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Alkaloids from *Piper nigrum* Exhibit Antiinflammatory Activity via Activating the Nrf2/HO1 Pathway

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In the present study, ten alkaloids, namely chabamide (1), pellitorine (2), retrofractamide A (3), pyrroperine (4), isopiperolein B (5), piperamide C9:1 (8*E*) (6), 6,7dehydrobrachyamide B (7), 4,5-dihydropiperine (8), dehydropipernonaline (9), and piperine (10), were isolated from the fruits of *Piper nigrum*. Among these, chabamide (1), pellitorine (2), retrofractamide A (3), isopiperolein B (5), and 6,7dehydrobrachyamide B (7) exhibited significant inhibitory activity on lipopolysaccharide-induced nitric oxide (NO) production in RAW264.7 cells, with IC_{50} values of 6.8, 14.5, 30.2, 23.7, and 38.5 μM, respectively. Furthermore, compound 1 inhibited lipopolysaccharide-induced NO production in bone marrow-derived macrophages with IC_{50} value of 9.5 μM. Consistent with NO inhibition, treatment of RAW264.7 cells with chabamide (1), pellitorine (2), and 6,7-dehydrobrachyamide B (7) suppressed expression of inducible NO synthase and cyclooxygenase-2. Chabamide (1), pellitorine (2), and 6,7dehydrobrachyamide B (7) induced heme-oxygenase-1 expression at the transcriptional level. In addition, compound 1 induced the nuclear translocation of nuclear factor-E2-related factor 2 (Nrf2) and upregulated the expression of Nrf2 target genes, NAD(P)H:quinone oxidoreductase 1 and γ-glutamyl cysteine synthetase catalytic subunit, in a concentration-dependent manner in RAW264.7 cells. These findings suggest that chabamide (1) from *P. nigrum* exert antiinflammatory effects via the activation of the Nrf2/heme-oxygenase-1 pathway; hence, it might be a promising candidate for the treatment of inflammatory diseases. Copyright © 2017 John Wiley & Sons, Ltd.

Keywords: Piper nigrum; alkaloid; chabamide; antiinflammation; heme oxygenase-1; Nrf2.

INTRODUCTION

The inflammatory process is the response to an injurious stimulus. It is a well-known symptom of both infectious diseases and non-infectious diseases, including cancers and autoimmune disorders (Zedler and Faist, 2006). In this process, activated inflammatory cells, including neutrophils, eosinophils, mononuclear phagocytes, and macrophages, produce various kinds of inflammatory mediator, including nitric oxide (NO), prostaglandin E2, and cytokines such as interleukin (IL)1β, IL-6, and tumor necrosis factorα. Excess or unregulated production of these mediators mediates or exacerbates a wide variety of diseases, such as sepsis, rheumatoid arthritis, atherosclerosis, and even cancer (Tabas and Glass, 2013).

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The nuclear factor-E2related factor 2 (Nrf2) belongs to the basic-leucine zipper transcription factor family, which regulates the cellular antioxidant response by inducing the transcription of protective genes, such as heme oxygenase (HO)1, NAD(P)H:quinone oxidoreductase 1 (NQO1), and γglutamyl cysteine synthetase catalytic subunit (GCLC) (Jaramillo and Zhang, 2013). The activation of Nrf2 has been shown to be a key step in the cellular response against several common diseases, including inflammation, aging, diabetes, cardiovascular disease, acute pulmonary injury, neurodegenerative diseases, and cancer (Motohashi and Yamamoto, 2004; Lau *et al*., 2008; Kensler *et al*., 2007). Under basal conditions, Kelchlike ECHassociated protein-1 (Keap-1) binds to Nrf2 and carries it to the Keap-1–Cul3–E3 ubiquitin ligase complex. This complex regulates the level of Nrf2 protein to maintain it at a low level. In the presence of endogenous and exogenous stresses, Keap-1 can be modified, allowing Nrf2 to be released from the complex, translocated into nucleus, and dimerize with the small Maf protein to form the Nrf2–Maf heterodimer that regulates the transcription of target genes (Jaramillo and Zhang, 2013). Heme-oxygenase-1 is the major

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antiinflammatory and anti-oxidative enzyme that is regulated by activating Nrf2 (Kapturczak *et al*., 2004; Paine *et al*., 2010). Heme oxygenase catalyzes the rate-limiting enzymatic step of heme degradation and produces carbon monoxide, ferrous iron, and biliverdin, which is converted into bilirubin via biliverdin reductase (Paine *et al*., 2010). Heme-oxygenase-1 maintains cellular homeostasis and plays an important protective role in tissues to reduce oxidative injury and attenuate the inflammatory response (Abraham and Kappas, 2008; Paine *et al*., 2010). Expression of HO-1 or treatment with carbon monoxide suppresses production of the proinflammatory cytokines and chemokines in activated macrophages (Morse *et al*., 2003; Nakahira *et al*., 2006). Thus, targeting the Nrf2/HO-1 pathway with natural phytochemicals could be a potential strategy for the prevention or treatment of inflammatory diseases (Motterlini and Foresti, 2014).

