Original Article

Okanin, a chalcone found in the genus *Bidens*, and 3-penten-2-one inhibit inducible nitric oxide synthase expression via heme oxygenase-1 induction in RAW264.7 macrophages activated with lipopolysaccharide

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Excess production of nitric oxide by activated macrophages *via* inducible nitric oxide synthase leads to the development of various inflammatory diseases. Heme oxygenase-1 expression *via* activation of nuclear factor-erythroid 2-related factor 2 inhibits nitric oxide production and inducible nitric oxide synthase expression in activated macrophages. Okanin is one of the most abundant chalcones found in the genus *Bidens* (Asteraceae) that is used as various folk medications in Korea and China for treating inflammation. Here, we found that okanin (possessing the α-β unsaturated carbonyl group) induced heme oxygenase-1 expression *via* nuclear factor-erythroid 2-related factor 2 activation in RAW264.7 macrophages. 3-Penten-2-one, of which structure, as in okanin, possesses the α-β unsaturated carbonyl group, also induced nuclear factor-erythroid 2-related factor 2-dependent heme oxygenase-1 expression, while both 2-pentanone (lacking a double bond) and 2-pentene (lacking a carbonyl group) were virtually inactive. In lipopolysaccharide-activated RAW264.7 macrophages, both okanin and 3-penten-2-one inhibited nitric oxide production and inducible nitric oxide synthase expression via heme oxygenase-1 expression. Collectively, our findings suggest that by virtue of its α-β unsaturated carbonyl functional group, okanin can inhibit nitric oxide production and inducible nitric oxide synthase expression *via* nuclear factor-erythroid 2-related factor 2-dependent heme oxygenase-1 expression in lipopolysaccharide-activated macrophages.

Key Words: okanin, heme oxygenase-1, inducible nitric oxide synthase, nuclear factor-erythroid 2-related factor 2, α-β unsaturated carbonyl group

Okanin (chemical structure shown in Fig. 1) is one of the most abundant chalcone [1,3-diaryl-2-propen-1-one] compounds found in the genus *Bidens* (Asteraceae) that has been used as various folk medications in Korea and China for treating inflammation, malaria, hypertension, diabetes, peptic ulcer, snake bite and smallpox.[1–3] Although ethnopharmacological studies carried out with plants of the genus *Bidens* have demonstrated anti-inflammatory activity,[4–10] whether okanin would also have anti-inflammatory activity is not yet investigated.

Activated macrophages play a pivotal role in a variety of inflammatory diseases *via* the excess production of pro-inflammatory cytokines and the prolonged expression of inducible pro-inflammatory enzymes, such as inducible nitric oxide synthase (iNOS).[11,12] The inflammatory enzyme iNOS, once expressed in activated macrophages, can generate a large amount of nitric oxide (NO) for a long period.[11,12] The free radical NO has been implicated as an important inflammatory mediator in the process of macrophage-mediated inflammation.[12] However, uncontrolled/excess NO production by activated macrophages leads to the development of various inflammatory diseases.[12,13] Hence, pharmacological inhibition of NO production and/or iNOS expression is a promising strategy for reducing the potentially harmful pro-inflammatory activity of macrophages.[13]

Heme oxygenase-1 (HO-1) is an inducible enzyme that catalyzes the rate-limiting step in the conversion of free heme into carbon monoxide, free iron, and biliverdin, which is subsequently catabolized into bilirubin by biliverdin reductase.[14] In addition to its primary role in heme degradation, HO-1 has been also recognized to play other important roles in resolution of inflammation, which has been demonstrated in HO-1 knockout mice and a human case of genetic HO-1 deficiency.[15,16] Particularly, HO-1 and its enzymatic metabolites are the critical regulators of inflammation, with activated macrophages acting as the critical targets.[14–16] Nuclear factor-erythroid 2-related factor 2 (Nrf2) is a redox sensitive transcription factor that is critical for induction of the gene encoding HO-1.[17] Under normal conditions, Nrf2 is sequestered in the cytoplasm by forming a complex with the negative regulator of Nrf2, Klech-like ECH-associated protein 1 (Keap1).[17] This complex is disrupted by several naturally occurring compounds, and Nrf2 is liberated and translocated to the nucleus where it binds to antioxidant response element (ARE) sequences in the *ho-1* gene promoter.[18,19]

