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Induction of heme oxygenase-1 mediates the anti-inflammatory effects of the ethanol extract of *Rubus coreanus* in murine macrophages

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Abstract

Foods of plant origin, especially fruits and vegetables, draw increased attention because of their potential benefits to human health. The aim of the present study was to determine *in vitro* anti-inflammatory activity of four different extracts obtained from the fruits of *Rubus coreanus* (aqueous and ethanol extracts of unripe and ripe fruits). Among the four extracts, the ethanol extract of unripe fruits of *R. coreanus* (URCE) suppressed nitric oxide (NO) and prostaglandin E_2 (PGE_2) production in lipopolysaccharide (LPS)-stimulated RAW264.7 murine macrophages. We also demonstrated that URCE by itself is a potent inducer of heme oxygenase-1 (HO-1). Inhibition of HO-1 activity by tin protoporphyrin, a specific HO-1 inhibitor, suppressed the URCE-induced reductions in the production of NO and PGE_2 as well as the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2). Our data suggest that URCE exerts anti-inflammatory effects in macrophages via activation of the HO-1 pathway and helps to elucidate the mechanism underlying the potential therapeutic value of *R. coreanus* extracts.

Keywords: *Rubus coreanus*; Heme oxygenase-1; Inducible nitric oxide synthase; Prostaglandin E_2; Inflammation; Macrophages

The inflammatory response protects the host against tissue injury and microbial invasion. This response should be short-lived, as failing to do so results in pathogenesis of many immune-related diseases like inflammatory bowel disease, multiple sclerosis, and rheumatoid arthritis. An appropriate inflammatory response depends upon careful control by a number of mediators, including eicosinoids, oxidants, cytokines, and lytic enzymes, secreted by the inflammatory cells such as macrophages and neutrophils [1–3]. Among the inflammatory mediators, reactive oxygen species, nitric oxide (NO) in particular, initiates a wide range of toxic oxidative reactions causing tissue injury [4,5]. NO is produced by nitric oxide synthase (NOS). Of the three major classes of NOS, the neuronal and endothelial isoforms are constitutively expressed, generating low NO concentrations, while expression of the inducible NOS (iNOS) is stimulated by bacterial products or inflammatory cytokines, leading to the production of high levels of NO [4]. As NO is involved in various pathological processes, including inflammation [4,5], suppression of NO production could be a target of potential anti-inflammatory drugs.

Heme oxygenase (HO) is the rate-limiting enzyme in heme catabolism with consequent generation of biliverdin, free iron, and carbon monoxide (CO). Three mammalian HO isoforms have been identified, one of which, HO-1, is a stress-responsive protein endowed with important cytoprotective effects [6]. Interestingly, expression of HO-1 in

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macrophages is an element of the repair processes that occur during the resolution of inflammation that leads to healing and tissue repair [6]. Accumulating evidence has indicated that both mouse and human deficiencies of HO-1 expression have a phenotype of an increased inflammatory state [7,8]. In addition, it has been reported that HO-1 induction negatively regulates iNOS expression [9]. Several lines of evidence also demonstrated that down-regulation of iNOS-dependent NO production by HO-1 appears to be mediated by the product, CO [10,11].

The discovery of many new drugs against various diseases has been inspired by the use of fruits and vegetables in traditional medicine. *Rubus* is one of a hundred genera in family Rosaceae, subfamily Rosoideae, tribr Potentilleae; there are 250 species of *Rubus* established across the world. Extracts of the leaves and fruit of *Rubus* species have been used in various countries as natural remedies to treat diabetes, infections, colic, and burns [12]. Specifically, *Rubus coreanus* is a perennial shrub distributed in the southern part of the Korean peninsula, and unripe fruit of *R. coreanus* has been used in traditional herbal medicine for the treatment of diabetes mellitus and sexual disinclination [13].