Piper species have been used as both a traditional medicine and a seasoning in China, India, Vietnam, Latin America, and West Indies for thousands of years. These plants have been used to treat bronchitis, gastrointestinal diseases, rheumatism, and a chronic inflammation condition (Parmar *et al*., 1997). As the most widely used spice in the world, *Piper nigrum* L. (Piperaceae), commonly known as black pepper, has been employed in the traditional medicine of many countries. Similar to other *Piper* species, alkaloids are the main chemical constituents of *P. nigrum*. Piperine, the major chemical constituent of *P. nigrum*, has been extensively investigated and demonstrated that it has diverse activities, such as bioavailability enhancement (Allameh *et al*., 1992; Singh *et al*., 2015), protective activity in hepatoma cells (Singh *et al*., 1994), regulation of obesity-induced dyslipidemia (Shah *et al*., 2011), and antiinflammatory activity (Tasleem *et al*., 2014; Singh and Duggal, 2009). Meanwhile, there are limited studies regarding the bioactivities of the minor compounds in this plant. In the present study, we isolated ten alkaloids from the $CHCl_3$ fraction and investigated the antiinflammatory effect of these alkaloids and their underlying mechanism.

MATERIALS AND METHODS

Plant materials. The dried fruits of *P. nignum* was purchased from the Kwangmyungdang Medicinal Herbs Co. Ltd (Lot No.: K4041201406) of Ulsan-si, Korea, and identified by Professor Byung-Sun Min, Catholic University of Daegu, Korea. A voucher specimen (CUD-1007-1) is deposited at the Herbarium of the College of Pharmacy, Catholic University of Daegu, Korea.

General experimental procedures. The 1D and 2D NMR spectra were determined using a Varian Unity Inova 400 MHz and a Bruker Ascend™ 500-MHz spectrometer. Mass spectra were recorded using a JEOL JMS-AX 300L spectrometer. Silica gel (Merck, 63–200 μm particle size) and RP-C_{18} (Merck, 75 μm particle size) were used for column chromatography (CC). Thin layer chromatography was performed using Merck silica gel 60 F_{254} and RP-C_{18} F_{254} plates.

Extraction and isolation of alkaloids from *P. nigrum*. The dried fruits of *P. nigrum* (1 kg) were extracted with MeOH (3 × 2 L) at room temperature. After the solvent was evaporated under reduced pressure, the crude MeOH extract (100 g) was suspended in distilled water (0.5 L) and extracted with $CHCl_3$. The $CHCl_3$ extract was evaporated *in vacuo*, yielding 35 g of residue. Subsequently, this fraction was subjected to CC over silica gel (*n*hexane: acetone, 9:1 → 0:1) to yield nine fractions (PN1–PN9). The fractions PN6–8 were re-crystallized from acetone to yield whitish crystalline needles of **10** (13.30 g). Fraction PN2 was fractionated by RP-C_{18} CC (MeOH : H_2O, 1:2 → 1:1) resulting in **2** (37 mg). Fraction PN4 was fractionated by silica gel CC ($CHCl_3$: acetone, 8:1) to yield **5** (51.2 mg). After removing piperine from the combination of fractions PN6–8 by re-crystallization, the residue was chromatographed over silica gel CC (*n*-hexane : acetone, 9:1) to obtain 15 sub-fractions. Sub-fraction PN6.2 was subjected to RP-C_{18} CC (MeOH : H_2O, 1:1) furnishing **8** (15.8 mg) and **9** (7.2 mg). Fraction PN6.3 was subjected to silica gel CC ($CHCl_3$: EtOAc, 40:1) to obtain 16 sub-fractions. Fraction PN6.3.8 was fractionated by silica gel CC (*n*-hexane : acetone, 20:1) to yield ten sub-fractions. Sub-fraction PN6.3.8.6 was further purified by RP-C_{18} CC (MeOH : H_2O, 1:1) resulting in the isolation of **3** (16.8 mg). Fraction 6.3.12 was subjected to RP-C_{18} CC (MeOH : H_2O, 1:1) furnishing **1** (21.4 mg), **6** (5.0 mg), and **7** (3.5 mg). Fraction PN6.13 on silica gel CC (*n*-hexane : EtOAc, 1:1) yielded **4** (47.1 mg).

Cell culture. RAW264.7 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and were maintained in Dulbecco's Modified Essential Medium supplemented with penicillin (100 units/mL)–streptomycin (100 mg/mL) and 10% heat-inactivated fetal bovine serum (Cambrex, Charles City, IA, USA). The cells were maintained in a humidified atmosphere under 5% CO_2 at 37 °C.

Isolation of bone marrow-derived macrophages and culture. Six-week-old mice were sacrificed and bone marrow cells obtained from femurs and tibias by flushing the bone marrow cavities with culture medium. The marrow plugs were dispersed by passing a 25-gauge needle through them, and the cells were suspended by vigorous pipetting and washed. Subsequently, the cells were resuspended in modified eagle's medium containing 10% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 2 mM L-glutamine, and 10 ng/mL macrophage-colony stimulating factor (Prospec, East Brunswick, NJ, USA). Following incubation at 37 °C and 5% CO_2 overnight, non-adherent cells were collected, seeded, and incubated for 3 days with modified eagle's medium containing 30 ng/mL M-CSF which media was subsequently replaced every 2 days. Adherent cells were washed and seeded at plates for the experiments. Animal study in this paper was in accordance with procedures approved by the Institutional Animal Care and

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Use Committee of Catholic University of Daegu (protocol number IACUC-2016-044).

