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Anti-Inflammatory Effect of Heme Oxygenase 1: Glycosylation and Nitric Oxide Inhibition in Macrophages

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Flavonoids including the aglycones, hesperetin (HT; 5,7,3′-trihydroxy-4′-methoxy-flavanone), and naringenin (NE; 5,7,4′-trihydroxy flavanone) and glycones, hesperidin (HD; 5,7,3′-trihydroxy-4′-methoxy-flavanone 7-rhamnoglucoside) and naringin (NI; 5,7,4′-trihydroxy flavanone 7-rhamno glucoside), were used to examine the importance of rutinose at C7 on the inhibitory effects of flavonoids on lipopolysaccharide (LPS)-induced nitric oxide production in macrophages. Both HT and NE, but not their respective glycosides HD and NI, induced heme oxygenase 1 (HO-1) protein expression in the presence or absence of LPS and showed time and dose-dependent inhibition of LPS-induced nitric oxide (NO) production and inducible nitric oxide synthase (iNOS) expression in RAW264.7, J774A.1, and thioglycolate-elicited peritoneal macrophages. Additive inhibitory effect of an HO-1 inducer hemin and NE or NI on LPS-induced NO production and iNOS expression was identified, and HO enzyme inhibitor tin protoporphyrin (SnPP) attenuated the inhibitory effects of HT, NE, and hemin on LPS-induced NO production. Both NE and HT showed no effect on iNOS mRNA and protein stability in RAW264.7 cells. Removal of rutinose at C7 of HD and NI by enzymatic digestion using hesperidinase (HDase) and naringinase (NIase) produce inhibitory activity on LPS-induced NO production, according to the production of the aglycones, HT and NE, by high-performance liquid chromatography (HPLC) analysis. Furthermore, the amount of NO produced by LPS or lipoteichoic acid (LTA) was significantly reduced in HO-1-overexpressing cells (HO-1/RAW264.7) compared to that in parental cells (RAW264.7). Results of the present study provide scientific evidence to suggest that rutinose at C7 is a negative moiety in flavonoid inhibition of LPS-induced NO production, and that HO-1 is involved in the inhibitory mechanism of flavonoids on LPS-induced iNOS and NO production. J. Cell. Physiol. 202: 579–590, 2005.

Flavonoids extensively exists in plants, fruits, and vegetables, and it is estimated that humans intake about 20–1,000 mg of flavonoids daily (Middleton et al., 2000; Birt et al., 2001). Hydroxylation, methoxylation, and glycosylation have been found to occur in the metabolism of flavonoids, and the bioactivity of flavonoids have been shown to be associated with the types of modification experienced (Middleton, 1998; Ko et al., 2004). Previous studies demonstrated that flavonoids are present in foods as glycoside conjugates, which show greater hydrophilic properties than aglycoside. An in vivo absorption assay indicates that mammalian and microbial glucosidases might remove the sugar moiety from flavonoid glycoside before absorption. Additionally, glycoside derivatives of flavonoids are more easily absorbed than aglycones in humans (Hollman et al.,

Abbreviations: HO-1, heme oxygenase 1; NO, nitric oxide; LPS, lipopolysaccharide; LTA, lipoteichoic acid; SnPP, tin protoporphyrin; iNOS, inducible nitric oxide synthase; CO, carbon monoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NBT, nitroblue tetrazolium; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography.

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1999). Williamson et al. (1996) also showed that quercetin glucoside exhibited more-preferential absorption than quercetin, and exhibited several biological activities including antioxidant activity and induction of quinone reductase in hepalclc seven cells. Results of an in vitro study suggested that deglycosylation of flavonoid mono-glucosides such as querectin-4-glucoside and naingenin-7-glucoside by extracts from the small intestine greatly facilitate expression of their bioactivities (Choudhury et al., 1999). In human intestinal anaerobic bacterium, which produce α-rhamnosidase and β-glucosidase and transform flavonoid rhamnoglucosides, such as rutin, hesperidin (HD), poncrin, and naringin (NI), to their aglycones (Kim et al., 1996). These data indicate that flavonoids are extensively modified in vivo, and that the modification status is closely related to their bioactivities.

Production of nitric oxide (NO) is regulated by nitric oxide synthase (NOS) in the conversion of L-arginine to L-citrulline, and there are at least three types of NOS including inducible nitric oxide synthase (iNOS), eNOS, and nNOS which have been identified. Both nNOS and eNOS are constitutive forms (cNOS) and Ca^{+2}-dependent, and a small amount of NO produced by cNOS plays an important role in maintaining normal physiological functions. However, activation of iNOS occurs in several human diseases such as inflammation, diabetes, stroke, and sepsis (Nathan, 1992; Marletta, 1993). Both lipopolysaccharide (LPS) and lipoteichoic acid (LTA) are derived from Gram-negative and Gram-positive bacteria, and induce NO production by activation of iNOS gene expression (Kengatharan et al., 1996; Jungi et al., 1999). Therefore, NO production induced by LPS or LTA through iNOS induction may reflect the degree of inflammation and may provide a measure for assessing the effect of drugs on the inflammatory process. Heme oxygenase (HO) is the rate-limiting enzyme in the oxidative degradation of heme into bilirubin, iron, and carbon monoxide (CO), whereas HO-2 and HO-3 are constitutively expressed and heme oxygenase 1 (HO-1) is the inducible form that provides protection against oxidative stress (Ryter and Tyrrell, 2000; Ryter et al., 2002). HO-1 is highly upregulated by various stress stimuli, including heme, heavy metals, UV light, LPS, heat shock, and hyperoxia (Ryter and Choi, 2002). HO-1-deficient mice develop anemia with low serum iron levels along with an overload of iron in the liver and kidney causing oxidative damage and chronic inflammation (Ishizaka et al., 2002; Chin et al., 2003). Accordingly, overexpressing of HO-1 protects coronary endothelial cells against the toxic effects of heme proteins and pulmonary epithelial cells against hyperoxia (Suttner et al., 1999; Malaguarnera et al., 2002). Vicente et al. reported that monocytes/macrophages cells participated in the negative regulation of inflammatory responses, and played an anti-inflammatory role with HO-1 in the response (Vicente et al., 2003). Our previous study demonstrated that the inflammatory molecules, NO and PGE_2, participated in HO-1 induction in LPS/IFN-γ-treated macrophages and protecting cells from UV irradiation-induced cell death (Chen et al., 2002a). Those data suggest that HO-1 induction participates in anti-inflammation and damage protection process, however, the effect of HO-1 on LPS-induced NO production is still undefined.

Flavonoids have been demonstrated to exhibit several biological activities such as antioxidant, anti-inflammatory, and antitumor properties (Guengerich and Kim, 1990; Ko et al., 2002; Shen et al., 2004a,b). Several previous studies including our own indicated that flavonoids are able to inhibit LPS-induced inducible NO and PGE_2 production through blocking iNOS and COX-2 gene expressions (Shen et al., 2002). Although, NO inhibition by flavonoids has been extensively studied, the effect of glycosylation and the role of HO-1 plays in NO inhibition by flavonoids have not been delineated. In the present study, flavonoids and their respective flavonoid glycosides, including hesperetin (HT; 5,7,3′-trihydroxy-4′-methoxy-flavanone) and HD (5,7,3′-trihydroxy-4′-methoxy-flavanone 7-rhamnoglucoside), and naringenin (NE; 5,7,4′-trihydroxy flavanone) and NI (5,7,4′-trihydroxy flavanone 7-rhamnoglucoside) were used to examine the inhibitory effects of NO on LPS-treated macrophages. Data from the present study show that the aglycones, HT and NE, but not the flavonoid glycosides, HD and NI, inhibited LPS-induced NO production in accordance with the induction of HO-1 expression. Relationship between glycosylation, HO-1 induction and NO inhibition was proposed in the present study.

MATERIALS AND METHODS

Cells

RAW264.7 and J774A.1 are mouse macrophage cell lines, and were obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Gibco-BRL, Gaithersburg, MD) supplemented with antibiotics (100 U/ml of penicillin A and 100 U/ml of streptomycin), and 10% heat-inactivated fetal bovine serum (FBS) (Gibco-BRL) and maintained in a 37°C humidified incubator containing 5% CO_2.

