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Nitric Oxide and Prostaglandin E_2 Participate in Lipopolysaccharide/Interferon-γ-Induced Heme Oxygenase 1 and Prevent RAW264.7 Macrophages From UV-Irradiation-Induced Cell Death

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Abstract Induction of heme oxygenase (HO)-1 during inflammation has been demonstrated in many cell types, but the contribution of inflammatory molecules nitric oxide (NO) and prostaglandin E_2 (PGE_2) has remained unresolved. Here we show that NO donors including sodium nitroprusside (SNP) and spermine nonoate (SP-NO), and PGE_2 significantly stimulate HO-1 expression in RAW264.7 macrophages, associated with alternative induction on NO and PGE_2 in medium, respectively. NO donors also show the inductive effect on cyclo-oxygenase 2 protein and PGE_2 production. In the presence of lipopolysaccharide and interferon-γ (LPS/IFN-γ), HO-1 protein was induced slightly but significantly, and SNP, SP-NO, and PGE_2 enhanced HO-1 protein induced by LPS/IFN-γ. L-Arginine analogs *N*-nitro-L-arginine methyl ester (L-NAME) and *N*-nitro-L-arginine (NLA) significantly block HO-1 protein induced by LPS/IFN-γ associated with a decrease in NO (not PGE_2) production. And, NSAIDs aspirin and diclofenase dose dependently inhibited LPS/IFN-γ-induced HO-1 protein accompanied by suppression of PGE_2 (not NO) production. PD98059 (a specific inhibitor of MEKK), but not SB203580 (a specific inhibitor of p38 kinase), attenuated PGE_2 (not SP-NO) induced HO-1 protein. Under UVC (100 J/m^2) and UVB (50 J/m^2) irradiation, PGE_2 or SP-NO treatment prevents cells from UVC or UVB-induced cell death, and HO-1 inhibitor tin protoporphyrin (SnPP) reverses the preventive effects of PGE_2 and SP-NO. The protective activity induced by PGE_2 on UVC or UVB irradiation-induced cell death was blocked by MAPK inhibitor PD98059 (not SB203580). These results demonstrated that inflammatory molecules NO and PGE_2 were potent inducers of HO-1 gene, and protected cells from UV-irradiation-induced cell death through HO-1 induction. J. Cell. Biochem. 86: 331–339, 2002.

Key words: nitric oxide; prostaglandin E_2; heme oxygenase; lipopolysaccharide; UV irradiation

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 constitutive expressed and HO-1 is the inducible form that provides protection against oxidative stress [Nath et al., 1992; Yet et al., 2001]. HO-1 null mice and HO-1 deficient human showed that HO-1 is an important molecule in the host's defense against oxidative stress and have the potent anti-inflammatory properties because both HO-1 deficient mice and humans have a phenotype of an increased inflammatory state [Poss and Tonegawa, 1997; Yachie et al., 1999]. Enhancement of HO-1 protein by using agonists prevented the development of hypoxic

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pulmonary hypertension and indicated that CO might be involved in the vasodilating and antiproliferative action. Exogenous administration of HO-1 by gene transfer protects the lung cells from free radicals induced lethal effects and showed the potent anti-inflammatory effects in the lung [Otterbein et al., 1999].

Septic shock and sepsis syndrome caused death in hospitalized patients mediated by excessive stimulation of immune cells, particularly monocytes and macrophages [Ledingham and McArdle, 1978; Hollenberg et al., 2000]. During the sepsis, infectious organisms induced uncontrolled production of pro-inflammatory molecules including nitric oxide (NO), prostaglandin E_2 (PGE_2), tumor necrosis factor-α, and interleukin-1β was detected. Lipopolysaccharide (LPS) and lipoteic acid (LTA), the constituents of Gram negative and Gram positive, respectively, are the leading causes of sepsis. Experimental administration of LPS or LTA to macrophages or mice mimics the same inflammatory responses as sepsis [Bucher et al., 1997]. After LPS or LTA administration, there is a rapid increase in the NO and PGE_2 productions through induction of nitric oxide synthase (NOS) and cyclo-oxygenase (COX) activities [Chen et al., 2000, 2001a]. Both NOS and COX have constitutive and inducible isoforms encoded by two unique genes located on different chromosomes [Wada et al., 2000]. iNOS and COX-2 are inducible forms of NOS and COX enzymes and are triggered by cytokines, LPS, or LTA. Recent studies indicated that molecular mechanism of NO and PGE_2 during inflammation is a complex process, and both NO and PGE_2 played as secondary messengers in modulation of inflammatory signaling transduction [Notoya et al., 2000].