Measurement of nitric oxide and the cell viability assay. RAW264.7 cells or bone marrow-derived macrophages were seeded in 24-well plates at a density of 5×10^5 cells/well. The plates were pretreated with various concentrations of the test compounds for 30 min and then incubated for a further 24 h the presence or absence of 1 μg/mL lipopolysaccharide (LPS) (Lee *et al*., 2015a). Tin protoporphyrin (SnPP) or copper protoporphyrin (CuPP) was added to the plates along with the test compounds. Nitrite concentration in the culture supernatant was measured by the Griess reaction. The nitrite level in each sample was calculated from a standard curve generated with sodium nitrite. Cell viability was measured using the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT)-based colorimetric assay. The reduction of MTT to formazan within the cells was quantitated by measurement of the OD_{570} against OD_{630}.

Western blot analysis. Western blot analysis was performed as described previously (Nguyen *et al*., 2016). Briefly, cells were lysed in lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 5 mM sodium orthovanadate, 1% NP-40, and protease inhibitor cocktail (BD Biosciences, New Jersey, USA). In certain experiments, the cytoplasmic and nuclear fractions were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit (Thermo Fisher Scientific, Rockford, IL). Fifty micrograms of protein per lane was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane and probed with the appropriate antibodies. The signal was detected by using an enhanced chemiluminescent system (Intron, Seongnam, Korea).

Real-time quantitative polymerase chain reaction. RAW264.7 cells were stimulated with 1 μg/mL LPS in the presence of various concentrations of the test alkaloids. The cells were collected and total RNA was isolated using RNeasy Mini Kit from Qiagen (Santa Clarita, CA). One microgram of total RNA was used to synthesis the first stranded cDNA using Superscript real-time polymerase chain reaction (PCR) kit (Invitrogen, Carlsbad, CA, USA). Real-time quantitative PCR (qPCR) was performed to measure the mRNA expression levels of HO-1, NQO1, and GCLC as described previously (Nguyen *et al*., 2016). For amplification of HO-1, NQO1, and GCLC, the following primers were used: HO-1, 5′-CGC AAC AAG CAG AAC CCA-3′ (sense) and 5′-GCG TGC AAG GGA TGA TTT CC-3′ (antisense); NQO1, 5′-CGC CTG AGC CCA GAT ATT GT-3′ (sense) and 5′-GCA CTC TCT CAA ACC AGC CT-3′ (antisense); GCLC, 5′-GTC TGA CAC GTA GCC TCG GTA A-3′ (sense) and 5′-TGG CCA CTA TCT GCC CAA TT-3′ (antisense).

Statistical analysis. Statistical analyses were performed by one-way analysis of variance, followed by the Fisher least significant difference test. $P < 0.01$ was considered statistically significant. The results are presented as the mean ± standard error of mean.

RESULTS

Isolation of compounds from *P. nigrum*

In our studies in discovering bioactive compounds with antiinflammatory activity, we investigated the alkaloids from *P. nigrum*. Repeated CC of the $CHCl_3$-soluble fraction of *P. nigrum* on silica gel and RP-C_{18} led to the isolation of ten alkaloids. These alkaloids were elucidated as chabamide (**1**), pellitorine (**2**), retrofractamide A (**3**), pyrroperine (**4**), isopiperolein B (**5**), piperamide C9:1 (8*E*) (**6**), 6,7-dehydrobrachyamide B (**7**), 4,5-dihydropiperine (**8**), dehydropipernonaline (**9**), and piperine (**10**) by comparison with the published spectroscopic data (Fig. 1) (Ku *et al*., 2013; Srinivas and Rao, 1999; Yang *et al*., 2002; Pedersen *et al*., 2009; Ahmad *et al*., 1995; Tuntiwachwuttikul *et al*., 2006; Kayamba *et al*., 2013;

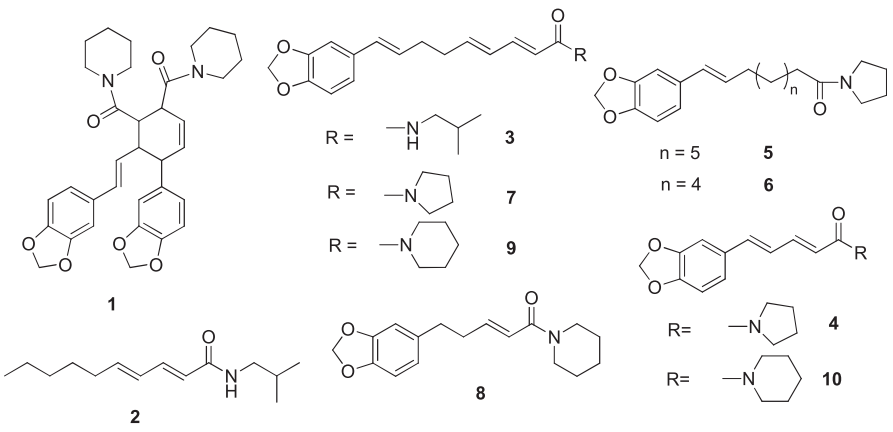

Figure 1. Chemical structures of isolated compounds from the fruits of *P.nigrum.*

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Rukachaisirikul *et al*., 2002; Singh *et al*., 1969; Berger and Sicker, 2009).