Agents

The four structurally related flavonoids of HT, HD, NE, and NI were obtained from Sigma Chemical (St. Louis, MO). LPS (*Escherichia coli*, serotype 055:B5), LTA (*Bacillus subtilis*), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), hemin, and tin protoporphyrin (SnPP) were purchased from Sigma Chemical. All chemical solvents were purchased from Merck KGaA, Darmstadt, Germany. The antibodies of anti-HO-1, anti-iNOS, and anti-α-tubulin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell viability assay

MTT was used as an indicator of cell viability as determined by its mitochrondrial-dependent reduction to formazone. Cells were plated at a density of 10^5 cells/well into 96-well plates for 12 h, followed by treatment with different concentrations of each compound for a further 16 h. Cells were washed with PBS twice, and MTT (50 mg/ml) was added to the medium for 4 h. Then, the supernatant was removed, and the formazone

crystals were dissolved using isopropanol. The absorbance was read at 600 nm with an enzyme-linked immunosorbent assay (ELISA) analyzer (Dynatech MR-7000; Dynex Technologies, Chantilly, VA) (Chen et al., 2002b).

The culture of peritoneal macrophages cells from Balb/c mice

Resident peritoneal macrophages were harvested after thioglycolate-induced inflammatory responses for 3 days, and macrophages cells were collected by phosphate buffer saline (PBS) i.p. in Balb/c mice. Cells were washed and adjusted to a density of 5×10^5 cells/well with RPMI medium (Gibco-BRL) in 24-well plates. The RPMI medium was supplemented with antibiotics (100 U/ml of penicillin A and 100 U/ml of streptomycin) and 10% heat-inactivated fetal bovine serum (Gibco-BRL) and cell was incubated at 37°C for 4 h to allow attachment of macrophages. At the end of 4 h, the supernatant was removed, and fresh RPMI medium was added for incubation (Shen et al., 2002).

Nitrite assay

Cells were plated at a density of 5×10^5 cells/ml in 24-well plates for 12 h, followed by treatment with LPS (50 ng/ml) and different concentrations of the indicated compounds such as flavonoids, hemin, or SnPP for a further 12 h. The amount of NO production in the medium was detected with 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% naphthylethylendiamine dihydrochloride in water). The absorbance of the mixture at 530 nm was determined with an ELISA plate reader (Dynatech MR-7000; Dynatech Laboratories), and the nitrite concentration was determined using a dilution of sodium nitrite as a standard. Both phenol red-free and-containing medium have been used in our lab. And, we found that phenol red showed no interference on Griess reaction for NO detection. The standard $NaNO_2$ was prepared in the phenol red-containing medium as references for quantification of NO amount in medium under different treatment.

Western blotting

Total cellular extracts were prepared according to our previous papers, separated on 8–12% SDS–polyacrylamide minigels, and transferred to immobilon polyvinylidenedifluoride membranes (Millipore Corporation, Bedford, MA). Membranes were incubated with 1% bovine serum albumin and then with anti-iNOS, anti-HO-1 or anti-α-tubulin antibodies (Santa Cruz Biotechnology) overnight at 4°C. Expression of the protein was detected by staining with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma Chemical) (Lee et al., 2002; Chen et al., 2004).

RT-PCR

RAW264.7 cells were treated with indicated compounds (200 μM) and LPS (50 ng/ml) for 4 h or others time points and washed out with ice-cold PBS. Total RNA was isolated by total RNA extraction kit (Amersham Pharmacia, Buckinghamshire, UK), and the total RNA concentration was detected using a spectrophotometer. Total RNA (1 μg) was converted to cDNA with oligo d(T). PCR was performed on the cDNA using the following sense and antisense primers, respectively, for iNOS: CCCTTCCGAAGTTTCTGGCAGCAGC and GGCTGTCAGAGAGCCTCGTGGCT TTGG; and GAPDH: TGAAGGTCGGTGTGAACGGATTTGGC and CATGTAGGC CATGAGGTCCACCAC. PCR of the cDNA was performed in a final volume of 50 μl containing PCR primers, oligo (d)T, total RNA, and DEPC H_2O by RT-PCR Beads Kit (Amersham Pharmacia). The amplification sequence protocol was 95°C for 30 sec, 56°C for 30 sec, 72°C for 45 sec for 30 cycles. The PCR products were separated by electrophoresis on 1.2% agarose gels and visualized by ethidium bromide staining.

Deglycosylation by HDase and naringinase (NIase)

HDase and NIase were dissolved in Tris-HCl buffer (5 mM, pH 4.0). The reaction-buffer (Tris-HCl 5 mM, pH 4.0) contained each compound and then HDase or NIase were respectively added and incubated at 40°C for 30 or 60 min. High-performance liquid chromatography (HPLC) (PU1580, Jasco Co., Tokyo) analysis used an RP-18 column (5 μM, Merck), with acetonitrile:water (32:68, v/v) at a flow of 0.8 ml/min. Samples were detected by their absorbance at 280 nm (VU1575, Jasco). For the cell culture assay, the reaction-buffer pH was adjusted to 7.0–7.4 using NaOH and sterilized using a 0.22 μM filter, according, 0.5 ml of the reaction-buffer and 0.5 ml of the culture medium were added to the cells culture assay (Habelt and Pittner, 1983; Romero et al., 1985; Day et al., 1998; Spencer et al., 1999).

iNOS protein and mRNA stability assay

In mRNA stability assay, RAW264.7 cells were treated with LPS (50 ng/ml) for 4 h, and LPS was washed out with ice-cold PBS. LPS-treated cells were then incubated with actinomycin D (10 ng/ml) in the presence or absence of HT or NE (200 μM) for 0, 8, 16, 24 h and expression of iNOS and GAPDH mRNA was analyzed by RT-PCR. For iNOS protein stability assay, LPS-treated cells were then incubated with cycloheximide (0.5 μg/ml) in the presence or absence of HT or NE (200 μM) for 0, 8, 16, 24 h and expression of iNOS protein and α-tubulin was analyzed by Western blotting.

Establishment of HO-1 transfectants

pCMV-HO-1, a constitutive expression vector, carries full-length human HO-1 cDNA under the control of the CMV promoter/enhancer sequence. We transfected RAW264.7 cells with pCMV-HO-1 or pCMV-neo using the Transfast™ transfection reagent (Promega Co., Madison, WI). After 48 h, cells were trypsinized and replaced in DMEM with 10% FBS and 400 μg/ml G418. G418-resistant cells were selected and expanded. No significant morphological changes in RAW264.7 cells with HO-1 overexpression, compared with neo-controlled or parental RAW264.7 cells. Folds of HO-1 protein induction in HO-1/RAW were measured by

densitometry analysis and expression as a ratio of HO-1/α-tubulin in the study.

Statistical analysis

Values are expressed as the mean ± SE. The significance of the difference from the respective controls for each experimental test condition was assayed by using Student's *t*-test for each paired experiment. A P value <0.05 or 0.01 was regarded as indicating a significant difference.

RESULTS

HT and NE, but not HD or NI, induce HO-1 expression in RAW264.7, J774A.1, and primary thioglycolate-elicited macrophages

The chemical structures of HT, HD, NE, and NI were shown in Figure 1. As shown the structural difference between HT/HD and NE/NI is that HD and NI possess a rutinose at the respective C7 of HT and NE. Results of Western blotting showed that the aglycones, HT and NE, but not the glycones, HD and NI, induced HO-1 protein expression in time- and dose-dependent manners in RAW264.7 macrophages (Fig. 2A). In the same part of the experiment, both HT and NE were able to induce HO-1 protein expression in another two macrophages J774A.1 and primary peritoneal macrophages; however, no significant change in HO-1 protein was found in either HD- or NI-treated cells (Fig. 2B,C). In addition, viability of the indicated flavonoid-treated cells was examined by the MTT assay, and results showed that HT, HD, NE, and NI, at a dose of 200 μM, produced no cytotoxic effects in these three cells (data not shown). Results of Figure 2 suggest that the aglycone flavonoids, HT and NE, but not the glycones, HD and NI, are effective HO-1 inducers in RAW264.7, J774A.1, and peritoneal macrophages cells.