Induction of HO-1 gene expression by LPS has been demonstrated in several previous studies [Oshiro et al., 1999; Immenschuh et al., 1999a]. However, little is known about the contribution of NO and PGE_2 on LPS-induced HO-1 gene expression. Using the well-established model of LPS-induced inflammation in RAW264.7 macrophages in our previous studies [Chen et al., 2001a,b], we study whether NO and PGE_2 involved in the modulation of HO-1 gene expression in the presence or absence of LPS/IFN-γ. Results of this study demonstrated that both NO and PGE_2 participate in the induction of HO-1 gene expression in the presence or absence of LPS/IFN-γ, and show the preventive effects on UV-irradiation-induced cell death. We proposed that NO and PGE_2 played as secondary messengers in the modulation of HO-1 gene expression during inflammation to protect cells from damage.

MATERIALS AND METHODS

Cells

RAW264.7, a mouse macrophage cell line, was obtained from the American Type Culture Collection. Cells were cultured in RPMI-1640 medium 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) and maintained at 37°C humidified incubator containing 5% CO_2.

Agents

Antibodies including anti-COX-2, anti-iNOS, anti-α-tubulin, and anti-HO-1 were purchased form Santa Cruz (Santa Cruz, CA). *N*-nitro-L-arginine (NLA), sodium nitroprusside (SNP), *N*-nitro-L-arginine methyl ester (L-NAME), indomethacin, PGE_2, LPS, interferon-γ (IFN-γ), naphylethylenediamine dihydrochloride, and sulfanilamide were purchased from Sigma (St. Louis, MO). Spermine nonoate (SP-NO) was purchased from Molecular Probes, Inc. (Eugene, OR).

Cell Viability

Cell viability was assessed by MTT staining as described by Mosmann [1983]. Briefly, RAW 264.7 macrophages were plated at a density of 10^4 cells/well into 24-well plates under different treatment for 24 h. At the end of the treatment, 20 μl of MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) (10 mg/ml) was added, and cells were incubated for a further 4 h. Cell viability was obtained by scanning with an ELISA reader (Molecular Devices) with a 600-nm filter.

Nitrite Assay

The nitrite concentration in the medium was measured as an indicator of NO production according to the Griess reaction [Chen et al., 2001a]. One hundred microliters of each supernatant was mixed with the same volume of Griess reagent (1% sulfanilaminde in 5% phosphoric acid and 0.1% naphthylethylendiamine dihydrochloride in water). Absorbance of the mixture at 550 nm was determined with

an ELISA plate reader (Dynatech MR-7000; Dynatech Laboratories).

Western Blots

Total cellular extracts were prepared according to our previous studies [Chen et al., 1999b, 2001c], separated on SDS–polyacrylamide min gels (8% for iNOS and COX-2; and 10% for HO-1 and α-tubulin), and transferred to immobilon polyvinylidene difluoride membrane (Millipore). The membrane was incubated with 1% bovine serum albumin and then incubated with anti-iNOS, anti-COX-2, anti-HO-1, and anti-α-tubulin antibodies, respectively (Santa Cruz, CA). Expression of iNOS, COX-2, HO-1, and α-tubulin were detected by NBT and BCIP staining (Sigma Chemical Co).

Measurement of PGE_2 Production

RAW264.7 cells were cultured in 6-well plates and were incubated with different treatment for 12 h. One hundred microliters of supernatant of culture medium were collected for the determination of PGE_2 concentrations by ELISA (Cayman Enzyme Immunoassay kit).

Statistical Analysis

Results are expressed as the means ± SEM of indicated number of experiments. Statistical significance was estimated using Student's *t* test for paired observations. A possibility value of less than 0.05 was considered significant.

RESULTS

Induction of HO-1 Protein by NO Donors and PGE_2

In order to elucidate the effects of NO and PGE_2 on HO-1 gene expression, RAW264.7 macrophages were treated with SNP (400 μM), SP-NO (200 μM), and PGE_2 (20 μg/ml) alone; and the expressions of iNOS, COX-2, and HO-1 proteins were detected by Western blotting using specific antibodies. Figure 1A shows that NO donors SNP and SP-NO are potent inducers on HO-1 and COX-2 proteins without change on iNOS protein expression. Both SNP and SP-NO showed the significant induction on PGE_2 and NO production in medium measured by ELISA and Griess reaction, respectively. In the same part of the experiment, adding PGE_2 showed the inductive effect on HO-1, but not iNOS and COX-2, protein, associated with the increased amount of PGE_2, not NO, in the culture medium. The data of Figure 1 suggested that NO and PGE_2 were potent inducers on HO-1 gene expression.