In this study, we examined the effects of four different extracts of *R. coreanus* on LPS-induced inflammatory responses in RAW264.7 murine macrophages. We demonstrated that the ethanol extract of unripe *R. coreanus*, URCE, induces the expression of HO-1, thereby inhibiting expression inflammatory mediators such as NO and PGE_2, as well as iNOS and COX-2 expression. Our present data indicate that induction of HO-1 in response to URCE may contribute to the reduction of inflammatory responses.

Materials and methods

Chemicals and reagents. All reagents were from obtained Sigma–Aldrich (St. Louis, MO) unless otherwise indicated. CellTiter 96® Aqueous One Solution, Griess reagent, and Access RT-PCR system were purchased from Promega (Madison, WI). PGE_2 enzyme-linked immunosorbent assay (ELISA) kits were manufactured by R&D (Minneapolis, MI). Cell extraction buffer was obtained from Biosource International (Camarillo, CA). Antibodies against iNOS and COX-2 were purchased from BD Biosciences (San Jose, CA). Rabbit polyclonal antibody against HO-1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Tin protoporphyrin (SnPP) was obtained from Porphyrin Products Inc. (Logan, UT).

Preparation of Rubus coreanus extracts. Ripe and unripe fruits of *R. coreanus* Miq. (Rosaceae) were collected from Hyeong Seuong (Gangwondo, Korea) on August 10, 2005 and July 10, 2005, respectively. The plant was identified and authenticated by emeritus professor Hyung Jun Ji, Department of Natural Products Science, Seoul National University, Republic of Korea. A voucher specimen (B 1003) of the plant was deposited at the Regional Innovation Center at Hallym University. We prepared the following four extracts of *R. coreanus* fruits: ethanol (URCE) and aqueous (URCW) extracts of unripe fruits, and ethanol (RCE) and aqueous (RCW) extracts of completely ripened fruits. In brief, the shade-dried unripe (50 g) or the lyophilized ripe (50 g) fruits were pulverized and extracted three times with 1 L of 95% ethanol or distilled water to give the following extract yields: URCE, 14.0%; URCW, 31.6%; RCE, 49.0%; and RCW, 54.4%.

Cell culture, cytotoxicity assay, and nitrite, and PGE_2 measurement. RAW264.7 murine macrophages were obtained from Korea Cell Bank (Seoul, Korea), and cultured in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO_2. Cell viability was determined with 0, 50, 100, 200, and 400 μg/ml of various *R. coreanus* extracts described our previous report [14]. The nitrite and PGE_2 in cell culture medium were measured as described our previous report [14].

Immunoblotting analysis. Cells were lysed by using cell extraction buffer supplemented with protease inhibitors. Immunoblot assay was carried out as described previously [14].

RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was isolated at times indicated using RNeasy kit from Quagen according to manufacturer's instructions. RT-PCR was performed by Acess RT-PCR system according to manufacturer's instructions with primers specific for the HO-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers used correspond to the mouse HO-1 (5′-GCTTGTTGCGCTCATTCTCC-3′ and 5′-GCCACCAAGGAGGT ACACATA-3′) and mouse GAPDH (5′-TGAAGGTCGGTGTGAACG CATTTGGC-3′ and 5′-CATGTAGGCCATGAGGTCCACCAC-3′). The amplified products were separated by electrophoresis on a 1.5% agarose gel and detected under UV light.

Determination of bilirubin in culture medium. RAW264.7 cells were plated a density of 5×10^5 cells/well into 24-well plates and treated with various concentrations of URCE or hemin (50 μM) for 12 h. The culture supernatant (500 μl) was then collected in a tube and 250 mg of barium chloride was added. After vortexing, 0.75 ml of benzene was added and the tubes mixed again. The benzene phase containing the extracted bilirubin was separated from the aqueous phase by centrifugation for 30 min at 13,000*g*. Bilirubin was determined spectrophotometrically as a difference in absorbance between 450 and 600 nm ($\varepsilon_{450} = 27.3\ mM^{-1}\ cm^{-1}$) [15].