Recently, a series of naturally occurring compounds from medicinal plants have been reported to induce HO-1 expression in different cell types, and in some of these studies, HO-1 has been shown to mediate their anti-inflammatory properties through inhibition of NO production and iNOS expression.[20–24] In the present study, we have also reported that okanin inhibited NO production and iNOS expression through Nrf2-dependent HO-1

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expression in RAW264.7 macrophages activated with the endotoxin lipopolysaccharide (LPS).

Materials and Methods

Chemicals and reagents. Previously, we had isolated okanin from the ethanol extract of the flowers of *Bidens bipinnata* L.[(25)] and this compound was used in this study. 3-Penten-2-one, 2-pentanone, 2-pentene, Dulbecco's modified Eagle's medium (DMEM), hemin, LPS (*Escherichia coli* 055:B5), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT), *N*-acetyl-*L*-cysteine (NAC) were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies against iNOS, HO-1, Nrf2, lamin B and β-actin and small interfering RNA (siRNA) products against Nrf2 and HO-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All other reagents used were of analytical grade.

Cell culture. RAW264.7 macrophages were obtained from the American Type Culture Collection (Manassas, VA). The cells were cultured in DMEM supplemented with 2 mM glutamine, antibiotics (100 U/ml of penicillin A and 100 U/ml of streptomycin) and 10% heat-inactivated fetal bovine serum (Gibco/BRL, Rockville, MD) and maintained at 37°C in a humidified incubator containing 5% CO_2.

Cell viability assay. Cell viability was determined by a modified MTT reduction assay. MTT is a pale yellow substance that is reduced by living cells to yield a dark blue formazan product. This process requires active mitochondria, and even fresh dead cells do not reduce significant amounts of MTT. RAW264.7 macrophages were cultured in a 96-well flat-bottom plate at concentration of 5×10^5 cells/ml. After 12 h of preconditioning, the cells were treated with various concentrations of okanin for 24 h. Thereafter, culture medium was aspirated and 100 µl of MTT dye (1 mg/ml in phosphate-buffered saline) was added; the cultures were incubated for 4 h at 37°C. The formazan crystals produced through dye reduction by viable cells were dissolved using acidified isopropanol (0.1 N HCl). Index of cell viability was calculated by measuring the optical density of color produced by MTT dye reduction at 570 nm.

Nitrite assay. The nitrite concentration in the medium was measured as an indicator of NO production according to the Griess reaction. One hundred microliters of each supernatant was mixed with the same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water); absorbance of the mixture at 550 nm was determined with an ELISA plate reader.

Western blot analysis. RAW264.7 macrophages were incubated with or without reagents. They were harvested, washed ice-cold phosphate-buffered saline (PBS) and kept on ice for 1 min. The suspension was mixed with buffer A (10 mM HEPES, pH 7.5, 10 mM KCl, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 5 µg/ml aprotinin, 5 µ/ml pepstatin, and 10 µg/ml leupeptin) and lysed by three freeze-thaw cycles. Cytosolic fraction was obtained by centrifugation at $12,000 \times g$ for 20 min at 4°C. The pellets were re-suspended in buffer C (20 mM HEPES, pH 7.5, 0.4 M NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 5 µg/ml aprotinin, 5 µg/ml pepstatin, and 10 µg/ml leupeptin) on ice for 40 min and centrifuged at $14,000 \times g$ for 20 min at 4°C. The resulting supernatant was used as soluble nuclear fraction. Protein content was determined with BCA protein assay reagent (Pierce, Rockford, IL). Total cellular or nuclear fractions were separated on 10% SDS-polyacrylamide gels, and transferred to the nitrocellulose membranes (Amersham Biosciences, Inc., Piscataway, NJ). The membrane was then blocked in blocking buffer containing 20 mM sodium phosphate buffer, pH 7.6, 150 mM NaCl, 0.1% Tween 20, and 5% nonfat dry milk for 1 h at room temperature. Thereafter, the membrane was incubated with antibodies against HO-1 (1:1000 dilution), Nrf2 (1:500 dilution), iNOS (1:1000 dilution), lamin B (1:1000 dilution)