Effect of the isolated alkaloids on nitric oxide production and inducible nitric oxide synthase expression in lipopolysaccharide-stimulated RAW264.7 macrophages

Cells were stimulated with 1 μg/mL LPS for 24 h in the presence of compounds **1**–**10**, and the levels of NO in the culture supernatants were measured using Griess reaction. Compounds **1**, **2**, **3**, **5**, and **7** attenuated LPS-induced NO production, with IC_{50} values of 6.8 ± 0.7, 14.5 ± 1.9, 30.2 ± 3.5, 23.7 ± 3.3, and 38.5 ± 4.6 μM, respectively, whereas the other alkaloids did not show significant inhibitory activities in this cell system (Table 1). The $CHCl_3$ extract also suppressed LPS-induced NO production, with IC_{50} values of 32.4 ± 5.2 μg/mL. Celastrol was as a positive control compound, and it inhibited the production of NO in a concentration-dependent manner with an IC_{50} value of 1.0 μM (Lee *et al.*, 2010). The cytotoxic effects of the isolated compounds (**1**–**10**) were evaluated by the MTT assay. None of the isolated alkaloids significantly affected the viability of RAW 264.7 cells following 24 h of treatment in the presence or absence of LPS, even at a concentration of 20 μM (data not shown).

To determine whether chabamide (**1**), pellitorine (**2**), and 6,7dehydrobrachyamide B (**7**) suppress the expression of inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) in LPSstimulated RAW264.7 cells, cells were stimulated with LPS in the presence of these compounds at various concentrations (3–30 μM), and iNOS and COX-2 expression was determined by Western blot analysis. Cells treated with these compounds decreased the expression levels of iNOS and COX-2 protein in a concentration-dependent manner (Fig. 2). Our data showed that compounds **1**, **2**, and **7** exhibited significant inhibitory activities against LPS-induced iNOS and COX-2 expression in a concentration-dependent manner. Among them, compound **1** exhibited the most potent inhibitory activity against LPS-induced iNOS and COX-2 expression.

Table 1. Inhibitory effect of isolated compounds on nitric oxide production in RAW264.7 cells

Compounds	IC_{50}, μM [a]	Compounds	IC_{50}, μM [a]
1	6.8 ± 0.7	**7**	38.5 ± 4.6
2	14.5 ± 1.9	**8**	>50
3	30.2 ± 3.5	**9**	45.6 ± 5.4
4	>50	**10**	42.8 ± 4.9
5	23.7 ± 3.3	Celastrol[b)]	1.0 ± 0.1
6	>50		

[a]50% inhibition concentrations are expressed as the mean ± SEM (n = 3). RAW264.7 cells were pretreated with the indicated concentrations of alkaloids for 30 min, followed by stimulation with lipopolysaccharide (1 μg/ml). After 24-h incubation, the amounts of nitric oxide in the culture supernatants were determined using Griess reaction.

[b]Positive control.

Effect of alkaloids on heme-oxygenase-1 expression in RAW264.7 cells

Heme-oxygenase1 expression plays a vital role in cytoprotective response in diverse pathological conditions because of its antioxidant and antiinflammatory properties. Induction of HO1 is a protective response of tissue injury in models of allograft rejection and vascular inflammation through either the prevention of oxidative damage or immunomodulatory effects (Kapturczak *et al*., 2004). Heme-oxygenase-1 expression inhibits the production of inflammatory cytokines such as iNOS, COX-2, IL-6, and tumor necrosis factor-α (Kapturczak *et al*., 2004). To investigate the antiinflammatory mechanism of the active compounds, we evaluated whether the alkaloids isolated from *P. nigrum* could mediate the induction of HO-1. RAW264.7 cells were treated with the ten alkaloids at a concentration of 30 μM, and the expression level of HO-1 was determined by Western blot analysis. Among ten alkaloids, chabamide (**1**), pellitorine (**2**), and 6,7-dehydrobrachyamide B (**7**) at a concentration of 30 μM induced HO-1 protein expression, while the other compounds did not affect HO-1 expression (Fig. 3a). These results suggest that chabamide (**1**), pellitorine (**2**), and 6,7-dehydrobrachyamide B (**7**) might exert antiinflammatory effects via the induction of antiinflammatory HO-1.