The aglycones, HT and NE, but not the glycones, HD and NI, inhibit LPS-induced NO production and iNOS protein expression in RAW264.7, J774A.1, and peritoneal macrophages

Both glycones and aglycones including HT/HD and NE/NI were used to examine their effects on LPS-induced NO production in RAW264.7, J774A.1, and peritoneal macrophages. Macrophages were incubated with the indicated compounds of HT, HD, NE, or NI in the presence of LPS and the amount of NO produced in the medium was estimated by the Griess reaction. In RAW264.7, J774A.1, and peritoneal macrophages, LPS (50 ng/ml for RAW264.7, 100 ng/ml for J774A.1, and 5 μg/ml for peritoneal macrophages) treatment for 12 h induced NO production of 47 μM in RAW264.7 cells, 25 μM in J774A.1, and 60 μM in peritoneal macrophages, respectively. Neither HT, HD, NE, or NI alone affected the production of NO in cells in the absence of LPS (data not shown). In the presence of different doses of the aglycones, HT or NE, together with LPS for 12 h, a concentration-dependent inhibition of nitrite production was detected in RAW264.7 and J774A.1 macrophages with reducing iNOS protein expression; however, the glycones, HD and NI, showed no obvious inhibition on LPS-induced NO production (Fig. 3A,B). Similarly, HT and NE, but not HD or NI, at a dose of 200 μM, inhibited LPS-induced NO production and iNOS protein expression in peritoneal macrophages (Fig. 3C). An increase in HO-1 protein was observed in HT- or NE-treated these cells in the presence of LPS. These results indicate that the aglycone flavonoids, HT and NE, significantly inhibited LPS-induced iNOS expression and NO production with induction of HO-1 protein expression.

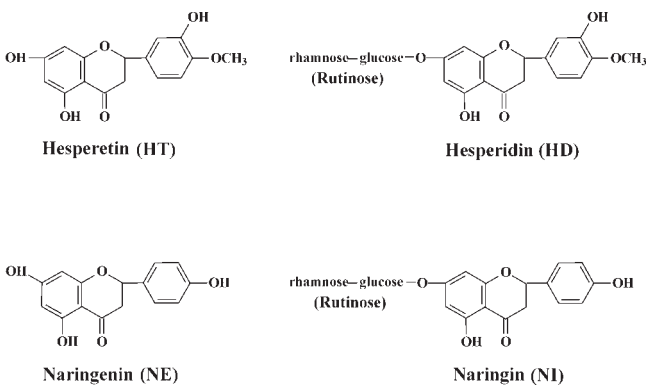

Fig. 1. Chemical structures of hesperetin (HT), hesperidin (HD), naringenin (NE), and naringin (NI).

The aglycones, HT and NE, inhibit LPS-induced iNOS mRNA expression but no interference on mRNA and protein stability of *iNOS* gene

We further elucidate the effect of HT and NE on LPS-induced iNOS gene expression at mRNA level by RT-PCR assay. As illustrated in Figure 4A, HT and NE, but not HD and NI, at the dose of 200 μM, significantly decreased LPS-induced iNOS mRNA expression. Additionally, study if HT and NE inhibition of iNOS mRNA and protein expression via inducing their degradation was performed. As shown in Figure 4B, stability of iNOS mRNA in control-, HT- or NE-treated RAW264.7 cells was examined by RT-PCR assay. It indicated that HT and NE did not change the stability of iNOS mRNA compared with control group (Fig. 4B). Similar results were derived from iNOS protein stability assay by Western blotting (Fig. 4C). These data suggested that HT and NE inhibited LPS-induced iNOS gene expression at protein and mRNA level, but no change on their stability in cells.

Elevation of HO-1 expression by hemin inhibits LPS-induced NO production and iNOS expression in RAW264.7, J774A.1, and peritoneal macrophages

In order to study the relationship between HO-1 protein and LPS-induced NO production, an HO-1 inducer, hemin, was used in this study. In the absence of LPS, hemin concentration-dependently induced HO-1 protein expression in RAW264.7, J774A.1, and peritoneal macrophages (Fig. 5A–C; upper part). In the presence of LPS (50 ng/ml for RAW264.7, 100 ng/ml for J774A.1, and 5 μg/ml for peritoneal macrophages), hemin also showed a similar concentration-dependent induction of HO-1 protein expression as that in the

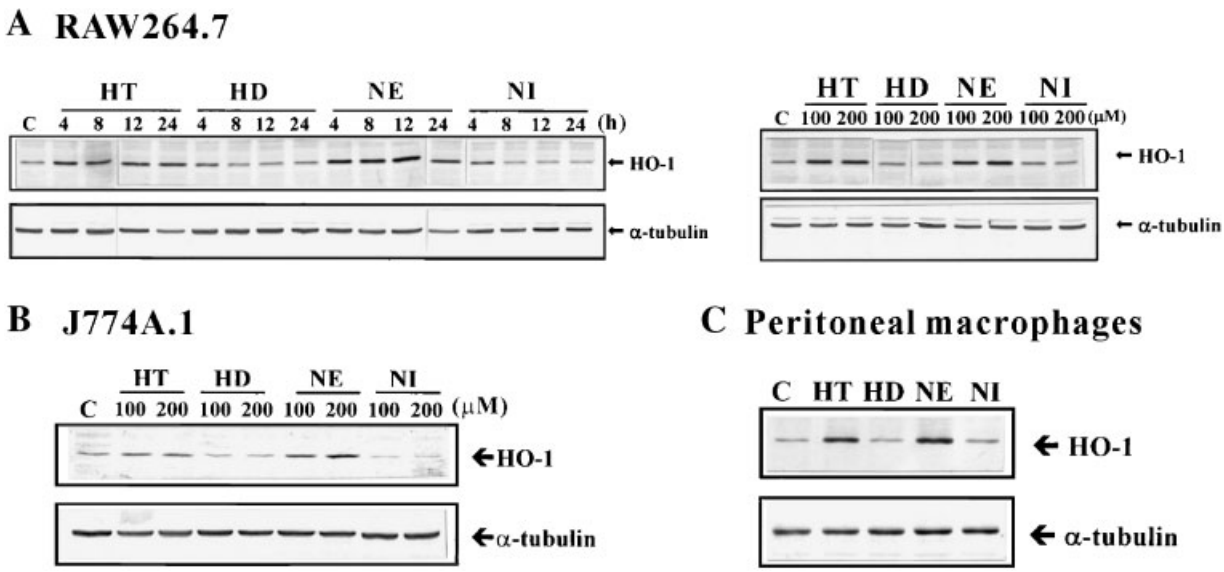

Fig. 2. Induction of heme oxygenase 1 (HO-1) expression by HT and NE in macrophage cells. **A**: Time-dependent induction of HO-1 protein by HT and NE, but not HD and NI, in RAW264.7 cells. Left part: Cells were treated with HT, HD, NE, and NI (200 μM) for 4, 8, 12, and 24 h, and the expression of HO-1 protein was analyzed by Western blotting. HT and NE, at different doses, induce HO-1 protein expression in RAW264.7 cells. Right part: RAW264.7 cells were treated with different concentrations (100 and 200 μM) of the indicated compounds for 12 h, and the expression of HO-1 protein was analyzed by Western blotting. **B**: Induction of HO-1 expression by HT and NE in J774A.1 macrophage cells. Cells were treated with different concentrations (100 and 200 μM) of the indicated compounds for 12 h, and the expression of HO-1 protein was analyzed. **C**: Induction of HO-1 expression by HT and NE in thioglycolate-elicited peritoneal macrophage cells. Cells were treated with 200 μM of the indicated compounds for 12 h, and the expression of HO-1 protein was analyzed. α-Tubulin was used as an internal control. C, control.

absence of LPS. Interesting, hemin dose-dependently inhibited LPS-induced iNOS protein expression and NO production in these three cells (Fig. 5A–C, middle and lower parts). Results of the MTT assay showed that hemin exhibited no cytotoxicity in cells (data not shown).