or β-actin (1:1000 dilution) at 4°C overnight. The membrane was then washed four times with PBS-Tween 20 buffer and further incubated with secondary antibodies for 1 h at room temperature. Specific bands were detected using enhanced chemiluminescence detection system (Amersham Biosciences), and the membrane was exposed to X-ray film.

Transfection of siRNA. RAW264.7 macrophages were grown in 6- or 12-well plates and transiently transfected with Nrf2 siRNA or HO-1 siRNA mixed with siRNA transfection reagent (Santa Cruz Biotechnology) according to the manufacturer's instructions. After incubation at 37°C and 5% CO_2 for 30 h, cells were treated with reagents. The samples were then prepared for Western blot analysis.

Nrf2-ARE binding assay. The amount of Nrf2 available in the nucleus to bind to AREs was determined using the ELISA-based TransAM™ Nrf2 Kit (Active Motif, Carlsbad, CA). Briefly, nuclear extracts were added to wells containing the immobilized consensus ARE oligonucleotide. A primary antibody against Nrf2 was added to each well. Then a secondary antibody conjugated to horseradish peroxidase that binds to the primary antibody was added to each well. The signal was detected at 450 nm, and Nrf2-ARE binding was reported as optical density (OD) units at 450 nm.

Statistical analysis. Data are expressed as means ± SE. One-way analysis of variance procedures were used to assess significant differences among treatment groups. For each treatment showing a statistically significant effect, the Newman-Keuls test was used for comparisons of multiple group means. The criterion for statistical significance was set at $p < 0.05$ or 0.01.

Results

Okanin and 3-penten-2-one, but not 2-pentanone and 2-pentene, can induce HO-1 expression. The chemical structures of okanin and other three synthetic compounds tested in this study are shown in Fig. 1. Both okanin and 3-penten-2-one contain an α-β unsaturated carbonyl group in the central five-carbon chain. In comparison with 3-penten-2-one, 2-pentanone and 2-pentene lack a double bond and a carbonyl group, respectively. 3-penten-2-one, 2-pentanone and 2-pentene were used to explore a possible mechanism of action of okanin. In RAW264.7 macrophages, okanin and 3-penten-2-one, but not 2-pentanone and 2-pentene, exhibited cytotoxicity at more than 40 µM (data not shown).

