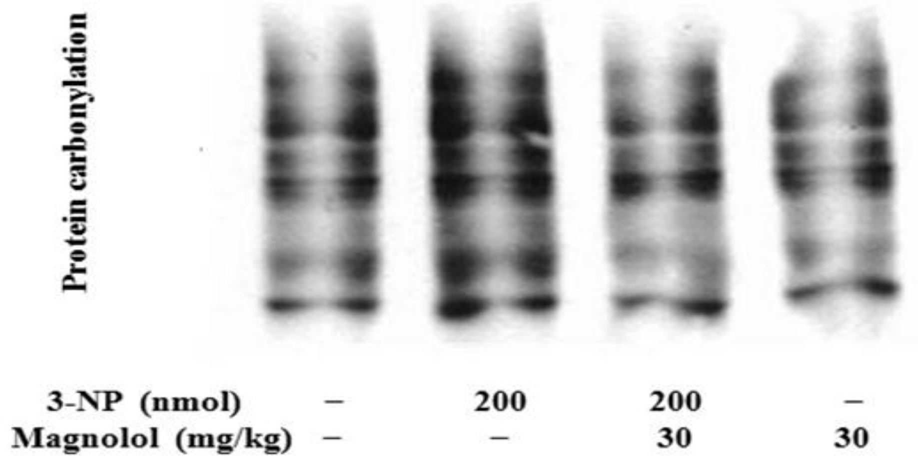
Fig. 6. Protective effect of magnolol on the reduction of striatal GAD and TH contents induced by 3-NP injection

The GAD and TH levels in the striata were determined by Western blotting 4 days after 3-NP injection. The GAD and TH contents of the lesioned side (L) to those of the intact side (I) were reduced in the 3-NP-injected mice, whereas these contents in lesioned side were restored in the magnolol-treated mice (n = 5). * indicates $p < 0.05$ compared with the control and ** indicates $p < 0.05$ compared with the 3-NP treated group.

A.



B.



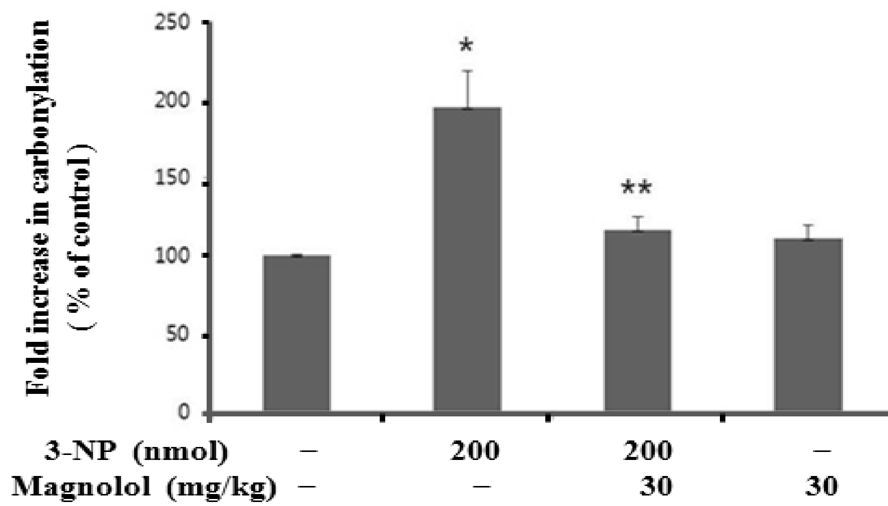
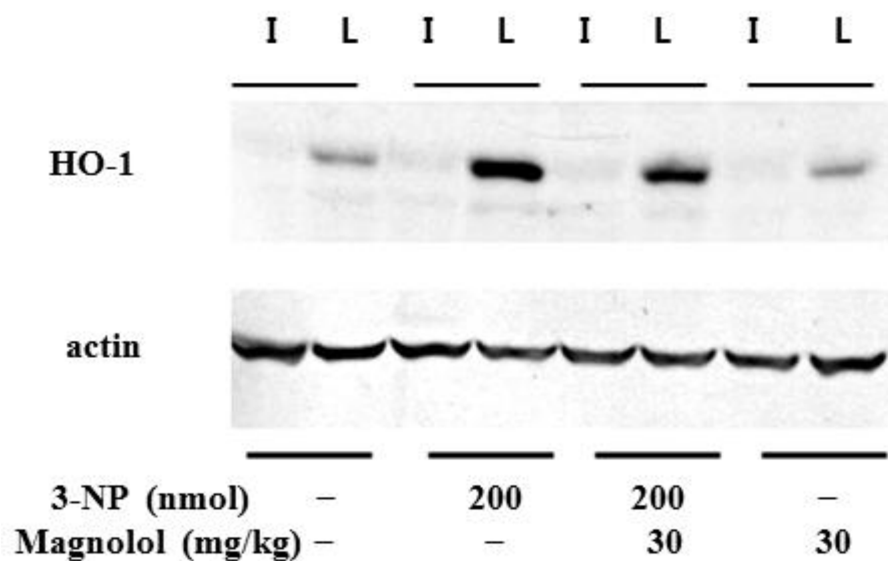