HT and NE act in parallel with hemin to inhibit LPS-induced iNOS protein expression and NO production with HO-1 induction

Results described above show that HT and NE, inhibition of LPS-induced NO production involves

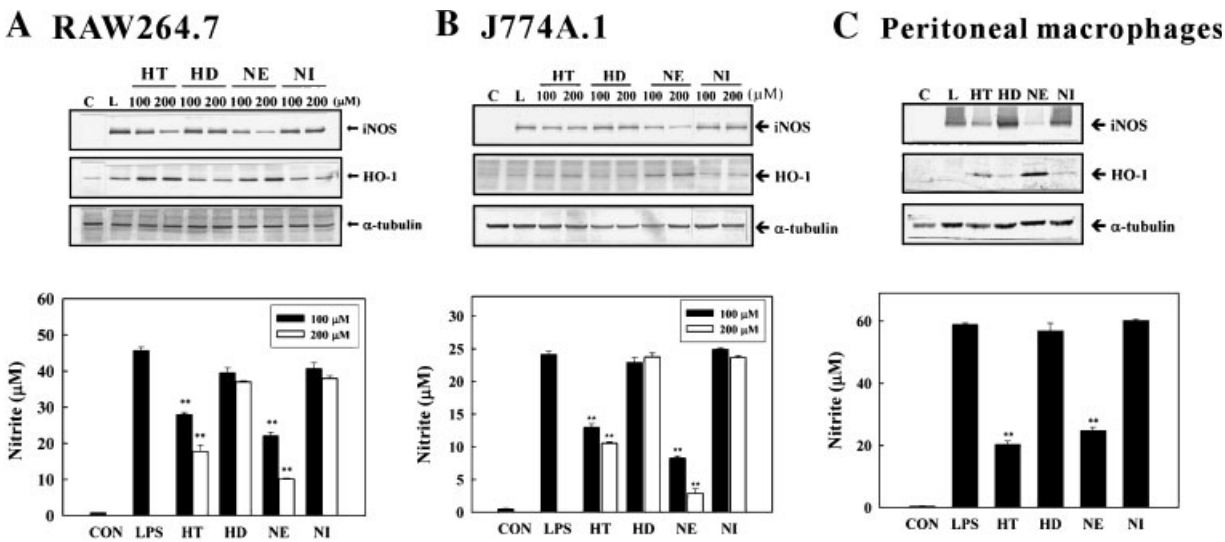

Fig. 3. HT and NE inhibition of lipopolysaccharide (LPS)-induced inducible nitric oxide synthase (iNOS) and nitric oxide (NO) production, associated with increasing HO-1 protein expression in RAW264.7, J774A.1, and peritoneal macrophage cells. **A**: RAW264.7 cells were treated with LPS (50 ng/ml) in the presence of different concentrations (100 and 200 μM) of HT, HD, NE, and NI for 12 h. **B**: J774A.1 cells were treated with LPS (100 ng/ml) in the presence of different concentrations (100 and 200 μM) of the indicated compounds for 12 h. **C**: Peritoneal macrophage cells were treated with LPS (5 μg/ml) in the presence of 200 μM of the indicated compounds for 12 h. Upper part: iNOS, HO-1, and α-tubulin protein expression were detected by Western blotting. Lower part: NO production in the medium under different treatments in different cell types was measured by the Griess reaction. Data were obtained from three independent experiments and are expressed as the mean ± SE. $^{**}P < 0.01$ indicate a significant difference from the LPS-treated group, as analyzed by Student's *t*-test.

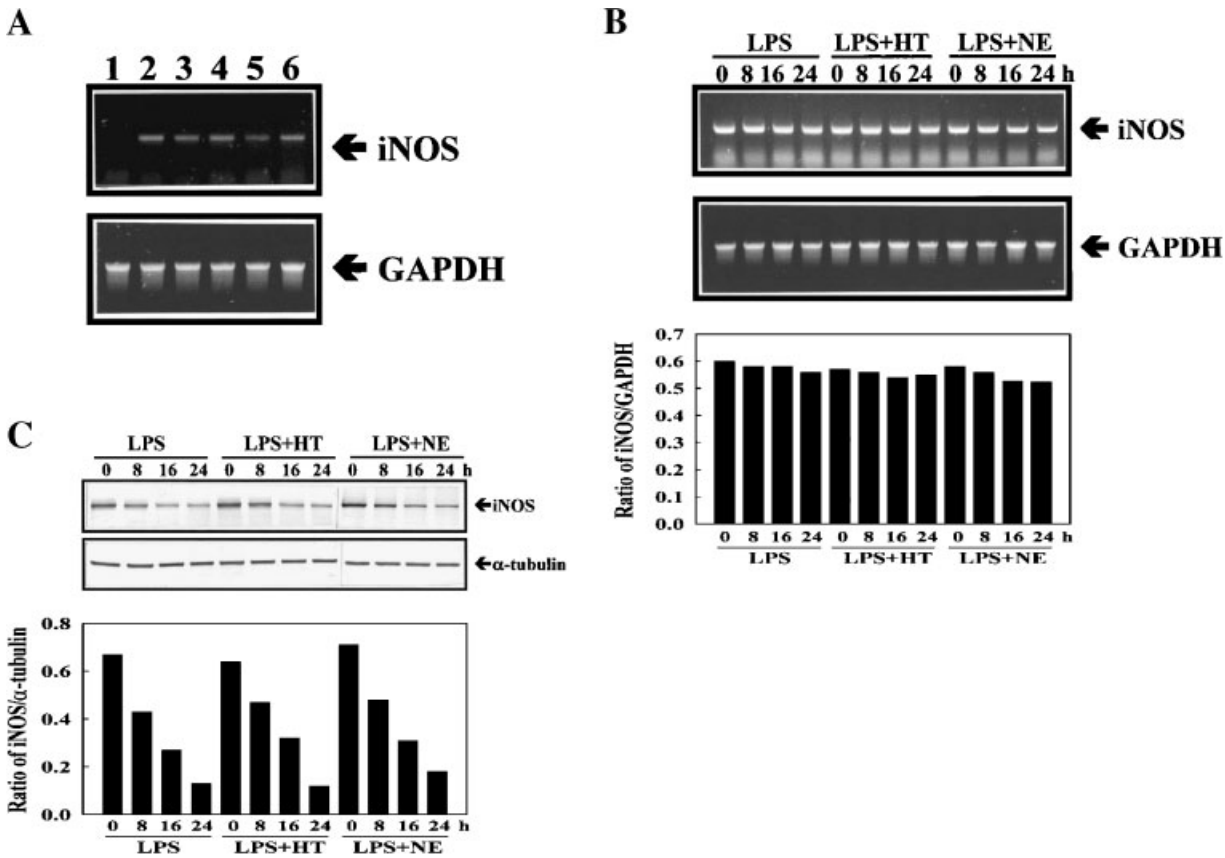

Fig. 4. HT and NE inhibition of LPS-induced iNOS gene expression at mRNA level, but no interference on the stability of iNOS mRNA and protein. **A**: HT and NE inhibited LPS-induced iNOS mRNA expression in RAW264.7 cells. Cells were treated with HT, HD, NE, or NI in the present of LPS (50 ng/ml) for 4 h and the expression of iNOS mRNA was analyzed by RT-PCR. Equal amount of GAPDH was described as an internal control. Lane: 1, control; lane 2, LPS; lane 3, HT; lane 4, HD; lane 5, NE; lane 6, NI. **B**: HT and NE do not interfere in LPS induced iNOS mRNA stability. Cells were treated as described in Materials and Methods, and stability of iNOS mRNA was analyzed by RT-PCR. **C**: HT and NE do not interfere in iNOS protein stability. Cells were treated with LPS for 6 h and iNOS protein stability was evaluated by Western blotting as described in Materials and Methods. Band intensity of iNOS, GAPDH, and α-tubulin were quantified by a densitometry analysis, and the data was expressed as a ratio of iNOS/GAPDH or iNOS/α-tubulin.