Treatment of RAW264.7 macrophages with non-cytotoxic concentrations of okanin (0.5–10 µM) for 6 h resulted in a
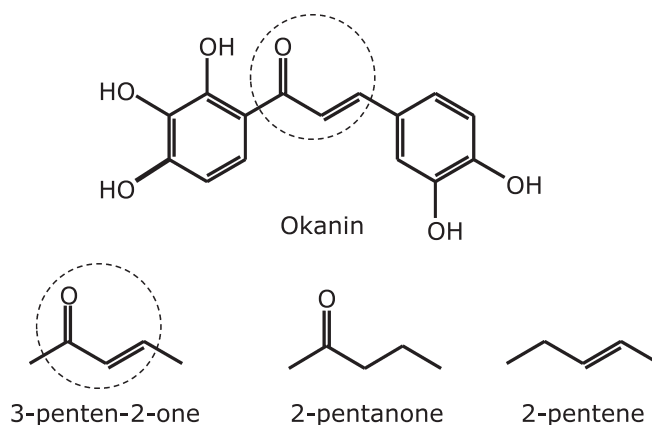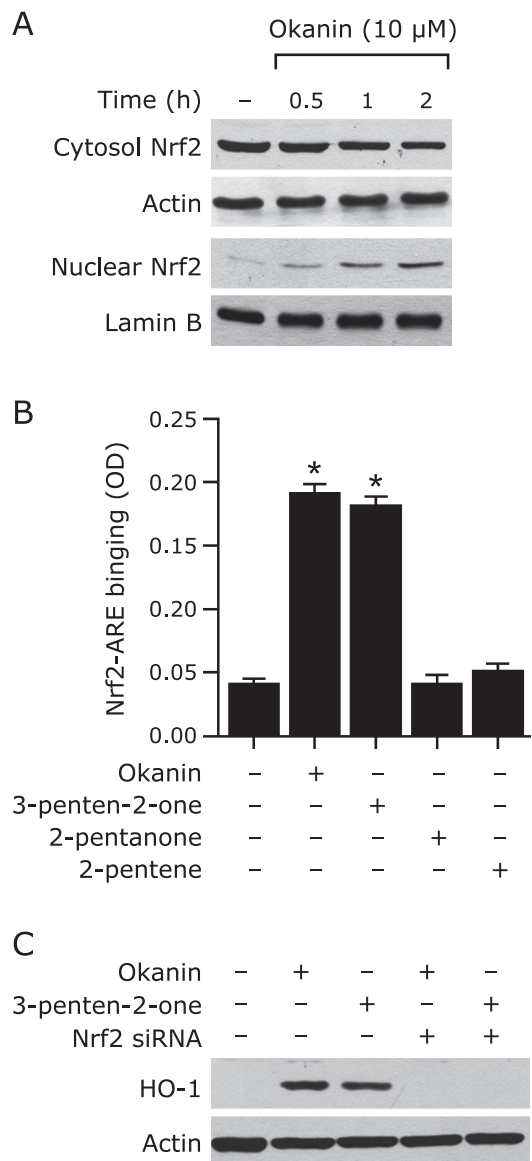

Fig. 1. Chemical structures of okanin, 3-penten-2-one, 2-pentanone and 2-pentene. In comparison with 3-penten-2-one, 2-pentanone and 2-pentene lack a double bond and a carbonyl group, respectively. The α-β unsaturated carbonyl group is marked with dotted circles.
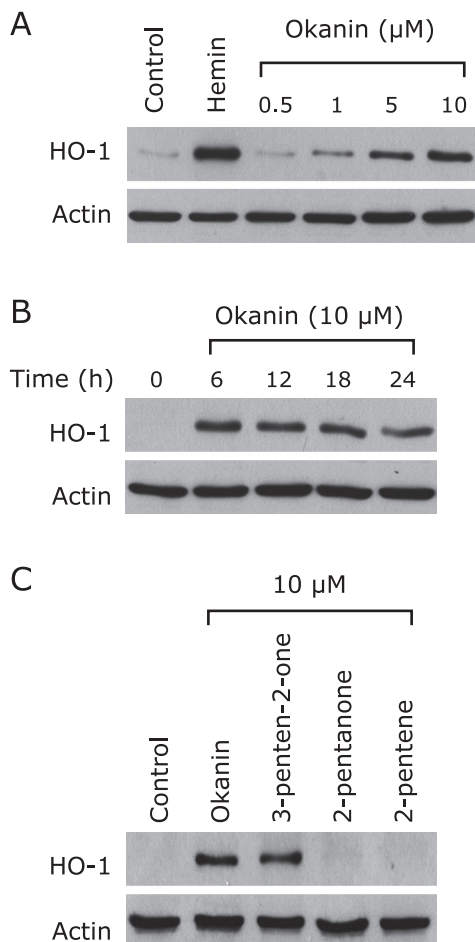
Fig. 2. Effects of okanin, 3-penten-2-one, 2-pentanone and 2-pentene on HO-1 expression. (A) RAW264.7 macrophages were incubated for 6 h without or with 10 μM of hemin, a well-known inducer of HO-1, or indicated concentrations of okanin. (B) RAW264.7 macrophages were incubated without or with 10 μM of okanin for indicated time periods. (C) RAW264.7 macrophages were incubated for 6 h without or with 10 μM of okanin, 3-penten-2-one, 2-pentanone, or 2-pentene. Western blot analysis for HO-1 expression was performed as described under Materials and Methods. Blots shown are representative of three independent experiments.

