Magnolol protects PC12 cells from hydrogen peroxide or 6-hydroxydopamine induced cytotoxicity

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ABSTRACT — *Magnoliae Cortex* contains a range of bioactive components including terpenes (e.g. α-, β- and γ-eudesmol), phenylpropanoids (e.g. honokiol and magnolol) and alkaloids (e.g. magnocurarine). We recently reported that pretreatment of PC12 cells with *Magnoliae Cortex* extract significantly suppresses cytotoxicity induced by H₂O₂ or 6-hydroxydopamine (6-OHDA) through the induction of drug-metabolizing and antioxidant enzymes. In this study, we investigated whether honokiol and magnolol, which are known to be active components of *Magnoliae Cortex*, induce drug-metabolizing enzymes and antioxidant enzymes in PC12 cells. We also examined the cytoprotective effect of honokiol and magnolol against H₂O₂ or 6-OHDA induced cell death in PC12 cells. Our results revealed that honokiol and magnolol induced both NAD(P)H:quinone oxidoreductase 1 (NQO1) and catalase enzyme activities in a concentration-dependent manner. Pretreatment of PC12 cells with magnolol suppressed toxicity induced by H₂O₂ or 6-OHDA. However, pretreatment of PC12 cells with honokiol showed only a suppressive effect on toxicity induced by H₂O₂. Our results suggest that the cytoprotective effect of *Magnoliae Cortex* extract on PC12 cells is mainly attributable to magnolol and only partially to honokiol.

Key words: Catalase, Honokiol, Magnolol, NAD(P)H:Quinone oxidoreductase 1, Oxidative stress, PC12 cells

INTRODUCTION

The bark of various *Magnolia* species (*Magnolia obtusata* Thunberg, *Magnolia officinalis* Rehder et Wilson, and *Magnolia officinalis* Rehder et Wilson var. Biloba Rehder et Wilson) is referred to as *Magnoliae Cortex* (Koboku in Japanese) in herbal medicine. *Magnoliae Cortex* contains many bioactive components, such as sesquiterpenoids (β-eudesmol), phenylpropanoids (honokiol and magnolol) (Fig. 1) and alkaloids (magnocurarine) (Tang and Eisenbrand, 1992). Indeed, *Magnoliae Cortex* elicits various pharmacological effects and can act as a muscle relaxant (Watanabe et al., 1983), sedative (Yang et al., 2007), anxiolytic compound (Kuribara et al., 1998) and antiulcer agent (Li et al., 2005). β-Eudesmol, a major component of *Magnoliae Cortex*, blocks H₃ receptors and inhibits gastric acid secretion as well as exhibiting antitumor activity (Nogami et al., 1986; Kimura et al., 1991). Honokiol and magnolol are known to display anxiolytic activity by increasing the concentration of serotonin (Xu et al., 2008). In addition, herbal medicines containing *Magnoliae Cortex*, such as Saiboku-to, Shinpi-to and Hengekoboku-to, are used to treat digestive tract diseases and neurological disorders.

Oxidative stress is thought to be involved in the onset of neurological disorders. Reactive oxygen species and lipid peroxidation is now thought to be a major cause of the onset of neurological disorders. Oxidative stress in cells is also known to be suppressed by drug metabolizing enzymes, such as NAD(P)H: quinone oxidoreductase 1 (NQO1) and glutathione S-transferase, and antioxidant enzymes, such as catalase, superoxide dismutase, and glutathione peroxidase. It is possible that neurological disorders caused by oxidative stress are prevented by compounds that can induce drug-metabolizing and antioxidant enzymes (Van Muiswinkel and Kuiperij, 2005).

We have recently demonstrated that *Magnoliae Cortex* extract induces NQO1 and catalase in PC12 cells.
Nishiyama et al., 2019. Pretreatment of PC12 cells with Magnoliae Cortex extract was found to significantly suppress cytotoxicity induced by H2O2 or 6-hydroxydopamine (6-OHDA). Based on our findings, we proposed that the pretreatment of PC12 cells with Magnoliae Cortex extract leads to an enhancement of its cytoprotective effect. In the present study, we focus on honokiol and magnolol, which are the major bioactive compounds in Magnoliae Cortex (Fig. 1). Here, we aimed to establish whether honokiol and magnolol have the potential to induce drug-metabolizing and antioxidant enzymes to account for the observed protective effect of Magnoliae Cortex against H2O2 and 6-OHDA induced cytotoxicity.