HO-1 protein expression. In order to provide evidence to identify if HO-1 participates in HT- or NE-inhibited NO production, RAW264.7 macrophages were treated with HT and NE (100 μM) with or without hemin (20 and 50 μM) in the presence of LPS (50 ng/ml), and NO production, and iNOS and HO-1 protein expressions were analyzed. Hemin at doses of 20 and 50 μM induced HO-1 protein expression with slight inhibition of LPS-induced NO production (Fig. 5). Results in Figure 6A show that hemin, at doses of 20 and 50 μM, enhanced the inhibitory activities of HT and NE on LPS-induced iNOS protein expression in accordance with elevation of HO-1 protein expression. As the same part of the experiment, hemin at doses of 20 and 50 μM potentiated the inhibitory effects of HT and NE on LPS-induced NO production. These data suggest that the inhibitory effects of HE and NE paralleled the action of hemin on LPS-induced NO production and iNOS protein expression. In addition, SnPP, an HO-1 inhibitor, was used to investigate the role of HO-1 in the inhibition by HT and NE of LPS-induced NO production. Results in Figure 6B show that inhibitory effect of HT, NE, and hemin of LPS-induced NO production was partially but significantly reversed by the addition of SnPP. This indicates that HO-1 participated in NO inhibition by HT and NE.

Rutinoside at C7 is a negative moiety in the induction of HO-1 expression and inhibition of LPS-induced NO production by flavonoids

An interesting problem derived from previous results is why the aglycone flavonoids, HT and NE, but not the glycone flavonoids, HD and NE, possess the biological activities to induce HO-1 gene expression and inhibit LPS-induced NO production and iNOS protein expression. This suggests that rutinoside at C7 is critical for the biological activities of flavonoids. In order to delineate the importance of rutinoside in HO-1 induction and NO inhibition, removal of rutinoside from HD and NI by HDase and NIase, respectively, and the production of HT and NE was detected by HPLC analysis. Results in Figure 7A show that the different retention times of HT/HD and NE/NI as detected by HPLC, with the retention times for the aglycones, HT and NE, at around 6 min, and of the glycones, HD and NI, at around 3 min. Addition of HDase and NIase significantly removed the rutinoside from HD and NI, respectively, and

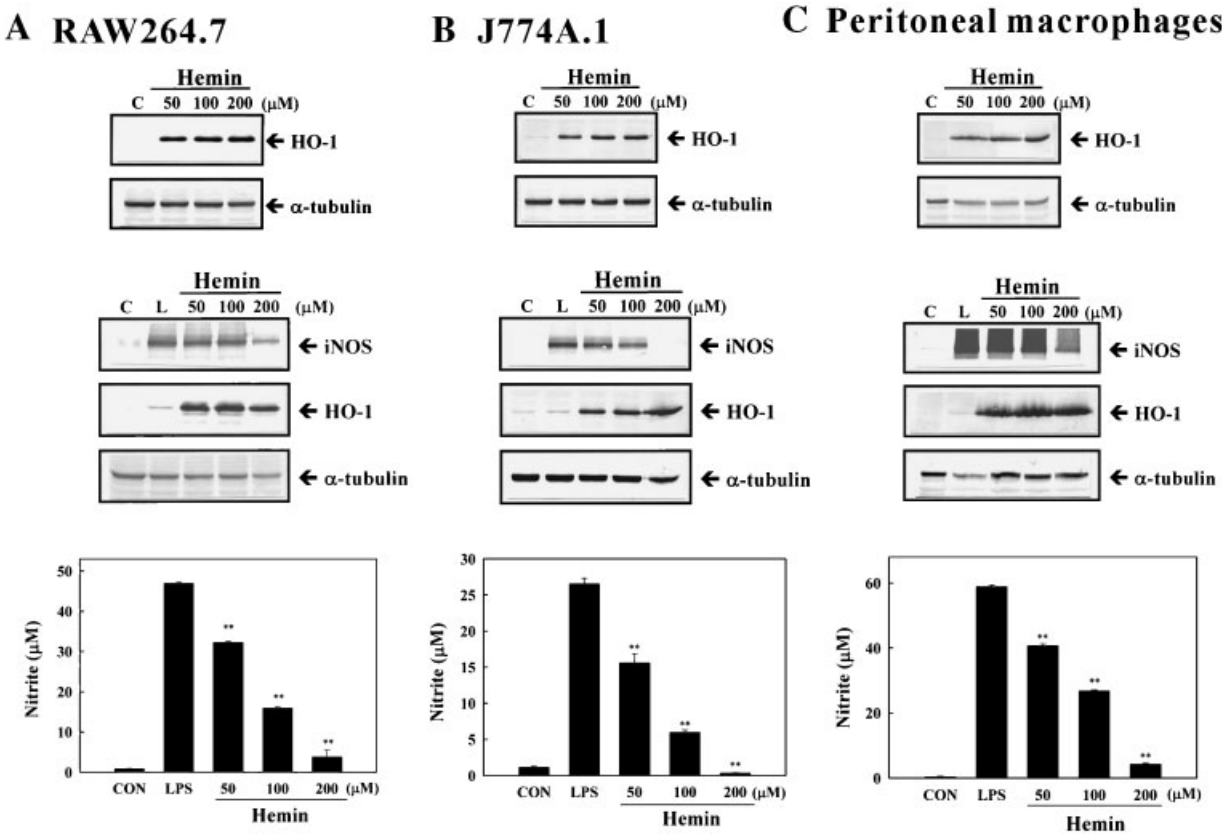

Fig. 5. Hemin, an HO-1 inducer, inhibits LPS-induced iNOS protein and NO production, with increasing HO-1 protein in RAW264.7, J774A.1, and peritoneal macrophage cells. Upper part: Hemin induction of HO-1 protein expression in the absence of LPS in RAW264.7 (**A**), J774A.1 (**B**), and peritoneal macrophages (**C**). Briefly, hemin (50, 100, and 200 μM) was added to each cell, and expressions of HO-1 protein were analyzed by Western blotting. Middle part: Hemin dose-dependently inhibited LPS-induced iNOS protein expression in RAW264.7 (**A**), J774A.1 (**B**), and peritoneal macrophages (**C**). Cells were treated with different doses of hemin in the presence of LPS, and the expression of iNOS, HO-1, and α-tubulin protein were analyzed by Western blotting. Lower part: Hemin inhibition of LPS-induced NO production in RAW264.7 (**A**), J774A.1 (**B**), and peritoneal macrophages (**C**). Cells were treated with different doses of hemin in the presence of LPS, and NO produced was measured. Data were obtained from three independent experiments and are expressed as the mean ± SE. $^{**}P < 0.01$ indicates a significant difference from the LPS-treated group, as analyzed by Student's *t*-test.

produced the aglycones, HT and NE, as determined by HPLC. Furthermore, RAW264.7 cells were treated with either the glycones, HD or NI, HDase, NIase, HD digested by HDase, or NI digested by NIase in the presence of LPS, and the expressions of iNOS, HO-1, and α-tubulin were examined. Results in Figure 7B show that HD, NI, HDase, and NIase alone caused no effect on HO-1 protein expression, LPS-induced iNOS expression, or NO production. Interestingly, the addition of the extract from HDase-digested HD or NIase-digested NI produced significant inhibition of LPS-induced iNOS expression and NO production with an increase in HO-1 protein. These data provide evidence suggesting that rutinoside at C7 has no effect on flavonoid inhibition of LPS-induced NO production or induction of HO-1 expression.