NO and PGE_2 Stimulated Synergistically on LPS/IFN-γ-Induced HO-1 Gene Expression

In order to demonstrate the effects of NO and PGE_2 on LPS/IFN-γ-induced HO-1, RAW264.7 macrophages were treated with LPS/IFN-γ (100 ng/ml of LPS and 20 ng/ml of IFN-γ) in the presence or absence of SNP, SP-NO, and PGE_2. As shown in Figure 2, LPS/IFN-γ showed the slight but significant induction on HO-1 protein

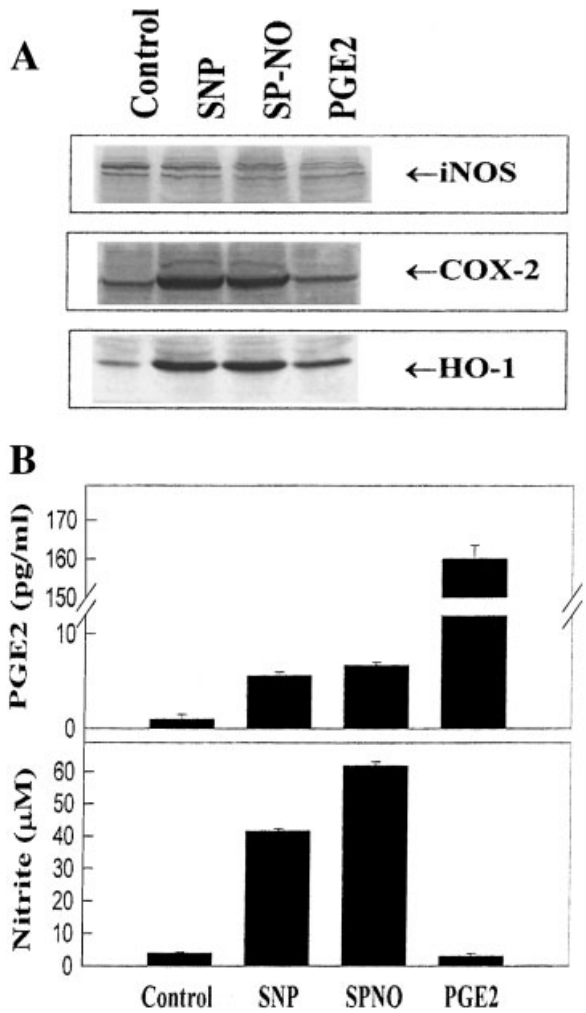

Fig. 1. Alternative inductive effects of SNP, SP-NO, and PGE_2 on iNOS, COX-2, HO-1 proteins, and NO and PGE_2 production in RAW264.7 cells. (**A**) Cells were treated with SNP (SNP; 400 μM), SP-NO (SPNO; 200 μM), and PGE_2 (PGE_2; 20 μg/ml) for 12 h, respectively, and the expression of iNOS, COX-2, and HO-1 proteins were detected by Western blotting using specific antibodies. (**B**) The amount of NO and PGE_2 in the medium was measured by Griess reaction and ELISA as described in Materials and Methods.