Data analysis. Data are presented means ± SEM from at least three independent experiments. Values were evaluated by one-way ANOVA, followed by Duncan's multiple range tests using the GraphPad Prism 4.0 software. SnPP-untreated and -treated groups were compared between themselves using Student's *t* test. Differences were considered significant at $P < 0.01$.

Results

Rubus coreanus extracts were not toxic to murine macrophages

Murine RAW264.7 macrophages were chosen for use in an investigation of the anti-inflammatory effects of the extracts of *R. coreanus*. Four different extracts of *R. coreanus* were prepared; ethanol extracts of unripe (URCE) and ripe fruits (RCE), and water extracts of unripe (URCW) and ripe fruits (RCW). We first examined whether the four extracts have cytotoxicity in RAW264.7 cells. No notable cytotoxicity was observed when the cells were exposed up to 400 μg/ml URCW, URCE, RCW, or RCE for 24 h (Fig. 1).

URCE profoundly inhibits LPS-induced production of nitrite and PGE_2

In order to assess the anti-inflammatory activities of the four extracts, RAW264.7 cells were challenged with LPS in the presence or absence of the four extracts, and the level of nitrite, a stable metabolite of NO, in the medium was measured. As shown in Fig. 2A, among the four extracts, only

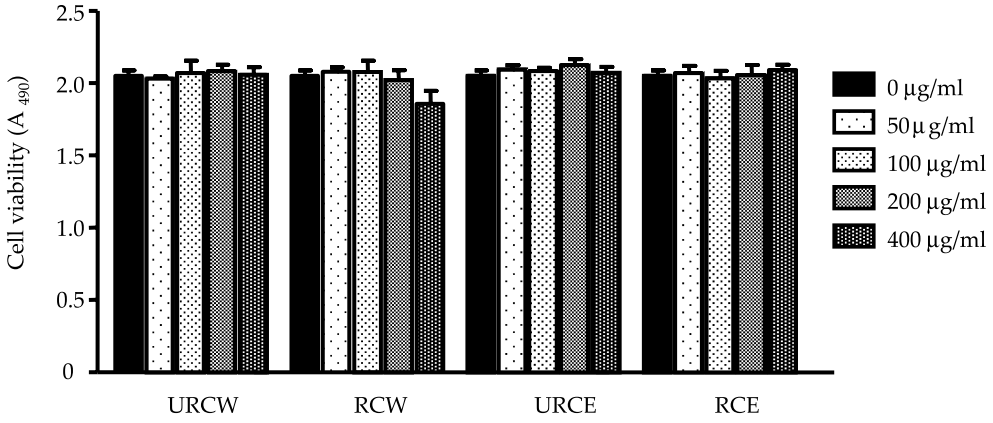

Fig. 1. Effects of *Rubus coreanus* extracts on viability of RAW264.7 murine macrophage. Cells were treated with various concentrations of different *R. coreanus* extracts for 24 h. Cell viability was determined as described in Materials and methods. The results are expressed as means ± SEM from four independent experiments. URCW, unripe *R. coreanus* water extract; RCW, ripe *R. coreanus* water extract; URCE, unripe *R. coreanus* ethanol extract; RCE, and ripe *R. coreanus* ethanol extract.