Increasing endogenous HO-1 protein exhibits resistance to LPS- and LTA-induced iNOS expression and NO production

In order to provide direct evidence to support HO-1 participates in flavonoid inhibition of NO production induced by LPS, HO-1-overexpressing RAW264.7 (HO-1/RAW264.7) cells were established as described in Materials and Methods. No significant morphological change was found between HO-1/RAW264.7, neo-transfected RAW264.7, and parental RAW 264.7 macrophages. Control vector (pCMV)-transfected RAW264.7 (neo-RAW264.7) cells showed the same HO-1 protein expression and NO production induced by LPS as that in the parental RAW264.7 cells (data not shown). Results in Figure 8A show that expression of the HO-1 protein increased about three-fold in HO-1/RAW264.7 cells compared with parental RAW264.7 macrophages. In the presence of LPS or LTA, iNOS protein and NO productions were dose-dependently induced in parental RAW264.7 and neo-RAW264.7 (data not shown) cells, but significantly reduced in HO-1/RAW264.7 cells. This indicates that HO-1 over-expression attenuates the NO production and iNOS protein expression by LPS or LTA (Fig. 8B,C). Additionally, HT and NE showed more-significant inhibition of LPS-induced NO production in HO-1/RAW264.7 cells than in parental RAW264.7 cells (Fig. 8D). HD and NI performed no significant inhibitory effect on LPS-induced NO production compared with HT and NE in both RAW264.7 cells and HO-1/RAW264.7

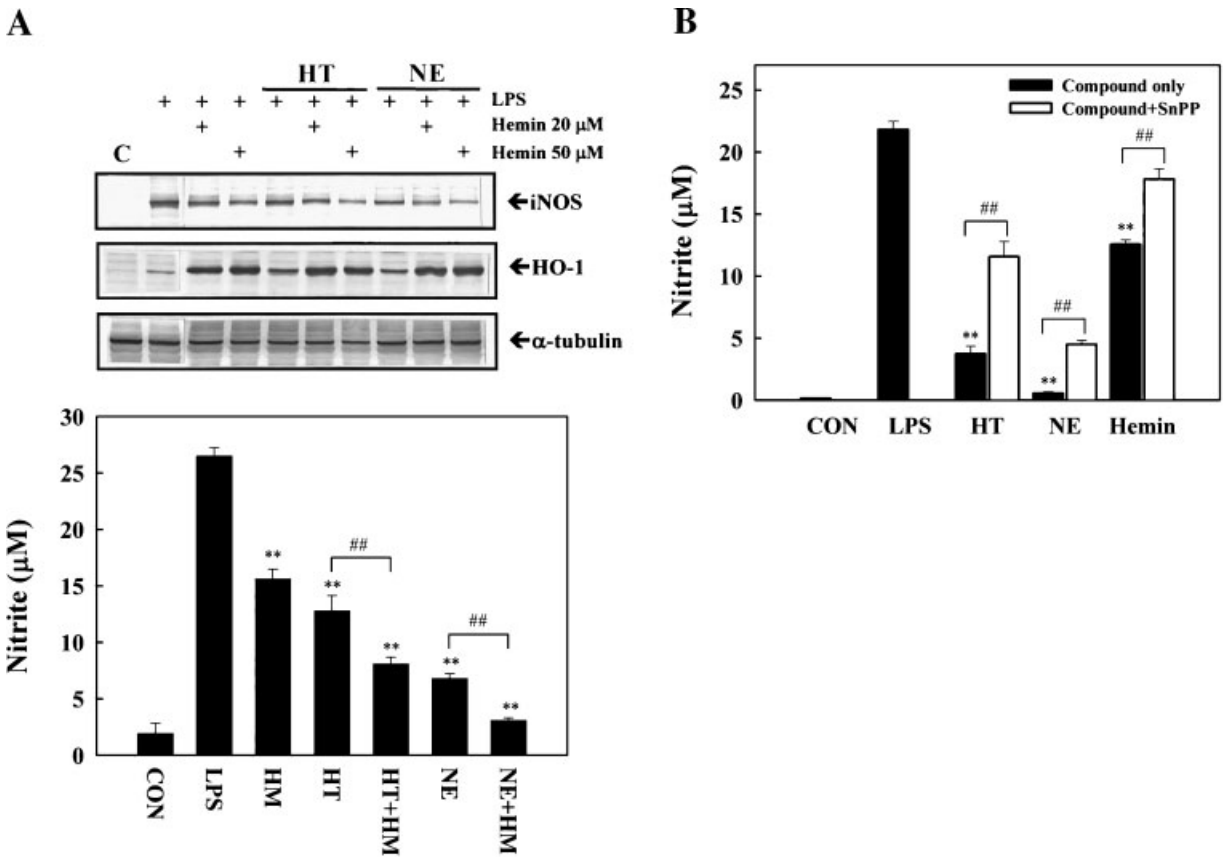

Fig. 6. Hemin pre-treatment enhances the inhibitory activities of HT and NE on LPS-induced NO production; HO activity inhibitor tin protoporphyrin (SnPP) attenuates the inhibitory effects of flavonoids on LPS-induced NO production in RAW264.7 cells. **A**: Upper part: RAW264.7 cells were treated with hemin (20 and 50 μM) with or without HT or NE (100 μM) in the presence of LPS (50 ng/ml) for 12 h. Expressions of iNOS, HO-1, and α-tubulin protein were examined by Western blotting. Lower part: The amount of NO produced was measured by the Griess reaction. Data were obtained from three independent experiments and expressed as the mean ± SE. **B**: RAW264.7 cells were treated with the aglycones, HT and NE (100 μM), and LPS (50 ng/ml) in the presence or absence of HO activity inhibitor SnPP (10 μM) for 12 h, and the amount of NO produced in the medium was determined by the Griess reaction. Data were obtained from six independent experiments, and are expressed as the mean ± SE. $^{\#\#}P < 0.01$ indicate significant difference from the indicated flavonoid-treated group, and $^{**}P < 0.01$ indicates a significant difference from the LPS-treated group, as analyzed by Student's *t*-test.

cells. These data provide a direct evidence to suggest that over-expression of HO-1 protein attenuates LPS or LTA-induced NO production and iNOS protein expression.

DISCUSSION

Data of the present study provides scientific evidence to link glycosylation, NO inhibition, and HO-1 induction of flavonoids in LPS-treated macrophages. Briefly, the flavonoid aglycones, HT and NE, but not their respective flavonoid glycoside, HD and NI, inhibited LPS-induced NO production in the presence of HO-1 induction. Conversion of HD/NI to HT/NE by enzyme digestion recovered the inhibition by HD/NI of LPS-induced NO production and iNOS expression with induction of HO-1 expression. Additionally, overexpression of HO-1 by transfection decreased NO production and iNOS protein expression induced by LPS, while the HO-1 inhibitor, SnPP, attenuated the inhibitory effect by HT/NE on NO production induced by LPS. These results suggest that glycoside is a negative moiety in flavonoid inhibition of LPS-induced NO production, and that HO-1 induction may be involved in anti-inflammatory responses.

Structure–activity relationships of flavonoids have been extensively studied. Numbers of OH moieties in the A and B rings, and C_2-C_3 double bonds in concert with a 4-oxo functionally of the C ring have been shown to be important determinants of the antioxidant activity of flavonoids (Yoshida et al., 1999; Dugas et al., 2000). However, the effect of glycosides flavonoid or flavonoids bioactivity studies were less found. In relation to NO inhibition of flavonoids, such as apigenin, wogonin, oroxylin A, luteolin, tectorigenin, and quercetin inhibited NO production, however, flavonoid glycosides, such as apiin, did not demonstrated significant inhibition (Liang et al., 1999; Kim et al., 1999b). The inhibitory effect of flavonoids on human platelet function and ROS production in stimulated human neutrophils was diminished by glycosylation (Limasset et al., 1993). Our previous study demonstrated that aglycones quercetin but not its glycoside rutin inhibited NO production induced by LPS in vitro. Interestingly, both quercetin and its glycoside rutin showed significant NO inhibition elicited by LPS in vivo (Shen et al., 2002). We also found that addition of flavonoid with rhamnoside showed less apoptosis-inducing activity in leukemia cells (Chen