Fig. 7. Protective effect of magnolol on oxidative stress induced by 3-NP injection

(A) lipid peroxidation (B) protein carbonylation. Injection of 3-NP in the striatum increased levels of MDA, protein carbonyl 1 day after 3-NP injection. However, treatment with magnolol effectively reduced these parameters. Values are expressed as mean ± SEM (n = 7). * indicates $p < 0.05$ compared with the control group and ** indicates $p < 0.05$ compared with the 3-NP-injected group.


I L I L I L I L
HO-1
actin
3-NP (nmol) – 200 200 –
Magnolol (mg/kg) – – 30 30

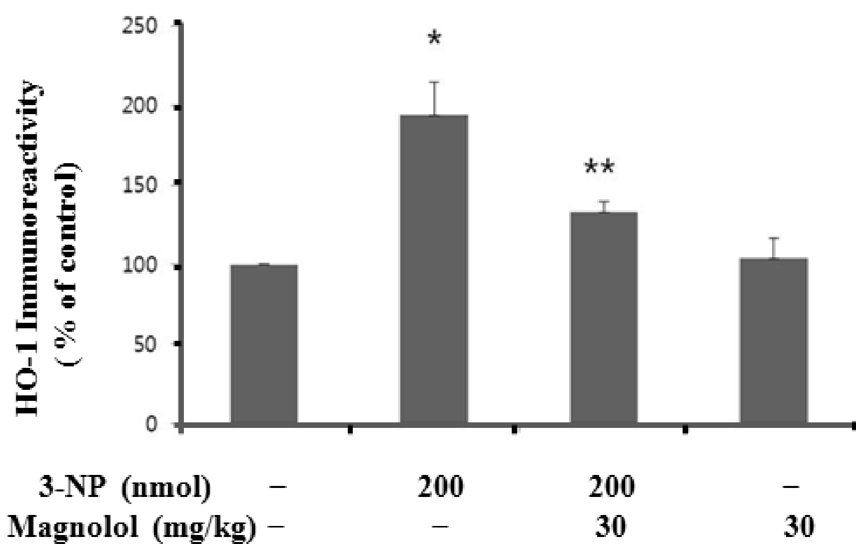

HO-1 Immunoreactivity
(% of control)
250
200
150
100
50
0
*
**
3-NP (nmol) – 200 200 –
Magnolol (mg/kg) – – 30 30

Fig. 8. Effect of magnolol on heme oxygenase (HO)-1 expression induced by 3-NP injection
Representative immunoblots show the expression of HO-1 protein in the striatum 1 day after 3-NP injection. Quantitative analyses of immunoblots reveal significant increases in HO-1 in the 3-NP-injected mice. Magnolol treatment effectively reduced 3-NP-induced HO-1 expression. Values are expressed as mean ± SEM (n = 5). * indicates $p < 0.05$ compared with the control group and ** indicates $p < 0.05$ compared with the 3-NP-injected group. (L: lesioned side; I: intact side of the striatum)