Fig. 3. Effects of okanin and 3-penten-2-one on Nrf2 activation. (A) RAW264.7 macrophages were incubated without or with 10 μM of okanin for indicated time periods. Cytosolic and nuclear extracts were isolated, and the levels of Nrf2 protein were determined by Western blot analysis. (B) RAW264.7 macrophages were incubated for 2 h without or with 10 μM of okanin, 3-penten-2-one, 2-pentanone, or 2-pentene. Nrf2 activation was assessed in nuclear extracts by analysis of Nrf2 binding to ARE. Data are expressed as mean ± SE from 3 to 4 experiments. *$p<0.05$ with respect to untreated control group. (C) RAW264.7 macrophages were transiently transfected with Nrf2 siRNA, and then exposed to 10 μM of okanin or 3-penten-2-one for 6 h. Western blot analysis for HO-1 expression was performed as described under Materials and Methods. Blots shown are representative of three independent experiments.

concentration-dependent increase in HO-1 protein levels, as confirmed by Western blot analysis (Fig. 2A). Induction of HO-1 expression by okanin was robust at 6 h and slightly decreased over time (Fig. 2B). Similarly, treatment with 3-penten-2-one for 6 h resulted in a marked increase in HO-1 protein levels (Fig. 2C). In contrast, neither 2-pentanone nor 2-pentene induced HO-1 expression (Fig. 2C).
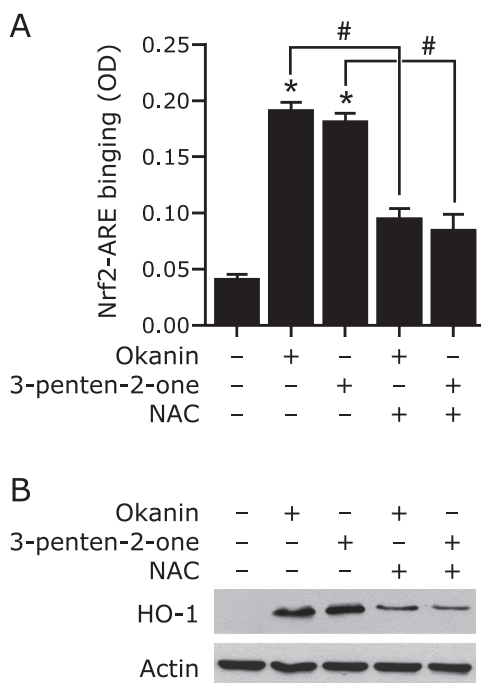
Okanin and 3-penten-2-one can induce Nrf2 activation that mediates HO-1 expression. A time-dependent increase in Nrf2 protein in the nucleus, along with a significant decrease in Nrf2 protein in the cytoplasm, was observed when RAW264.7 macrophages were incubated with 10 μM okanin (Fig. 3A). Similarly, 3-penten-2-one also increased levels of nucleus Nrf2 protein (not shown). To study Nrf2 activation, the nuclear extracts of RAW264.7 macrophages were isolated and the binding of Nrf2 to ARE was quantified by using a TransAM™ assay. Both okanin and 3-penten-2-one increased Nrf2 activation, whereas 2-pentanone and 2-pentene had no significant effect on Nrf2 activation (Fig. 3B). We next conducted the experiments of Nrf2 knockdown by using siRNA to investigate whether HO-1 expression would be

inhibited. HO-1 expression by okanin and 3-penten-2-one was prevented by siRNA against Nrf2 (Fig. 3C).