**MATERIALS AND METHODS**

**Materials**
Honokiol and magnolol were purchased from Nacalai Tesque Inc. (Kyoto, Japan). Rat adrenal medulla PC12 cells were obtained from American Type Culture Collection (Manassas, VA, USA). 6-OHDA and RPMI-1640 medium were purchased from Sigma-Aldrich (St Louis, MO, USA). Antibiotic-Antimycotic (100 ×) liquid, GlutaMAXTM-I Supplement were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Antibiotic-Antimycotic (100 ×) liquid, horse serum and GlutaMAXTM-I Supplement were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Antibiotic-Antimycotic (100 ×) liquid, horse serum and GlutaMAXTM-I Supplement were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Antibiotic-Antimycotic (100 ×) liquid, horse serum and GlutaMAXTM-I Supplement were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Antibiotic-Antimycotic (100 ×) liquid, horse serum and GlutaMAXTM-I Supplement were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Antibiotic-Antimycotic (100 ×) liquid, horse serum and GlutaMAXTM-I Supplement were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Antibiotic-Antimycotic (100 ×) liquid, horse serum and GlutaMAXTM-I Supplement were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Antibiotic-Antimycotic (100 ×) liquid, horse serum and GlutaMAXTM-I Supplement were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Antibiotic-Antimycotic (100 ×) liquid, horse serum and GlutaMAXTM-I Supplement were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Antibiotic-Antimycotic (100 ×) liquid, horse serum and GlutaMAXTM-I Supplement were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Antibiotic-Antimycotic (100 ×) liquid, horse serum and GlutaMAXTM-I Supplement were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Antibiotic-Antimycotic (100 ×) liquid, horse serum and GlutaMAXTM-I Supplement were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Antibiotic-Antimycotic (100 ×) liquid, horse serum and GlutaMAXTM-I Supplement were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Antibiotic-Antimycotic (100 ×) liquid, horse serum and GlutaMAXTM-I Supplement were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Antibiotic-Antimycotic (100 ×) liquid, horse serum and GlutaMAXTM-I Supplement were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Antibiotic-Antimycotic (100 ×) liquid, horse serum and GlutaMAXTM-I Supplement were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Antibiotic-Antimycotic (100 ×) liquid, horse serum and GlutaMAXTM-I Supplement were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Antibiotic-Antimycotic (100 ×) liquid, horse serum and GlutaMAXTM-I Supplement were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Antibiotic-Antimycotic (100 ×) liquid, horse serum and GlutaMAXTM-I Supplement were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Antibiotic-Antimycotic (100 ×) liquid, horse serum and GlutaMAXTM-I Supplement were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Antibiotic-Antimycotic (100 ×) liquid, horse serum and GlutaMAXTM-I Supplement were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Antibiotic-Antimycotic (100 ×) liquid, horse serum and GlutaMAXTM-I Supplement were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Antibiotic-Antimycotic (100 ×) liquid, horse serum and GlutaMAXTM-I Supplement were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Antibiotic-Antimycotic (100 ×) liquid, horse serum and GlutaMAXTM-I Supplement were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Honokiol and magnolol were purchased from Nacalai Tesque Inc. (Kyoto, Japan). Honokiol and magnolol were purchased from Nacalai Tesque Inc. (Kyoto, Japan). Honokiol and magnolol were purchased from Nacalai Tesque Inc. (Kyoto, Japan).

**Cytotoxicity of honokiol and magnolol**
Cytotoxicity was determined as previously described (Nishiyama et al., 2019). In brief, PC12 cells were plated at a density of 6.6 × 10⁴ cells per well in a collagen-coated 24-well cell culture dishes and cultured in RPMI 1640 medium supplemented with 1% Antibiotic-Antimycotic (100 ×) liquid, GlutaMAX™-I Supplement, 5% horse serum and 5% fetal bovine serum at 37°C under 5% CO₂ for 24 hr. After culturing, the cells were washed three times with 1 × PBS (-) and then incubated with honokiol or magnolol (0-15 µM in methanol solution) in serum-free medium for a further 24 or 48 hr. As a control, methanol was added to a final concentration of 0.1%. The cells were then cultured at 37°C under 5% CO₂ for 24 or 48 hr. After the incubation, the cell viability was measured according to the methods described below.

**Induction of enzyme activity by magnolol and honokiol**
Induction of enzyme activity was performed as previously described (Nishiyama et al., 2019). In brief, PC12 cells were seeded at a density of 2 × 10⁶ cells per dish (100 mm). After 24 hr of culture, the medium was removed and washed with 1 × PBS (-). The medium was changed to 10 mL of serum-free medium containing honokiol or magnolol (0-10 µM in methanol solution) and incubated for a further 24 hr. As a control, methanol was added to a final concentration of 0.1%. Cells were then cultured at 37°C under 5% CO₂ for 24 hr.