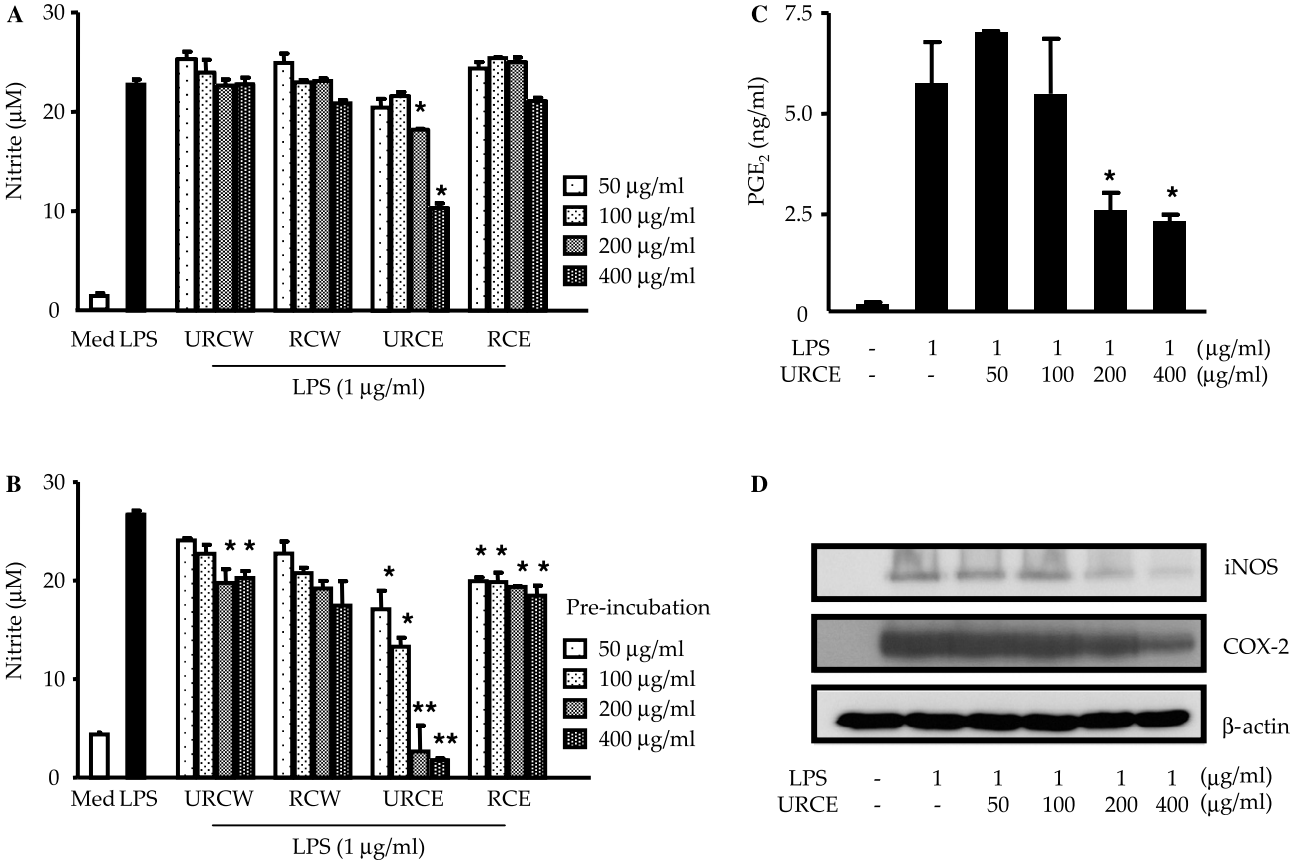

Fig. 2. Effects of *Rubus coreanus* extracts on LPS-induced NO and PGE_2 production in RAW264.7 cells. (A) Cells were treated with 0, 50, 100, 200, or 400 μg/ml of various *R. coreanus* extracts in the presence or absence of 1 μg/ml LPS for 18 h. (B,C) Cells were exposed to various *R. coreanus* extracts for 8 h, followed by 18 h culture in the presence of 1 μg/ml LPS. The concentrations of nitrite (B) and PGE_2 (C) were measured as described in Materials and methods. (D) Proteins (30 μg) of total cell lysates were resolved on 7% and 10% SDS–PAGE for iNOS and COX-2, respectively. Immunoblotting was performed as described in Materials and methods. β-Actin was used as a loading control. Significantly different from cells treated with 1 μg/ml LPS in the absence of extract, $^{*}P < 0.01$ and $^{**}P < 0.001$.