Nrf2 activation and HO-1 expression in RAW264.7 macrophages observed with okanin and 3-penten-2-one may be attributable to changes in the production of reactive oxygen species and the redox environment, and/or to direct thiol modification of Keap1 and other proteins.[26] To better characterize Nrf2 activation and HO-1 expression, we treated normal RAW264.7 macrophages

Fig. 4. Effects of NAC on Nrf2 activation and HO-1 expression by okanin and 3-penten-2-one. (A) RAW264.7 macrophages were treated with 10 μM of okanin or 3-penten-2-one together with 1 mM NAC for 2 h. Nrf2 activation was assessed in nuclear extracts by analysis of Nrf2 binding to ARE. Data are expressed as mean ± SE from 3 to 4 experiments. *$p<0.05$ with respect to untreated control group. #$p<0.05$. (A) RAW264.7 macrophages were treated with 10 μM of okanin or 3-penten-2-one together with 1 mM NAC for 6 h. Western blot analysis for HO-1 expression was performed as described under Materials and Methods. Blots shown are representative of three independent experiments.

with high concentrations of NAC, a thiol donor, along with okanin and 3-penten-2-one. The NAC treatment partially inhibited Nrf2 activation (Fig. 4A) and HO-1 expression (Fig. 4B) by okanin and 3-penten-2-one.

Okanin and 3-penten-2-one inhibit LPS-induced NO production and iNOS expression *via* HO-1 expression. RAW264.7 macrophages were pre-incubated for 6 h with okanin and activated with LPS. Okanin inhibited LPS-induced NO production (Fig. 5A) and iNOS expression (Fig. 5B) in a dose-dependent manner. Similarly, 3-penten-2-one also inhibited LPS-induced NO production (Fig. 5C) and iNOS expression (Fig. 5D). Unlike 3-penten-2-one, 2-pentanone and 2-pentene had no effect on LPS-induced NO production and iNOS expression (not shown). To explore a potential involvement of HO-1 expression in the observed inhibitory effects of okanin and 3-penten-2-one on LPS-induced NO production and iNOS expression, siRNA against HO-1 was used to inhibit cellular synthesis of HO-1 protein. As shown in Fig. 6, inhibition of HO-1 expression by siRNA significantly reversed the inhibitory effects of okanin and 3-penten-2-one on LPS-induced NO production and iNOS expression.
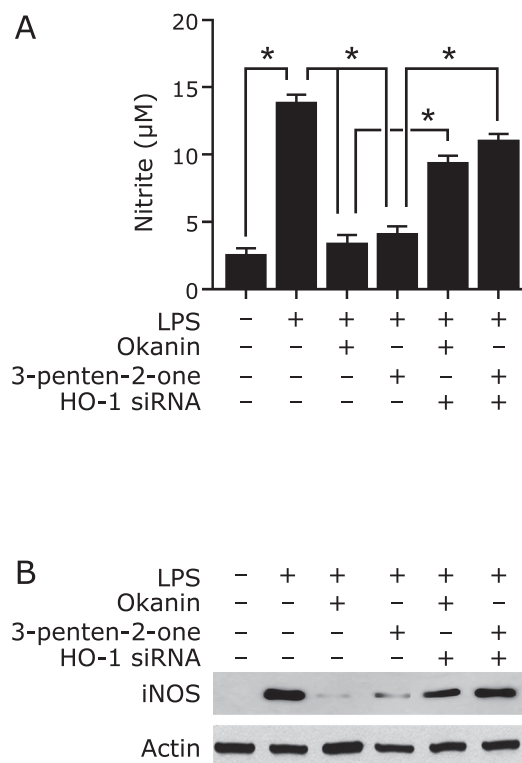
Discussion