**Enzyme source**
Enzyme sources were prepared as previously described (Nishiyama et al., 2019). In brief, after culturing the cells, the medium was removed and the cells were washed with 1 × PBS (-). Four ml of 1 × PBS (-) was added and cells were detached from the culture dish using a cell scraper. The resulting cell suspension was centrifuged at 500 × g for 3 min. The cell pellet was resuspended in 300 µL of 50 mM Tris-HCl buffer (pH 7.4) containing 2 mM EDTA and 125 mM sucrose and then subjected to ultrasonic treatment (10 sec). The resultant homogenate was centrifuged at 15,000 × g for 15 min, and the supernatant used as the enzyme source.

**Protein concentration**
Protein concentration was determined using the Bio-Rad Protein Assay according to the manufacturer’s instructions.

**NQO1 and catalase activities**
NQO1 and catalase activities were determined by previously described methods (Nishiyama et al., 2008, 2010).

![Fig. 1. Chemical structures of honokiol and magnolol.](image-url)
Hydrogen peroxide and 6-hydroxydopamine induced cytotoxicity

Cytotoxicity induction was carried out as previously described (Nishiyama et al., 2019). In brief, PC12 cells were seeded at a density of 6.6 × 10⁴ cells/well in a 24-well cell culture dish and cultured at 37°C under 5% CO₂ for 72 hr. After culturing, the medium was removed, and 300 µL of serum-free medium containing honokiol or magnolol (0, 5, and 10 µM) was added to each well. After culturing for 24 hr, 100 µL of serum-free medium containing H₂O₂ (250 µM or 500 µM) or containing 6-OHDA (100 µM or 200 µM) was added to each well. After 3 hr (H₂O₂) or 24 hr (6-OHDA) incubation, cell viability was measured according to the methods described below.

Determination of cell viability

Cell viability was determined by using a CellTiter-Glo® Luminescent Cell Viability Assay kit according to the manufacturer’s protocols and as previously described (Nishiyama et al., 2010).

Statistical analysis

Data represent the mean ± SD of three independent experiments. The statistical significance of differences was calculated using Dunnett’s test. Values of p < 0.05 were considered significant.

RESULTS

Cytotoxicity of honokiol and magnolol

Cytotoxicity of honokiol and magnolol were determined using a CellTiter-Glo® Luminescent Cell Viability Assay kit (Fig. 2). PC12 cells were exposed to honokiol or magnolol at various concentrations for 24 and 48 hr. No significant cytotoxicity was observed up to 15 µM of honokiol after 24 hr treatment. In addition, at 48 hr, no significant cytotoxicity was observed at a concentration of 5 µM honokiol. However, a decrease in cell viability was observed at a concentration of greater than 7.5 µM honokiol. At 24 and 48 hr incubation, cell viability was measured according to the methods described below.

Enzyme induction by honokiol and magnolol

The induction of NQO1 and catalase activities mediated by either honokiol or magnolol was examined (Fig. 3). NQO1 and catalase activities were increased with increasing concentrations of honokiol and magnolol. After treat-

Fig. 2. Cytotoxic effect of honokiol and magnolol on PC12 cells. PC12 cells were treated with 0-15 µM of honokiol or magnolol. Cell viabilities at 24 and 48 hr were measured using a CellTiter-Glo® Luminescent Cell Viability Assay kit as described in Materials and Methods.
ment at the highest concentration of honokiol (10 μM), the activities of NQO1 and catalase were 2.22 ± 0.03-fold and 1.95 ± 0.15-fold greater than that of the control, respectively. Similarly, after treatment of magnolol (10 μM), the activities of NQO1 and catalase were 2.11 ± 0.31-fold and 1.70 ± 0.16-fold, greater than that of the control, respectively.

Cytoprotective effect of honokiol and magnolol

Both H2O2, which is a reactive oxygen species, and 6-OHDA, which is a neurotoxic substance, are cytotoxic agents. The protective effect of honokiol and magnolol against H2O2- or 6-OHDA-induced cytotoxicity of PC12 cells was evaluated by cell viability assays (Fig. 4). PC12 cells were pretreated for 24 hr with 10 μM of magnolol and then exposed to 250 μM or 500 μM H2O2. The cell viability of 250 μM or 500 μM H2O2 treated PC12 cells increased by 1.3-fold and 2.9-fold compared with that of untreated cells, respectively. Similarly, cells pretreated with 10 μM of honokiol and then exposed to 250 μM or 500 μM H2O2 displayed cell viability that was 1.2-fold and 1.1-fold greater, respectively than that of untreated cells. However, the protective effect against 6-OHDA-induced toxicity in PC12 cells was only observed after pretreatment with magnolol. Cells pretreated with 10 μM of magnolol and then exposed to 100 μM or 200 μM 6-OHDA displayed cell viability that was 1.2-fold and 2.0-fold greater, respectively than the untreated group.