Discussion

3-NP, one of inhibitors of mitochondrial enzyme SDH, is a widely distributed plant and fungal neurotoxin known to induce damage to basal ganglia, hippocampus, spinal tracts and peripheral nerves in animals [1-3]. It has been reported that systemic administration of 3-NP can produce selective striatal lesions and produces delayed dystonia and abnormal choreiform movements in rats and humans [4-5]. Intrastriatal injection of 3-NP gives rise to reduction in markers for both striatal intrinsic neurons, such as GABA [28-29] and substance P [30], and dopaminergic afferent fibers [6] and these neuronal damage induced by 3-NP is due to the suppression of electron transport within the mitochondria [4]. There are lots of evidence that administration of 3-NP can produce selective striatal lesions occurred by secondary excitotoxic mechanisms [24], i.e., indirect activation of glutamate receptors [25]. Also it was reported that 3-NP induces protein oxidation in striatal and cortical synaptosomes and oxidative stress and neuronal DNA fragmentation in mouse striatum [27].

In the present study, I observed that treatment with 3-NP increased ROS production and protein carbonylation in cultured cells. Also 3-NP lead to cytotoxicity such as DNA condensation/fragmentation and increased the caspase-3 activity, expression of p53 and Bax protein. In *in vivo* study, intrastriatal injection of 3-NP produced significant striatal neuronal damage (TTC staining) and markedly reduced GAD, TH contents in lesioned side compared to sham-injected group 4 days after striatal 3-NP injection. And the levels of lipid peroxidation and protein oxidation in lesioned striatum were significantly increased 1 day after injection of 3-NP to mouse brains. These results are consistent with other previous reports that 3-NP induces oxidative stress and it might be an important mediator in 3-NP-induced neuronal damage [3, 4, 6].

I also evaluated that protective effect of magnolol on 3-NP-induced neuronal damage. Treatment with magnolol effectively decreased 3-NP-induced ROS generation and protein carbonylation. This decrease suggests that free radical scavenger magnolol can significantly eliminate the 3-NP-induced oxidative stress. And magnolol significantly reduced 3-NP-induced caspase-3 activity and apoptotic cell death. In animal study, treatment with magnolol significantly attenuates 3-NP-induced lipid peroxidation and protein oxidation in lesioned striatm 1 day after 3-NP injection. Treatment with magnolol effectively restored striatal neuronal damage induced by 3-NP injection. These present results showed that magnolol effectively protects 3-NP-induced oxidative stress and neuronal damage.

Magnolol, a component of *M. officinalis*, has been used in traditional herbal medicine to treat a variety of disorders including anxiety and nervous disturbances. And it could effectively scavenge hydroxyl radicals, 1-diphenylpicryl-2-hydrazyl (DPPH) radicals [14, 28]. Magnolol has exhibited approximately 1,000 times more potent than antioxidant effect than alpha-tocopherol (vitamin E) [31]. Magnolol significantly decreases amyloid beta (Aß) -induced ROS production and cell death [36] and protects neurons against chemical hypoxia by KCN in cultured cortical cells [13]. The protective effect of magnolol against neuron toxicity induced by glucose deprivation, excitatory amino acids and hydrogen peroxide in cultured cerebellar granule cells is also largely known [34]. Magnolol was shown to protect cortical neuronal cells from chemical hypoxia and to attenuate heat stroke-induced neuronal damage [29, 30]. In addition, exogenous and endogenous antioxidants such as taurine [8], vitamin C, epigallocatechin gallate (EGCG) [31] and melatonine [9, 32] effectively protect 3-NP-induced neuronal damage in rats. Taurine is an antioxidant that protects against neuronal damage caused by oxidative stress and vitamin C can directly scavenge free radicals in plasma and suppress their reactivity. EGCG reversed the behavioral, histological and cellular alterations against 3-NP toxicity. Melatonin prevented the increases in lipid peroxidation and total LDH activity, as well as the depletion of reduced glutathione and the reduction of antioxidative enzymes activities in cells incubated with 3-NP. Based on these previous reports and our results it can be suggested that magnolol can effectively attenuates 3-NP-induced oxidative stress and protects 3-NP-induced neuronal damage in cultured cells and mouse brain by reducing oxidative stress as a strong antioxidant.