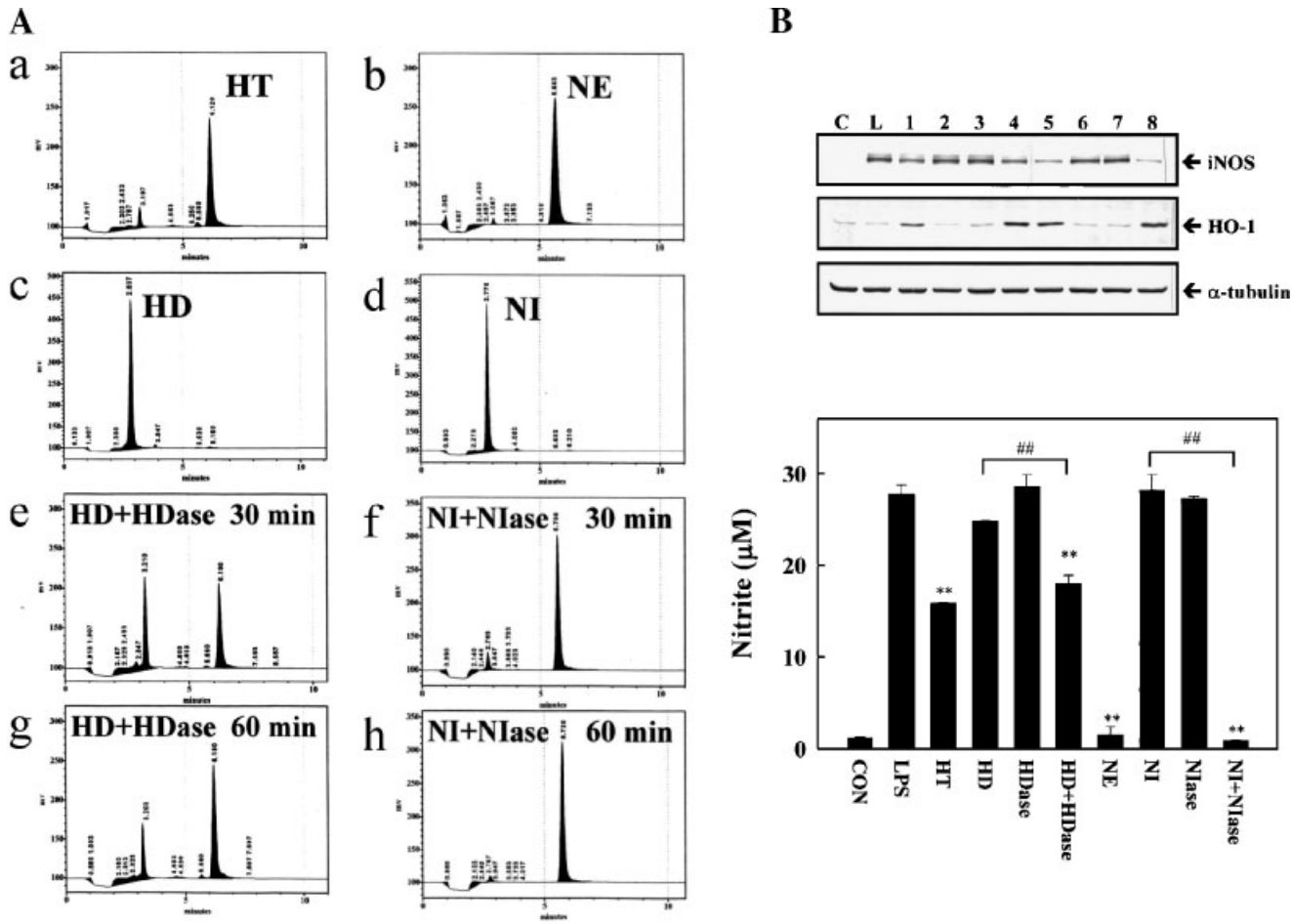

Fig. 7. Digestion of reutinoside at C7 of HD and NI by hesperidinase (HDase) and nariginase (NIase) resulted in inhibitory effects on LPS-induced iNOS expression and NO production by the production of HT and NE according to high-performance liquid chromatography (HPLC) analysis. **A**: HD and NI were incubated with or without HDase and naringinase (NIase) in Tris-buffer (pH 4.0) for 30 or 60 min, respectively, and the digested products were analyzed by HPLC and described in Materials and Methods. a: HT incubated for 60 min; b, NE incubated for 60 min; c, HD incubated for 60 min without HDase; d, NI incubated for 60 min without NIase; e, HD incubated for 30 min with HDase; f, NI incubated for 30 min with NIase; g, HD incubated for 60 min with HDase; h, NI incubated for 60 min with NIase. **B**: Upper part: RAW264.7 cells were treated Tris-buffer contained component in the presence of LPS (50 ng/ml) for 24 h, and expressions of iNOS, HO-1, and α-tubulin protein were detected by Western blotting. C, control; L, LPS; lane 1, HT incubated for 60 min; lane 2, HD incubated for 60 min; lane 3, HDase incubated for 60 min; lane 4, HD incubated for 60 min with HDase; lane 5, NE incubated for 60 min; lane 6, NI incubated for 60 min; lane 7, NIase incubated for 60 min; lane 8, NI incubated for 60 min with NIase. Lower part: NO production in the medium under different treatments was measured by the Griess reaction. $^{\#\#}P < 0.01$ indicates a significant difference from the indicated flavonoid-treated group, and $^{**}P < 0.01$ indicates a significant difference from the LPS-treated group, as analyzed by Student's *t*-test.

et al., 2003). In the present study, flavonoid aglycones (HT and NE) but not their respective flavonoid glycosides (HD and NI) inhibited LPS-induced NO production through blocking iNOS gene expression. Conversion of HD/NI, respectively to HT/NE by HDase and NIase recovered their inhibitory activities on LPS-induced NO production with the occurrence of the flavonoid aglycones, HT and NE. These data suggest that the addition of rhamnoside attenuates the NO inhibition of flavonoids in LPS-treated macrophages.

Previous studies showed that induction of the HO-1 protein protected cells from damage induced by in vitro and in vivo oxidative stresses (Clark et al., 2000; Yachie et al., 2003). An increasing amount of evidence has implicated involvement of HO-1 in the modulation of inflammatory responses; however, it has not yet been clearly delineated. Our recent studies demonstrated that HO-1 involved in LPS-induced responses and inhibited LPS-induced NO production in macrophages (Chen et al., 2002a; Lin et al., 2003). Additionally, induction of HO-1 suppressed venular leukocyte adhesion elicited by oxidative stress, and protected against the occurrence of atherosclerosis (Hayashi et al., 1999; Zhang et al., 2002). HO-1 was able to attenuate TNF-α-induced inflammatory injury in epithelial cells, and induction of HO-1 prevented systemic responses to hemorrhagic shock (Tamion et al., 2001; Kushida et al., 2002). These data suggested the beneficial biological effects of HO-1. In addition, several previous papers showed that activation of intracellular signaling pathway was important for HO-1 induction. Lee and Chau provided evidence the potent anti-inflammatory inter-