Considering that chabamide (**1**) was the most active compound inducing HO1 and inhibiting NO production, it was selected for further investigations. We next examined whether chabamide (**1**) inhibits LPS-induced NO production in bone marrow-derived macrophages. Chabamide (**1**) also attenuated NO production in LPS-stimulated bone-marrow-derived macrophage, with IC_{50} value of 9.5 ± 1.4 μM (Fig. 3b). To investigate the effect of chabamide (**1**) on HO-1 induction in RAW 264.7 cells, cells were treated with increasing concentrations of **1**, and expression level of HO-1 protein was determined by Western blot analysis. Our data showed that chabamide (**1**) increased HO-1 expression level in a concentration-dependent manner (Fig. 3c). A time course of HO-1 protein induction with 10 μM chabamide (**1**) also demonstrated that HO-1 protein expression level increased after 3 h of treatment, reached a peak at 9 h, and returned to basal level at 24 h (Fig. 3d). We also measured whether chabamide (**1**) induced the expression of HO-1 protein at the transcriptional level. RAW264.7 cells treated with increasing concentrations of compound **1** increased the level of mRNA expression in a concentration-dependent manner (Fig. 3e)

Chabamide (1) induces activation of Nrf2 in RAW264.7 cells

Because Nrf2 plays an important role in HO-1 expression, we further evaluated whether chabamide (**1**) upregulates Nrf2 activation. Our data revealed that treatment of RAW264.7 cells with chabamide (**1**) increased the nuclear translocation of Nrf2 in a concentration-dependent manner (Fig. 4a). To further confirm that chabamide (**1**) exerts antiinflammatory effect via the activation of Nrf2, the expression levels of other Nrf2 target genes (NQO1 and GCLC) were determined in the presence of chabamide (**1**) by using real-

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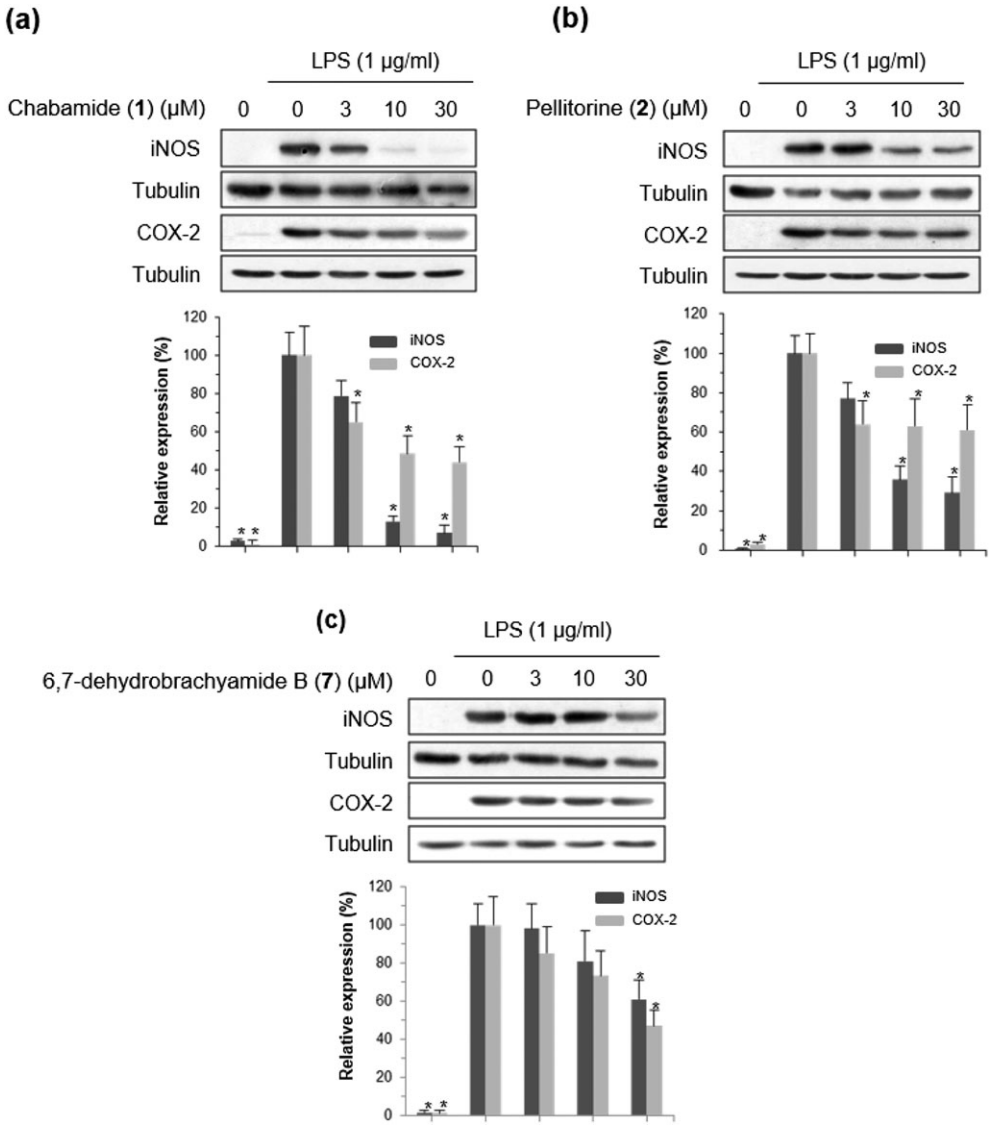

Figure 2. Effects of *P. nigrum* alkaloids on lipopolysaccharide (LPS)-induced expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). RAW264.7 cells were treated with indicated concentrations of chabamide (a), pellitorine (b), and 6,7-dehydrobrachyamide B (c), followed by stimulation with LPS (1 μg/mL). Subsequently, total lysates were prepared, and the expression level of iNOS and COX-2 was determined by Western blot analysis. Densitometry analyses of iNOS and COX-2 expression (normalized to α-tubulin) were expressed as the mean ± standard error of mean (SEM) of three independent experiments. $^*P < 0.01$ *versus* LPS only-treated control.

time qPCR analysis. Treatment of RAW264.7 cells with chabamide (**1**) increased the mRNA levels of GCLC and NQO1 in a concentration-dependent manner (Fig. 4b and c). These results suggest that chabamide (**1**) induces HO-1 expression by activating Nrf2.