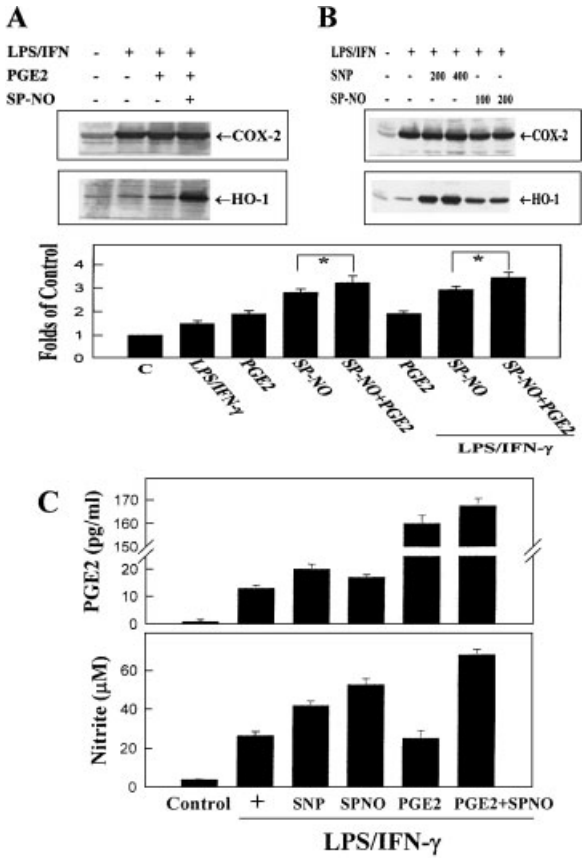

Fig. 2. Effects of SNP, SP-NO, and PGE_2 on LPS/IFN-γ (100 ng/ml for LPS and 20 ng/ml for IFN-γ)-induced HO-1, COX-2, NO, and PGE_2 in RAW264.7 cells. (**A**, **B**) RAW 264.7 macrophages were treated with SNP (200, 400 μM), SP-NO (100, 200 μM), and PGE_2 (20 μg/ml) in the presence or absence of LPS/IFN-γ for 12 h, and the expression of HO-1 and COX-2 proteins were analyzed by Western blotting using specific antibodies. (**Lower panel**) Band intensities of HO-1 protein under different treatments were analyzed by densitometry (IS-1000 Digital Imaging System) from three independent experiments, and described as folds of control, and the doses of PGE_2 and SP-NO are 20 μg/ml and 200 μM, respectively. $^*P < 0.05$ indicated significant difference between indicated two groups, as analyzed by Student's *t*-test. (**C**) The amount of NO and PGE_2 in medium under different treatments were detected using Griess reaction and ELISA as described in Figure 1. Unlabeled doses of SNP, SP-NO, and PGE_2 are 400 μM, 200 μM, and 20 μg/ml, respectively.

expression, and SNP, SP-NO, and PGE_2 stimulated LPS/IFN-γ-induced HO-1 protein. In the presence of SP-NO and PGE_2, synergistic stimulatory effect on LPS/IFN-γ-induced HO-1 protein was detected. However, SP-NO and SNP showed no inductive effect on LPS/IFN-γ-induced PGE_2 production and COX-2 protein, although both of them exhibited inductive effect on PGE_2 and COX-2 expression in the absence of LPS/IFN-γ (Fig. 1). These results indicated that NO and PGE_2 stimulated LPS/IFN-γ-induced HO-1 gene expression, and acted synergistically.

NO and PGE_2 Participated in LPS/IFN-γ-Induced HO-1 Gene Expression

In order to demonstrate whether NO and PGE_2 directly participated in LPS/IFN-γ-induced HO-1 gene expression, NOS inhibitors including *N*-nitro-L-arginine methyl este (L-NAME) and *N*-nitro-L-arginine (NLA), and COX inhibitors NSAIDs including aspirin and diclofenac were used to block NO and PGE_2 productions induced by LPS/IFN-γ, and the expression of iNOS, COX-2, and HO-1 proteins were analyzed. Results of Figure 3 showed that a prior addition of L-NAME (200, 400, 800 μM) or NLA (100, 200, 400, 800 μM) for 1 h significantly inhibited LPS/IFN-γ-induced NO (not PGE_2) production, accompanied by a dose dependent decrease on HO-1 protein induced by LPS/IFN-γ. However, iNOS, COX-2, and PGE_2 remained unchanged in L-NAME- and NLA-treated cells in the presence of LPS/IFN-γ. Results of Figure 4 showed COX inhibitors aspirin (200, 400, 800 μM) and diclofenac (10, 20, 40 μM) dose dependently blocked LPS/IFN-γ-induced PGE_2 (not NO) production, associated with dose dependent inhibition on HO-1 protein expression (Fig. 4). However, iNOS, COX-2, and NO production remained unchanged in aspirin- and diclofenac-treated cells in the presence of LPS/IFN-γ. These data demonstrated that NO and PGE_2 participated directly in LPS/IFN-γ-induced HO-1 gene expression.

MAPK Specific Inhibitor PD98059 Blocked PGE_2 but not SP-NO Induced HO-1 Protein

MAPK cascades have been described to be involved in inflammatory processes. In order to identify if MAPK cascades involved in the action of PGE_2 and SP-NO on HO-1 induction, PD98059 (a specific inhibitor of MAPK) and SB203580 (a specific inhibitor of p38 kinase) were used in this study. Result of Figure 5 showed that a prior treatment of macrophages with PD98059 or SB203580 (10, 20, 40 μM) for 1 h followed by SP-NO (400 μM) treatment showed no effect on HO-1 protein induced by SP-NO. Interestingly, PD98059, but not SB203580, inhibited PGE_2-induced HO-1 protein expression in a dose-dependent manner. It shows that SP-NO-mediated HO-1 expression does not require the activation of MAPK.