URCE effectively suppressed the LPS-induced nitrite production in a dose-dependent manner. However, no inhibitory effect of nitrite production was observed in RAW264.7 cells treated with URCW, RCW, or RCE. Interestingly, the inhibition of nitrite generation was much higher when RAW264.7 cells were pre-incubated with URCE for 8 h prior to the LPS challenge (Fig. 2B) compared to when the extract was added concurrently with LPS. Pretreatment of cells with higher concentrations (200 or 400 μg/ml) of URCE led to a decrease in nitrite production to the basal

level (Fig. 2B). Because, among the four extracts, only URCE profoundly inhibited nitrite production, we focused on URCE in the rest of the experiments.

Since PGE_2 is another inflammatory mediator, we investigated the effects of URCE on PGE_2 production in LPS-stimulated RAW264.7 cells. Similar to the findings related to nitrite accumulation, treatment of RAW264.7 cells with 1 μg/ml LPS led to a significant increase in PGE_2 production. Consistent with the nitrite accumulation, pre-incubation of cells with 200 or 400 μg/ml of URCE prior to LPS exposure resulted in a dramatic decrease in the secretion of PGE_2 (Fig. 2C).

Next, we carried out immunoblot analyses to determine whether the inhibitory effects of NO and PGE_2 production in URCE-treated cells were related to changes in the protein levels of iNOS and COX-2, respectively. The expression of iNOS and COX-2 was reduced in a URCE dose-dependent manner in LPS-stimulated RAW264.7 cells (Fig. 2D). These results indicated that the reduced levels of iNOS and COX-2 were responsible for the decreases in NO and PGE_2 production, respectively, in cells treated with URCE.

URCE-induced the gene HO-1 expression and bilirubin production

Based on observations that the inhibition of nitrite production was stronger in cells pretreated with URCE, we hypothesized that URCE-induced certain molecules to regulate inflammatory responses. Since HO-1 is known to contribute to inflammatory reactions [7–11], changes in HO-1 protein levels were examined in URCE-treated cells. Immunoblot analysis showed that URCE increased HO-1 protein levels in a dose-dependent manner in RAW264.7 cells (Fig. 3A). The stimulating action of 400 μg/ml URCE displayed time-dependence, occurring after 1 h exposure, peaking at 8 h, and being decreased at 18 h (Fig. 3B). In order to determine if induction of HO-1 gene expression by URCE appeared at the transcription level, RT-PCR using specific primer of HO-1 and GAPDH was performed. URCE-induced a time-dependent increase in HO-1 mRNA levels that was apparent 8 h after treatment, at 400 μg/ml URCE (Fig. 3C). Activation of HO-1 enzyme converts heme to bilirubin, an indicative of HO activity. In order to identify if there is activation of HO-1 enzyme activity in URCE-treated cells, production of bilirubin in cell culture media was measured by a $BaCl_2$ extraction method [15]. Results in Fig. 3D showed that bilirubin production in 200 or 400 μg/ml URCE significantly increased in RAW264.7 cells. Hemin, a HO-1 inducer, also profoundly induced the bilirubin production. These results indicated that URCE is a potent HO-1 inducer, and induction of HO-1 gene expression by URCE was examined at both the transcription and translation levels.