DISCUSSION

The enzyme-inducing ability of honokiol and magnolol was investigated. Both NQO1 and catalase in PC12 cells were induced in a concentration-dependent manner by treatment with honokiol or magnolol (Fig. 3). The enzyme induction effect of honokiol and magnolol was similar to the results using Magnoliae Cortex extract that we previously reported (Nishiyama et al., 2019). Therefore, we examined whether honokiol and magnolol are present in Magnoliae Cortex extract. Our results verified that Magnoliae Cortex extract contained both compounds (data not shown). We next examined whether pretreatment of PC12 cells with magnolol or honokiol suppresses the cytotoxic effect of H2O2 or 6-OHDA. Protective effects of magnolol were observed in PC12 cells against toxicity induced by H2O2 or 6-OHDA. Pretreatment of PC12 cells with honokiol showed a suppressive effect on H2O2-induced cytotoxicity. By contrast, 6-OHDA-induced cytotoxicity was not suppressed by honokiol (Fig. 4).

Although both honokiol and magnolol have the ability to induce NQO1 and catalase activity, there was a difference in their cytoprotective effects. These findings could be due to differences in the apoptosis-inducing potency of PC12 cells (Park et al., 2009; Wolf et al., 2007) and in the relative levels of cytotoxicity between honokiol and magnolol (Fig. 2). Because honokiol itself reduced cell viability together with 6-OHDA, any cell protection afforded by drug-metabolizing and antioxidant enzyme induction will be nullified. Thus, although both honokiol and magnolol have the ability to induce drug-metabolizing and antioxidant enzymes, only magnolol showed cytoprotective effects against toxicity induced by H2O2 or 6-OHDA.

Nuclear factor-E2-related factor 2 (Nrf2)/antioxidant response element (ARE) pathway plays an important role in regulating the expression of a series of drug-metabolizing enzymes. Indeed, this pathway regulates the expression of both NQO1 and GST. Polyphenols are known to activate Nrf2, and radicals derived from hydroxyl groups of polyphenols are thought to be important for Nrf2 activating activity. However, although honokiol and magnolol are polyphenolic compounds, they showed an induction
effect on NQO1, but not on GST. In addition, treatment of PC12 cells with sulforaphane (SFN) or \( t \)-butylhydroquinone (\( t \)-BHQ) that have Nrf2 activating activity also showed induction of NQO1 activity but no induction of GST activity (results not shown). These results suggest GST may be less responsive to Nrf2 in PC12 cells.

In the case of treatment of PC12 cells with SFN or \( t \)-BHQ, the induction of catalase was not observed (results not shown). The induction of catalase by honokiol and magnolol is thought to be related to induction of a pathway other than Nrf2/ARE because Nrf2-activating compounds, such as SFN and \( t \)-BHQ, did not induce catalase. It was thought that biphenyl compounds containing allyl groups, such as magnolol and honokiol, play an important role in the induction of catalase. Honokiol is known to exhibit anti-inflammatory activity by suppressing the phosphatidylinositol 3-kinase (PI3K)/Akt pathway (Venkatesan et al., 2007). In addition, catalase is known to be down-regulated by activation of the PI3k/Akt pathway (Kim and Cho, 2008). Regulation of catalase expression by honokiol and magnolol may be due to the repressive action of the PI3K/Akt pathway.

Honokiol and magnolol are structural isomers that differ in the position of one the two hydroxyl groups (Fig. 1). The relationship between the basic structures of honokiol and magnolol and their enzyme-inducing ability is intriguing. Further studies to examine the detailed induction mechanisms of honokiol and magnolol are warranted.

**Fig. 4.** Protection of PC12 cells against the cytotoxicity of H2O2 and 6-OHDA by pretreatment with honokiol and magnolol. The PC12 cells were pretreated with 0-10 \( \mu \)M honokiol or magnolol for 24 hr, followed by incubation with 0, 250 and 500 \( \mu \)M H2O2 or 0, 100 and 200 \( \mu \)M 6-OHDA for another 3 hr (H2O2) or 24 hr (6-OHDA). After incubation, cell viabilities were determined using a CellTiter-Glo® Luminescent Cell Viability Assay kit. Data represent the mean ± SD of three independent experiments. *, Significantly different from control cells at each concentration at \( p < 0.05 \).
Conflict of interest---- The authors declare that there is no conflict of interest.

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