Inhibition of heme-oxygenase-1 attenuates the inhibitory effects of chabamide (1) on nitric oxide production

A specific HO-1 inhibitor (SnPP) and an inactive compound (CuPP) were used to determine whether the induction of HO-1 could be responsible for the antiinflammatory effects of chabamide (**1**). As shown in Fig. 5, SnPP partially reversed the inhibitory effect of chabamide (**1**) on NO production in LPS-stimulated RAW264.7 cells. In contrast, CuPP did not affect the antiinflammatory effect of chabamide (**1**). These results suggest that Nrf2/HO-1 induction could be an underlying mechanism responsible for the antiinflammatory effect of chabamide (**1**) on LPS-induced inflammatory response in macrophages.

DISCUSSION

Increasing evidences have suggested that the induction of HO-1, a major antiinflammatory and anti-oxidative enzyme, which is regulated by Nrf2 activation, has a key role in inflammatory responses via the prevention of oxidative damage or via a local immunomodulatory influence on infiltrating inflammatory cells (Kapturczak *et al*., 2004). Although the exact mechanisms involved in the antiinflammatory effects of HO-1 remain unclear, one or more by-products of the HO-1 pathway have been evaluated as factors that could inhibit the inflammatory response (Abraham and Kappas, 2008; Ryter *et al*., 2002).

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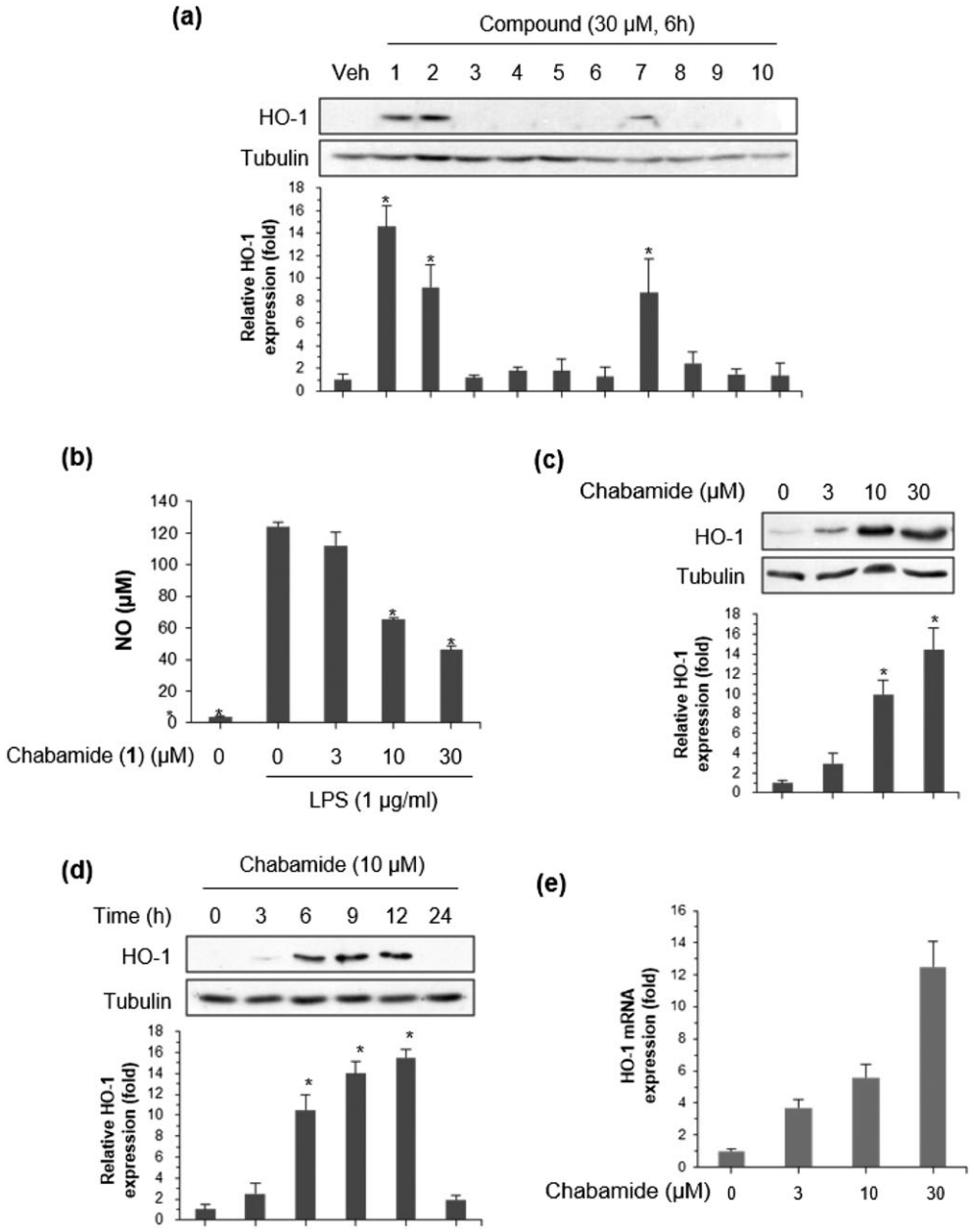