HO-1 mediates URCE suppression of LPS-stimulated inflammatory responses

Because pre-incubation with URCE dramatically suppressed the production of NO and PGE_2, and the expression of iNOS and COX-2 in LPS-stimulated cells, a

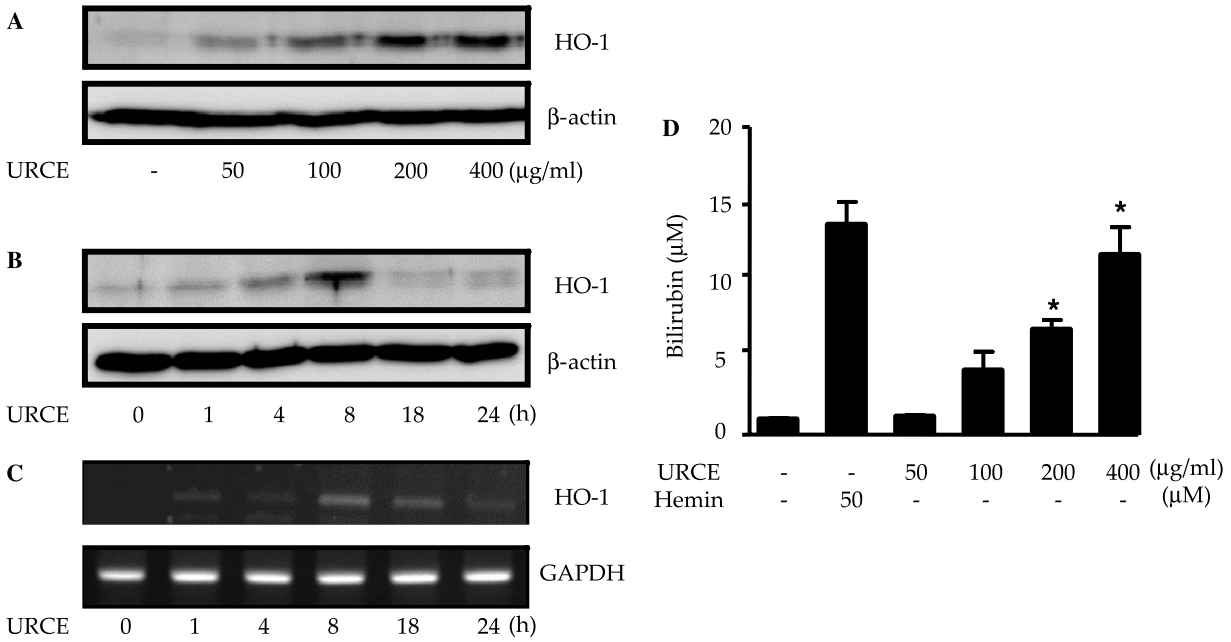

Fig. 3. Effects of URCE on HO-1 expression and bilirubin production in RAW264.7 cells. (A) Cells were treated with 0, 50, 100, 200, or 400 μg/ml URCE for 8 h. (B) Cells were treated with 400 μg/ml URCE for 1, 4, 8, 18, and 24 h. Total cell lysates (30 μg proteins) were resolved on 12% SDS–PAGE, and immunoblotting was performed as described in Materials and methods. β-Actin was used as a loading control. (C) Cells were treated with 400 μg/ml of URCE for 1, 4, 8, 18, and 24 h. Expression of HO-1 mRNA was examined by RT-PCR using specific primers for the HO-1. GAPDH was used as an internal control. (D) Cells were treated with indicated concentration of URCE or hemin (50 μM) for 12 h, and the amount of bilirubin in the culture supernatant was measured as described in Materials and methods. *Significantly different from 0 μg/ml of URCE, $P < 0.01$.