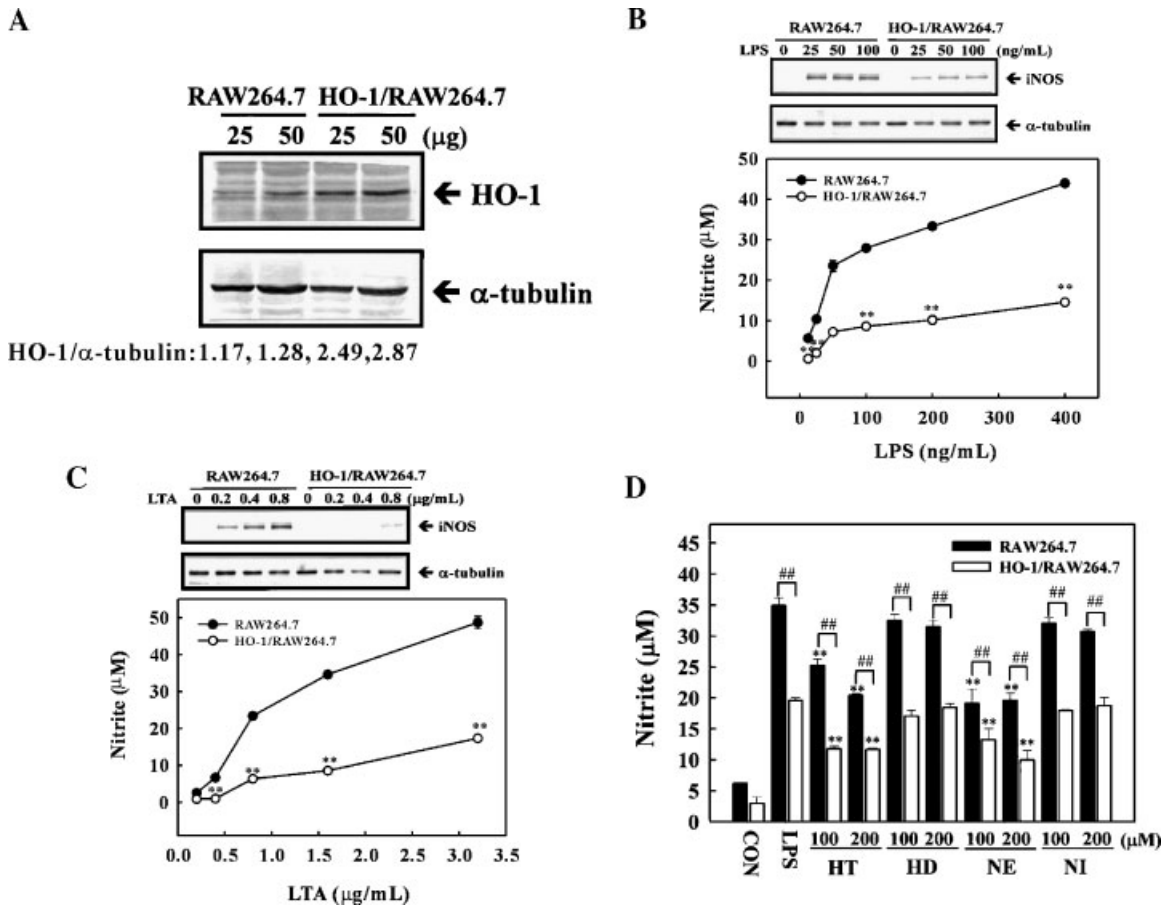

Fig. 8. HO-1/RAW264.7 cells potentiated the inhibitory activities of LPS- and lipoteichoic acid (LTA)-induced NO production. Transfection of HO-1-overexpressing cells (HO-1/RAW264.7) has been described in the section of Materials and Methods. **A**: Elevation of HO-1 protein level in HO-1-transfected RAW264.7 cells. Different amounts of total protein (25 and 50 μg) extracted from RAW 264.7 cells and HO-1/RAW 264.7 cells were applied to SDS–PAGE electrophoresis, and the level of HO-1 protein expression was detected by Western blotting. α-Tubulin was used as an internal control. Band intensity of HO-1 and α-tubulin protein were quantified by a densitometry analysis, and expressed as a ratio of HO-1/α-tubulin. **B**: Induction of iNOS protein expression induced by different doses of LPS in RAW264.7 cells. Upper part: RAW264.7 and HO-1/RAW264.7 cells were treated with different doses of LPS (25, 50, and 100 ng/ml) for 12 h, and expressions of iNOS and α-tubulin protein were detected by Western blotting. Lower part: Both RAW 264.7 and HO-1/RAW 264.7 cells were treated with different doses of LPS (12.5, 25, 50, 100, 200, and 400 ng/ml), and the amount of nitrite production in the medium was measured by Griess reaction. $^{**}P < 0.01$ indicates a significant difference from the data in RAW264.7 under LPS treatment. **C**: Induction of iNOS protein expression and NO production induced by different doses of LTA in RAW264.7 cells. Upper part: RAW264.7 and HO-1/RAW264.7 cells were treated with different dose of LTA (0.2, 0.4, and 0.8 μg/ml) for 12 h, and expressions of iNOS and α-tubulin protein were detected by Western blotting. Lower part: The amount of NO production induced by LTA (0.2, 0.4, 0.8, 1.6, and 3.2 μg/ml) in RAW 264.7 and HO-1/RAW 264.7 cells was measured by the Griess reaction. $^{**}P < 0.01$ indicates a significant difference from respective data in RAW264.7 on LTA-induced NO production. **D**: HT and NE show more potent inhibition on LPS-induced NO production HO-1/RAW264.7 than that in RAW264.7 cells. RAW 264.7 and HO-1/RAW 264.7 cells were treated with HT, HD, NE, and NI (100 and 200 μM) in the presence of LPS (50 ng/ml) for 12 h, and NO produced in the medium was detected by the Griess reaction. Data were obtained from six independent experiments, and expressed as the mean ± SE. $^{**}P < 0.01$ indicates a significant difference from the LPS-treated on each group, and $^{\#\#}P < 0.01$ indicates a significant difference from the respective group, as analyzed by Student's *t*-test.

leukin-10 (IL-10) induced expression of heme oxygenase-1 (HO-1) via a p38 mitogen-activated protein kinase-dependent pathway, and 15-deoxy-Delta 12,14-prostaglandin J2 exerted the anti-inflammatory effect in macrophages through a mechanism that involved the action of HO/CO (Lee and Chau, 2002; Lee et al., 2003). Chen et al. suggested that NO induced HO-1 gene expression via activation of ERK and p38 pathways (Chen and Maines, 2000). Our unpublished data indicated that activation of ERK but not JNK and p38 kinase involved in HO-1 induction by flavonoids (data not shown). In addition, several regulatory elements including activator protein 1 (AP-1), metal response element (MRE), and antioxidant response element (ARE) located at the promoter of *HO-1* gene and activation of MAPKs involved in HO-1 induction via increasing cytochrome P450 2E1 (CYP2E1) activity (Elbirt and Bonkovsky, 1999; Gong et al., 2003). Alam et al. (2000) demonstrated that activation of the p38 kinase pathway and Nrf2 involved in cadmium-induced HO-1 gene expression. It suggested that NE and HT induction of HO-1 might be through activation of MAPKs-mediated pathway to activate downstream molecules such as AP-1 or NF-κB.

Several natural products have been identified as HO-1 inducers including curcumin, quercetin, and baicalein.

Curcumin was able to protect endothelial cells from oxidative damage by inducing HO-1 gene expression, and our previous study demonstrated that NO inhibition of quercetin and baicalein occurred through HO-1 induction in LPS-treated RAW264.7 macrophages (Motterlini et al., 2000; Lin et al., 2003). However, a link between glycosylation, HO-1 induction and NO inhibition by natural products is still undefined. Glycosylation commonly occurs in the metabolism of flavonoids, and flavonoid glycosides have been shown to possess more-hydrophilic properties than do flavonoid aglycones. Kim et al. reported that flavonoid glycosides were metabolized to aglycones by human intestinal microflora producing α-rhamnosidase, exo-β-glucosidase, endo-β-glucosidase, and/or β-glucuronidase. Accordingly, rutin, HD, NI, and poncirin were transformed to their respective aglycones by bacteria producing α-rhamnosidase and β-glucosidase (Kim et al., 1999a). The in vitro hydrolytic capability of L-rhamnosidases on flavonoid glycoside was dependent on pH, temperature, and indicated compound properties in a reaction buffer (Hollman and Katan, 1998; Scaroni et al., 2002). Our previous data also suggested that the flavonoid glycosides, rutin and quercitrin, but not the flavonoid aglycone, quercetin, did not exhibit NO inhibition in LPS-treated macrophages in vitro; however, both rutin and quercitrin exhibited significant NO inhibition in vivo (Shen et al., 2002). These data indicated that in vivo metabolic activity may be involved in converting flavonoid glycoside to flavonoid aglycones, and that this might reverse their NO inhibitory activities. However, direct evidence is still lacking. Herein, removing the glycoside by enzyme digestion reversed the NO inhibitory activities of the flavonoid glycosides, HD/NI, with the production of the respective flavonoid aglycones, HT/NE, accompanied by HO-1 induction and iNOS inhibition as observed in flavonoid aglycones HT/NE-treated macrophages. This strongly supports glycoside being a negative moiety in the anti-inflammatory responses of flavonoids.

In conclusion, flavonoids have potential to be developed due to their beneficial biological effects, and results of the present study provide scientific evidence to indicate that flavonoid aglycones are an active form in the inhibition of NO production induced by LPS in accordance with HO-1 induction. Effects of additional substitutions such as methylation or complex glycosylation on the biological activities of flavonoids should be further elucidated.

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