Figure 3. Effect of 10 alkaloids from *P. nigrum* on heme-oxygenase (HO)1 expression. (a) RAW264.7 cells were treated with 30 μM indicated alkaloids. Total cell lysates were prepared, and the expression level of HO-1 was determined by Western blot analysis. Densitometry analyses of HO-1 expression (normalized to α-tubulin) were expressed as the mean ± SEM of three independent experiments. $*P < 0.01$ *versus* vehicle-treated control. (b) Bone marrow-derived macrophages were pretreated with indicated concentrations of chabamide for 30 min, followed by the stimulation with LPS (1 μg/mL). After 24-h incubation, the amount of nitric oxide (NO) in culture supernatants was determined. RAW264.7 cells were treated with the indicated concentrations of chabamide (c) or 10-μM chabamide for the indicated periods of time (d). Total cell lysates were prepared, and the level of HO-1 expression was determined by Western blot analysis. Densitometry analyses of HO-1 expression (normalized to α-tubulin) were expressed as the mean ± SEM of three independent experiments. $*P < 0.01$ *versus* vehicle-treated control. (e) RAW264.7 cells were treated with the indicated concentrations of chabamide. A real-time qPCR analysis was performed to determine the mRNA expression level of HO-1. Data represent mean ± SEM of three independent experiments. $*P < 0.01$ *versus* vehicle-treated control.

The fruits of *P. nigrum* have been widely used in traditional medicine of many countries due to their biological activities including antiinflammatory effects. The pharmacological activities including antiinflammatory activity of piperine, the first alkaloid isolated from the *Piper* species, have been extensively investigated (Lee *et al.*, 1984); however, the pharmacological activities of the minor compounds remain to be elucidated. In the current study, we investigated the antiinflammatory activity of the minor alkaloids isolated from *P. nigrum* and evaluated its underlying mechanism. Among the ten alkaloids isolated from *Piper*, chabamide (**1**) with isolation efficiency of 21.4 mg/kg from dried material significantly induced HO-1 expression at the concentrations that suppressed NO production and iNOS expression in LPS-stimulated RAW264.7 cells. This compound exerted the inhibition on NO production on both cloned (RAW264.7 macrophage) and naïve (bone marrow-derived macrophage) cells. Moreover, compound **1** increased the nuclear translocation of Nrf2 and expression of Nrf2 target genes such as NQO1 and GCLC. Our data also showed that inhibition of HO-1 activity by SnPP, a HO-1 inhibitor, abrogated the antiinflammatory activities of compound **1** in RAW264.7 cells. These

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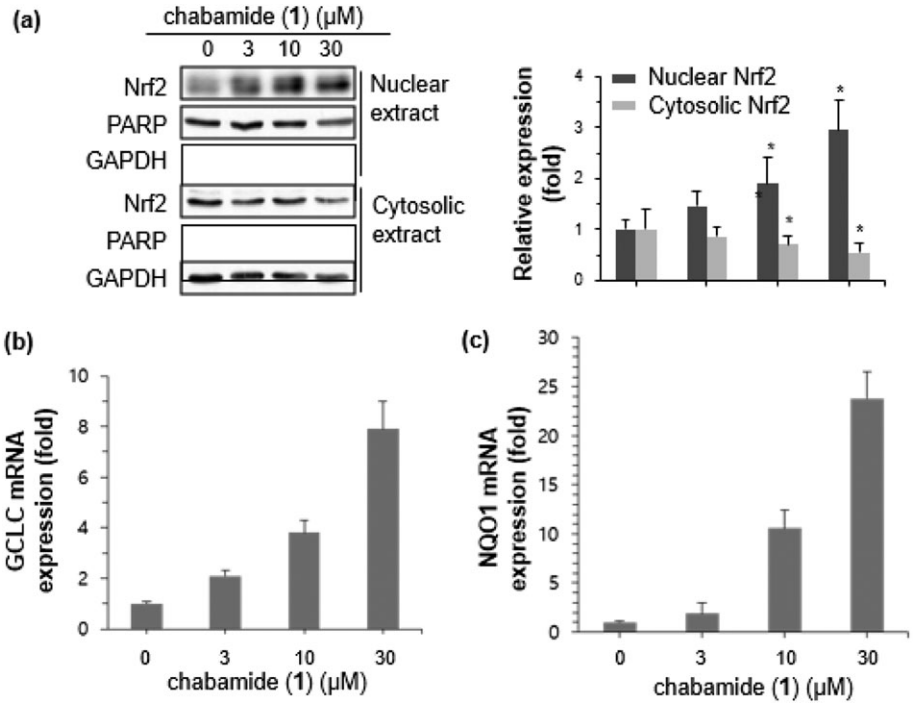

Figure 4. Chabamide induces nuclear factor-E2-related factor 2 (Nrf2) activation. (a) RAW264.7 cells were treated with the indicated concentrations of chabamide. Nuclear and cytoplasmic extracts were prepared, and the expression level of Nrf2 was determined by Western blot analysis. Poly(adenosine diphosphate-ribose) polymerase (PARP) was used as a nuclear marker and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a cytosolic protein marker. Densitometry analyses of Nrf2 expression (normalized to PARP or GAPDH) were expressed as the mean ± SEM of three independent experiments. $*P < 0.01$ *versus* vehicle-treated control. (b and c) RAW264.7 cells were treated with the indicated concentrations of chabamide, and then a real-time qPCR analysis was performed to determine the mRNA expression level of γ-glutamyl cysteine synthetase catalytic subunit (b) and NAD(P)H:quinone oxidoreductase 1 (c). Data represent mean ± SEM of three independent experiments. $*P < 0.01$ *versus* vehicle-treated control.