The present study demonstrates that okanin, one of the most abundant chalcones found in the genus *Bidens* (Asteraceae), induced the expression of the anti-inflammatory HO-1 in RAW264.7 macrophages and that HO-1 expression by okanin was associated with its inhibition of LPS-induced NO production and iNOS expression. We have also studied a possible mechanism
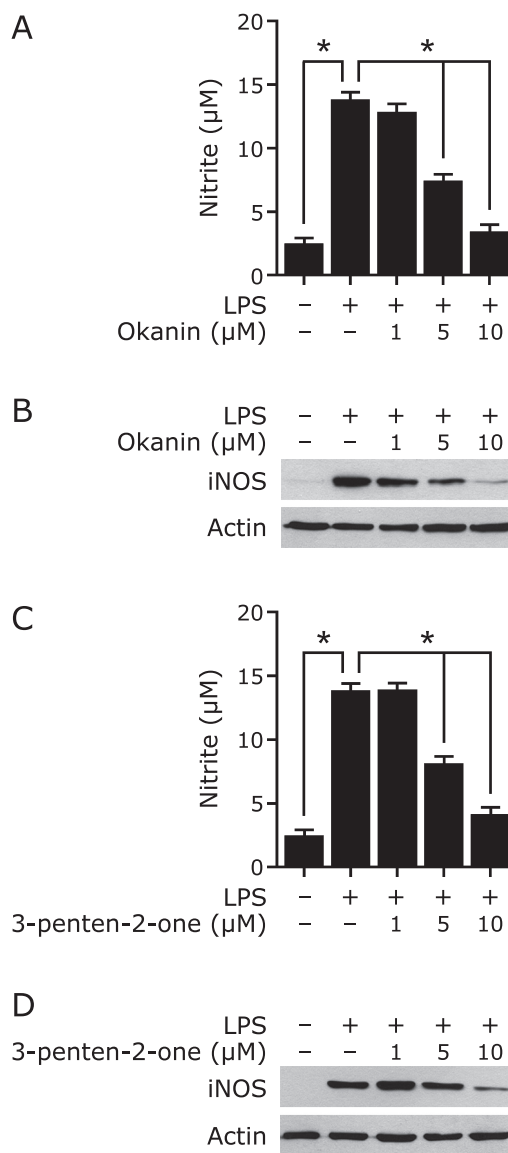
Fig. 5. Effects of okanin and 3-penten-2-one on NO production and iNOS expression. RAW264.7 macrophages were pre-incubated for 6 h without or with indicated concentrations of okanin or 3-penten-2-one, and then exposed to 1 μg/ml of LPS for 6 h (B, D) or 18 h (A, B). Nitrite assay for NO production (A, C) and Western blot analysis for iNOS expression (B, D) were performed as described under Materials and Methods. Blots shown are representative of three independent experiments. Data are expressed as mean ± SE from 3 to 4 experiments. *$p<0.05$.

behind the HO-1 expression by okanin, and demonstrated that HO-1 expression was dependent on Nrf2 activation.

From a chemical viewpoint, okanin consists of two hydroxy-phenyl rings linked by a three-carbon unit forming an α-β unsaturated carbonyl moiety (see Fig. 1). The α-β unsaturated carbonyl group (also known as classical Michael acceptor) has been shown to be a crucial structure of okanin, because both 2-pentanone (lacking a double bond) and 2-pentene (lacking a carbonyl group) were virtually inactive in inducing HO-1 expression; on the contrary, 3-penten-2-one (possessing an α-β unsaturated carbonyl group) had an ability to induce HO-1 expression (Fig. 2C). In fact, some phytochemicals carrying this reactive functional group, such as avicins,[27] curcumin,[28] costunolide,[29]

A

LPS	−	+	+	+	+	+
Okanin	−	−	+	−	+	−
3-penten-2-one	−	−	−	+	−	+
HO-1 siRNA	−	−	−	−	+	+

B

LPS	−	+	+	+	+	+
Okanin	−	−	+	−	+	−
3-penten-2-one	−	−	−	+	−	+
HO-1 siRNA	−	−	−	−	+	+

iNOS

Actin

Fig. 6. Effect of HO-1 siRNA on the inhibitory actions of okanin and 3-penten-2-one in NO production and iNOS expression. RAW264.7 macrophages transiently transfected with HO-1 siRNA were pre-incubated for 6 h without or with 10 μM of okanin or 3-penten-2-one, and then activated for 6 h (B) or 18 h (A) with 1 μg/ml of LPS. Nitrite assay for NO production (A) and Western blot analysis for iNOS expression (B) were performed as described under Materials and Methods. Blots shown are representative of three independent experiments. Data are expressed as mean ± SE from 3 to 4 experiments. *$p < 0.05$.

and dehydrocostus lactone,[30] have been reported to induce HO-1 expression. Moreover, 2'-hydroxychalcone, of which chemical structure is similar to that of okanin with the exception of four hydroxyl substituents on two aromatic rings, has been previously reported to induce HO-1 expression in RAW264.7 macrophages,[31] implying that okanin would have a chemical property resembling that of 2'-hydroxychalcone. Thus, we speculate that okanin may be effective in inducing HO-1 expression, at least in part, because it bears the α-β unsaturated carbonyl group.