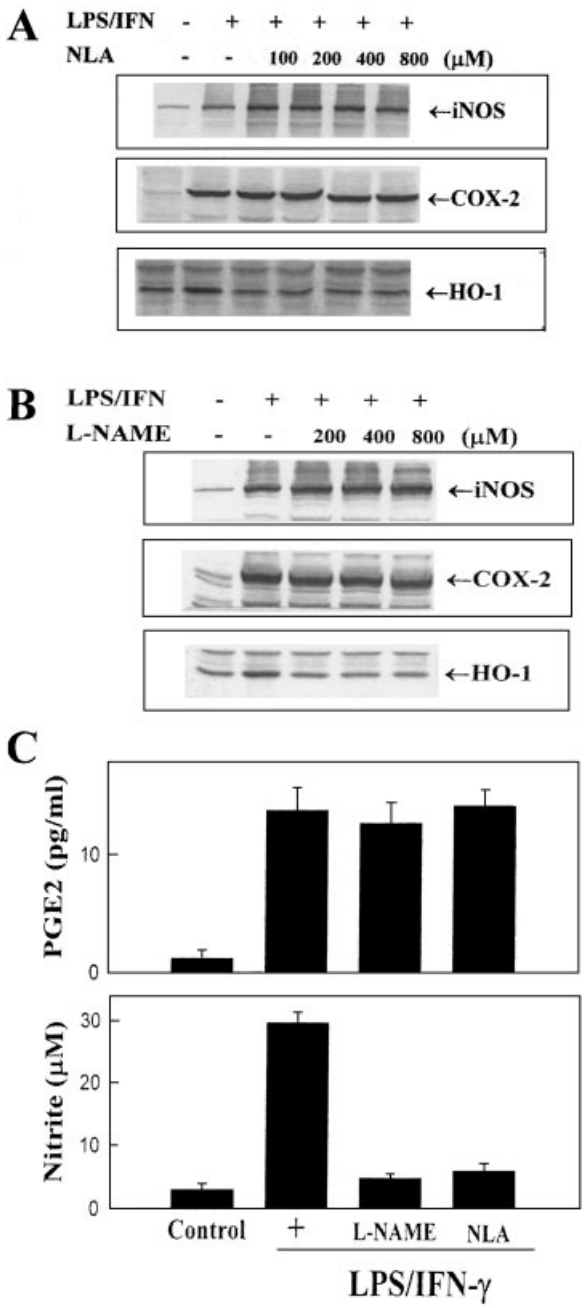

Fig. 3. Decrease of LPS/IFN-γ (100 ng/ml for LPS and 20 ng/ml for IFN-γ)-induced NO production by L-NAME and NLA attenuated HO-1 expression. (**A**, **B**) RAW264.7 macrophages were treated with different concentrations of NLA (100, 200, 400, 800 μM) or L-NAME (200, 400, 800 μM) for 1 h followed by LPS/IFN-γ treatment for a further 12 h. Expression iNOS, COX-2, HO-1 proteins were analyzed by Western blotting using specific antibodies. (**C**) The amount of NO and PGE_2 in medium under different treatments were detected using Griess reaction and ELISA as described in Figure 1. The doses of L-NAME and NLA for NO and PGE_2 are 800 μM.

Induction of HO-1 Protein by SP-NO and PGE_2 Protected Cells From UVC- and UVB-Irradiation-Induced Cell Death

Induction of HO-1 has been described to protect cells from damages. In order to examine if HO-1 protein induced by NO and PGE_2 showed the protective effects on UVC- or UVB-irradiation-induced cell death, RAW264.7 macrophages were treated with or without SP-NO (400 μM) or PGE_2 (20 μg/ml) for 6 h followed by UVC (100 J/m^2) or UVB (50 J/m^2) for a further 24 h. Viability of cells under different treatment was analyzed by MTT assay. Results of Figure 6 showed that UVC or UVB irradiation alone showed the cytotoxic effects on cells, and a prior treatment of cells with SP-NO or PGE_2 protected cells from UVC- or UVB-irradiation-induced cell death. Adding the HO inhibitor tin protoporphyrin (SnPP) with SP-NO or PGE_2 followed by UV irradiation reverses the protective effects induced by SP-NO and PGE_2. HO-1 inducer hemin prevented cells from UV irradiation induced cytotoxicity that was suppressed by SnPP as a positive control. The protective activity of PGE_2 on UV irradiation induced cell death was attenuated by PD98059 (not SB20 3580). These data demonstrated that both PGE_2 and SP-NO protect cells from UV irradiation induced cell death through HO-1 induction.