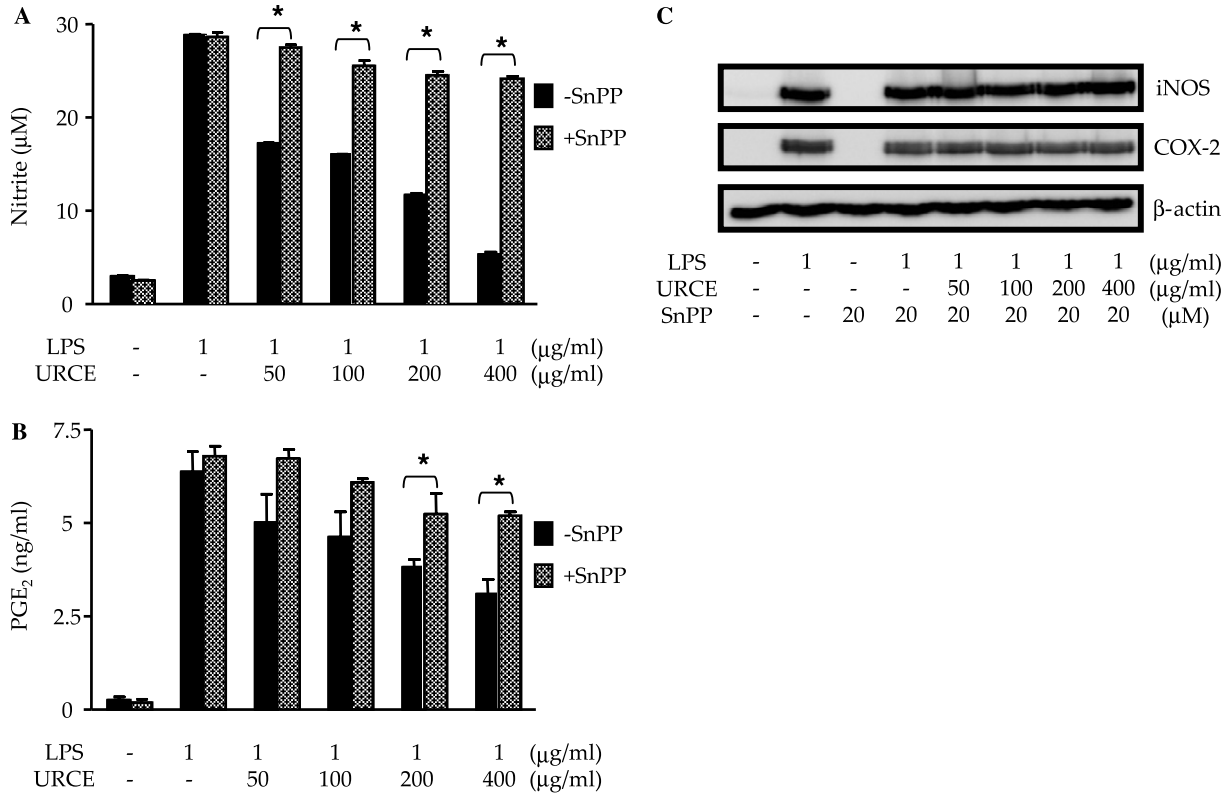

Fig. 4. Effects of SnPP on reduced production of NO and PGE_2 by URCE in LPS-stimulated RAW264.7 cells. (A,B) Cells were treated with indicated concentrations of URCE in the presence or absence of SnPP for 8 h, followed by 18 h culture in the presence of 1 μg/ml LPS. The concentrations of nitrite (A) and PGE_2 (B) in cell culture medium were measured as described in Materials and methods. (C) Proteins (30 μg) of total cell lysates were resolved on 7% and 10% SDS–PAGE for iNOS and COX-2, respectively. Immunoblotting was performed as described in Materials and methods. β-Actin was used as a loading control. *Significantly different from 0 μM SnPP at each concentration of URCE, $P < 0.001$.

process which was accompanied by the induction of HO-1 protein, we next examined whether the reduction in NO and PGE_2 production in URCE-treated cells is mediated through HO-1 induction by the utilization of an HO-1 inhibitor, SnPP. SnPP itself did not affect the production of NO or PGE_2 in LPS-stimulated RAW264.7 cells (Fig. 4A and B). However, SnPP drastically mitigated URCE-induced suppression of NO and PGE_2 production (Fig. 4A and B). In a similar context, the decreases in the expression of iNOS and COX-2 by URCE treatment were reversed by the inhibition of HO-1 activity (Fig. 4C). A concentration of 20 μM SnPP was chosen based on our preliminary experiments showing that it generates effects without cytotoxicity (data not shown). These results suggest that the activation of HO-1 mediates the inhibitory effects of URCE on LPS-induced inflammatory responses.