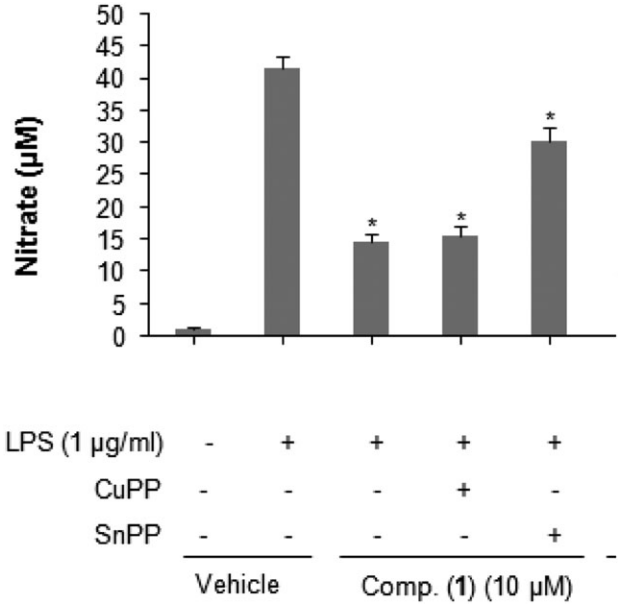

Figure 5. Inhibition of HO-1 attenuates the antiinflammatory activity of alkaloids from *Piper*. RAW264.7 cells were treated with chabamide in the presence of tin protoporphyrin or copper protoporphyrin and then stimulated with LPS (1 μg/mL). The amounts of NO in the culture supernatant were determined. Data represent mean ± SEM of three independent experiments. $*P < 0.01$ *versus* vehicle-treated control.

evidences suggest that the induction of HO-1 is at least partially responsible for the antiinflammatory effects of the *Piper* alkaloids.

Previously, chabamide (**1**) has been isolated from *Piper chaba* (Tuntiwachwuttikul *et al.*, 2006), but this is the first study that isolated this alkaloid from *P. nigrum*. Although its activities and underlying mechanism have not been established, chabamide (**1**) is an interesting compound with various pharmacological activities. Recently, the cytotoxic effect of compound **1** has been evaluated in K562 (a human leukemia cell line) cells with IC_{50} of 10.8 μM, and it was reported that compound **1** significantly inhibited cell proliferation by cell cycle arrest in the G0/G1 phase and increased the expression of apoptosis-related proteins. This provides the evidences that compound **1** might be a potential therapeutic agent for the treatment of leukemia (Ren *et al.*, 2015). In the present study, we showed that compound **1** exhibited antiinflammatory activity by inducing HO-1. The transcription factor Nrf2 plays a predominant role in HO-1 expression. Chabamide (**1**) induced the mRNA and protein expression of HO-1 in a concentration- and time-dependent manner. Moreover, chabamide (**1**) increased the nuclear translocation of Nrf2 as well as the expression of Nrf2 target genes such as NQO1 and GCLC, suggesting that chabamide (**1**) might exert antiinflammatory effect by activating the Nrf2/HO-1 pathway.

Several compounds, both natural and synthetic, exert effects on the inflammatory responses via Nrf2 signaling (Li *et al.*, 2014; Arlt *et al.*, 2013), contributing to antitumor, antioxidative, and antiinflammatory activities. Different skeletons of natural chemicals have been reported to be associated this pathway. Nguyen *et al.* showed that 7-methoxy-(9*H*-β-carbolin-1-yl)-(*E*)-1-propenoic acid, a β-carboline alkaloid isolated from *Eurycoma longifolia*, exerted antiinflammatory effects

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by activating the Nrf2/HO-1 pathway (Nguyen *et al.*, 2016). In addition, sappanone A, a homoisoflavanone compound isolated from the heartwood of *Caesalpinia sappan* induced the protein and mRNA expression of HO-1, increased the nuclear translocation of Nrf2, and upregulated the expression of Nrf2 target genes (Lee *et al.*, 2015b).

Although the isolation of bioactive compounds of *P. nigrum* has been reported previously, chabamide (**1**), a dimeric alkaloid, was isolated for the first time from this plant. Moreover, this is the first study that revealed the relationship between Nrf2 regulation and the antiinflammatory effect of this alkaloid. These findings revealed that the isolated alkaloids could be beneficial in the treatment of inflammation disease; however, further *in vivo* studies and clinical trials are required.

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Conflict of Interest

The authors declare no conflict of interest.

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