Recently, there are many reports that several flavonoids or natural antioxidants, such as α-mangostin and nordihydroguaiaretic acid (NDGA) activate the antioxidant pathway Nrf2/HO-1 in cerebellar granule neurons and protect them against 3-NP-induced neurotoxicity [31, 34] This statement is based on the fact that the protective effect of α-mangostin or NDGA was completely reversed by tin mesoporphyrin (SnMP; HO-1 inhibitor) suggesting that it was mediated by involving induction of HO-1. In fact, many reports have demonstrated that the induction of HO-1 expression represents an adaptive response that increases cell resistance to oxidative injury [31, 32]. Although magnolol is well known as polyphenol compound, this present study showed that magnolol itself did not induce HO-1 expression. But, treatment with magnolol reduced 3-NP-induced increase in HO-1 expression in cultured cells and mouse brains. From these results it can be suggested that the induction of HO-1 by 3-NP treatment is related to the adaptive protective response to 3-NP-induced oxidative stress and can be an oxidative stress marker.

In summary, our data demonstrated that magnolol can effectively protect 3-NP-induced oxidative stress and neuronal damage in cultured cells and mouse brains. And the protective

effect of magnolol against 3-NP-induced neuronal damage may be attributed to antioxidant activity. These results support that magnolol may be a potential candidate in preventing or reducing the metabolic impairment related disorders.

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국문초록

*Magnolia officinalis*로부터 추출된 magnolol은 항암, 항생균 그리고 항산화와 같은 여러 생물학적 활성이 있다고 알려져 있다. 그러나 현재까지 3-nirtopropionic acid (3-NP)로 인하여 유도되는 신경손상과 세포사에 대한 magnolol의 작용에 대해서는 알려진 바가 없다.

본 연구에서는 생쥐의 뇌조직과 배양세포에서 3-NP에 대한 magnolol의 보호작용 기전에 대하여 알아보고자 하였다.

사람의 신경아세포종인 SK-N-SH 세포주에 3-NP를 처리하면 3-NP는 핵의 응축과 분열, caspase-3 활성과 세포사를 초래하고, 활성산소의 생성와 단백질의 카보닐화를 증가시킴을 보였다. 그러나 magnolol은 3-NP에 의한 세포독성, 그리고 3-NP에 의한 활성산소의 발생과 단백질의 카보닐화를 효과적으로 감소시켰다. 한편 3-NP는 HO-1의 발현을 유의하게 증가시키는 반면, magnolol을 전처리한 경우 3-NP이 증가되는 HO-1의 발현을 감소시켰다. 그러나 SK-N-SH 세포주에서 magnolol 자체는 HO-1의 발현에 영향을 미치지 않았다. 동물실험에서는 3-NP를 생쥐의 오른쪽 선조체에 정위 주입함으로써 병변을 유발하였다. 3-NP 주입 후 4일에서 3-NP를 주입한 선조체에서 현저한 신경손상을 보이며(TTC 염색), 선조체 내 GAD (glutamic acid decarboxylase)와 TH (tyrosine hydroxylase)의 단백발현이 감소되는 결과를 보였다. 그러나 magnolol을 투여하면 3-NP에 의한 신경손상과 GAD, TH 단백발현의 감소를 유의하게 보호하였다. 또한 3-NP 주입 후 1일에서 malondealdehyde (MDA), 단백질의 카보닐화, 그리고 HO-1의 발현이 현저히 증가되었지만, magnolol을 투여하면 3-NP에 이들 지표가 유의하게 억제되는 결과를 보였다.

이상의 결과로부터 magnolol은 배양세포와 생쥐 모델에서 3-NP에 의한 신경세포의 손상을 항산화 작용을 통해 효과적으로 보호할 수 있음을 확인하였다. 이러한 결과는 magnolol은 대사장애와 연관된 신경손상을 방지하거나 억제하는데 적용할 수 있음을 제시하는 결과로 믿어진다.