Discussion

In the present study, we examined the potential involvement of HO-1 in the anti-inflammatory activity elicited by the ethanol extract of unripe *R. coreanus*. We found that URCE-induced HO-1 expression in RAW264.7 murine macrophages, and that this induction was correlated with a decrease in iNOS expression and production of NO, as well as the release of PGE_2 by LPS-stimulated macrophages. Inhibition of HO-1 activity by SnPP reversed these effects, which suggests that activation of HO-1 is involved in the inhibition of LPS-induced inflammatory responses by URCE.

In the present study, we found that the ability of URCE to suppress LPS-induced NO production was stronger when the RAW264.7 cells were pre-incubated with URCE than when URCE and LPS were simultaneously added to cell culture media. These results suggest that URCE induces mediators to control inflammation responses. A series of plant-derived components, including curcumin, carnosol, and resveratrol, have been reported to induce HO-1 and exert anti-inflammatory actions in different types of cells [16–18]. The present study examined whether URCE exhibits such anti-inflammatory effects by increasing HO-1 expression. HO-1 is well known as a member of the heat-shock protein group, which is involved in responses to a number of cellular injuries, including thermal and oxidant stresses. HO-1 catalyzes the rate-limiting step in the degradation of cellular heme, producing equimolar quantities of iron, CO, and biliverdin. The beneficial effects of HO-1 induction have been attributed to several factors, including the degradation of pro-oxidant heme [19], formation of biliverdin and bilirubin with their antioxidant properties [20], as well as the release of CO, which has anti-apoptotic and anti-inflammatory properties [21]. Although the exact

mechanisms involved in anti-inflammatory actions of the HO-1 system have not been fully elucidated, one or more of the HO-1 reaction products have been evaluated as possible factors. For example, CO has been shown to exert significant anti-inflammatory effects in several models of inflammatory tissue injury. CO has been shown to decrease the production of IL-6 [22] and NO [23], and increase the production of IL-10 [24] in LPS-stimulated macrophages. It should also be noted that biliverdin and/or bilirubin are capable of blocking key events in inflammation. Using the well-described rat model of LPS-induced shock, Otterbein et al. demonstrated that exposure to biliverdin imparts a potent defense against lethal endotoxemia systemically, as well as in the lungs, and effectively abrogates the inflammatory response [25]. Since URCE-induced the expression of HO-1 in RAW264.7 cells, the anti-inflammatory actions of URCE might be mediated by the products of HO-1 enzyme, CO, and/or biliverdin.

Our results indicate that inhibition of HO-1 activity by the treatment of SnPP, a HO-1 inhibitor, abrogates the inhibitory effects of URCE on LPS-induced production of NO and PGE_2 in murine macrophages. Moreover, SnPP treatment prevented URCE-induced decreases in the protein levels of iNOS and COX-2, major enzymes that produce NO and PGE_2, respectively. Because SnPP blocks HO-1 enzymatic activity, these data confirmed that URCE exerts anti-inflammatory actions via modulation of HO-1 expression.

Although the exact component(s) responsible for the induction of HO-1 and the anti-inflammatory effects of URCE are still unknown, niga-ichigoside F_1 (NIF_1) and 23-hydroxytormentic acid (23-HTA) isolated from methanolic extracts of *R. coreanus* were reported to have anti-inflammatory effects in a carrageenan-induced acute inflammatory animal model in addition to their anti-nociceptive actions [26,27]. Besides NIF_1 and 23-HTA, coreanoside F_1, suavissimoside, tannins, diterpenes, and catechins are functional compounds that exert various biological activities of *R. coreanus* [12,13]. Further studies are required in order to define the functional compounds that regulate the induction of HO-1 and the anti-inflammatory actions of URCE.

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