At present, the mechanism(s) by which the α-β unsaturated carbonyl group of okanin is capable of inducing HO-1 expression is not clear. It has been hypothesized that compounds containing the α-β unsaturated carbonyl group may cause the disruption of the Keap1-Nrf2 complex, most likely through interaction with the thiols present on Keap1, thereby resulting in Nrf2 activation.[32–34] This hypothesis is further supported by our observation that Nrf2 activation by okanin was partially abrogated by high concentrations of NAC (Fig. 4A). Presumably, the reaction of okanin with a large excess of NAC may result in reversible formation of the thiol-okanin product, allowing for a limited amount of free okanin to be available for interaction with Keap1. Additionally, there are

many experimental studies showing that α-β unsaturated carbonyl-containing compounds are capable of inducing HO-1 expression through Nrf2 nuclear translocation.[27,29,30,32] Thus, the present study examined whether okanin would also activate Nrf2 nuclear translocation in RAW264.7 macrophages. Indeed, both okanin and its core component (i.e., 3-penten-2-one) induced Nrf2 nuclear translocation, and in turn increased Nrf2-ARE biding activity. Moreover, Nrf2 activation by okanin was associated with HO-1 expression, because RAW264.7 macrophages in which the cellular synthesis of Nrf2 was suppressed by siRNA did not display any change in HO-1 expression following exposure to okanin (Fig. 3C). Our results, therefore, suggest that HO-1 expression by okanin may occur through the Nrf2-dependent pathway.

HO-1 represents a major protective factor because of its anti-oxidant and anti-inflammatory properties.[14–16] It has been shown that HO-1 expression has anti-inflammatory effects that are presumably mediated by its enzymatic metabolites, such as carbon monoxide and biliverdin/bilirubin.[35,36] In activated macrophages, HO-1 expression has been shown to inhibit the release of pro-inflammatory cytokines and the expression of pro-inflammatory enzymes, including iNOS.[36] Our data shows that knocking down HO-1 largely blocked the inhibition of okanin and its core component (i.e., 3-penten-2-one) on NO production and iNOS expression in LPS-activated RAW264.7 macrophages (Fig. 6), suggesting that the inhibitory effect of okanin on LPS-induced NO production and iNOS expression may be, at least in part, mediated through HO-1 expression. Our data are in agreement with other studies demonstrating that HO-1 expression in activated macrophages has anti-inflammatory effects[37–40] and, therefore, provide one of possible mechanisms that could explain the anti-inflammatory effects of medicinal plants of the genus *Bidens*.[1–10]

In summary, the results of the present study demonstrate that: (i) okanin, one of the most abundant chalcones found in medicinal plants of the genus *Bidens*, induces HO-1 expression through Nrf2-dependent pathway in RAW264.7 macrophages; (ii) the α-β unsaturated carbonyl functional group of okanin is crucial for Nrf2-dependent HO-1 expression; and (iii) okanin and its core component (i.e., 3-penten-2-one) inhibit LPS-induced NO production and iNOS expression by inducing HO-1 expression.

Conflicts of Interests

Abbreviations

ARE	antioxidant response element
DMEM	Dulbecco's modified Eagle's medium
HO-1	heme oxygenase-1
iNOS	inducible nitric oxide synthase
Keap1	Klech-like ECH-associated protein 1
LPS	lipopolysaccharide
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide
NAC	*N*-acetyl-*L*-cysteine
NO	nitric oxide
Nrf2	nuclear factor-erythroid 2-related factor 2
OD	optical density
PBS	phosphate-buffered saline
SE	standard error
siRNA	small interfering RNA

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