DISCUSSION

It is well known that NO and PGE_2 are important inflammatory molecules after LPS exposure, and induction of HO-1 gene expression by LPS also has been proposed previously [Camhi et al., 1995; Immenschuh et al., 1999b]. However, effects of NO and PGE_2 on HO-1 gene expression is still undefined. Results of this study demonstrated firstly that NO and PGE_2 were HO-1 inducers and involved in LPS/IFN-γ-induced HO-1 expression. Induction of HO-1 by NO and PGE_2 was able to prevent cells from UV irradiation induced cell death. We proposed that NO and PGE_2 played as secondary inflammatory molecules in stimulation of HO-1 gene expression during inflammation synergistically, and protected cells from cell death.

There is strong evidence to support that HO-1 is essential in maintaining cellular and tissue homeostasis in vitro and in vivo models of oxidant-induced injury [Kurata et al., 1996; Takahashi et al., 2000]. Despite convincing data indicating the protective function of HO-1 in

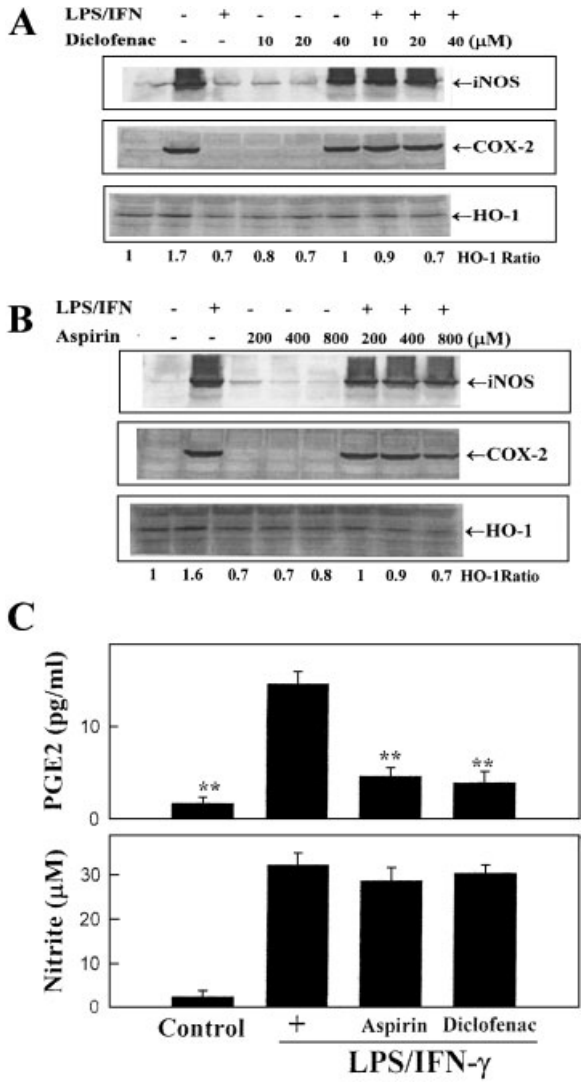

Fig. 4. Decrease of LPS/IFN-γ (100 ng/ml for LPS and 20 ng/ml for IFN-γ)-induced PGE_2 by COX inhibitors aspirin and diclofenac suppressed HO-1 expression. (**A**, **B**) RAW264.7 macrophages were treated with different concentrations of diclofenac (10, 20, 40 μM) or aspirin (200, 400, 800 μM) for 1 h followed by LPS/IFN-γ treatment for a further 12 h. Expression iNOS, COX-2, HO-1 proteins were analyzed by Western blotting using specific antibodies. Level of HO-1 protein expression was quantitated by densitometry analysis (IS-1000 Digital Imaging System), and described as means of three independent experiments (HO-1 ratio). (**C**) The amount of NO and PGE_2 in medium under different treatments were detected using Griess reaction and ELISA as described in Figure 1. The doses of aspirin and diclofenac used for NO and PGE_2 detection is 800 and 40 μM, respectively.

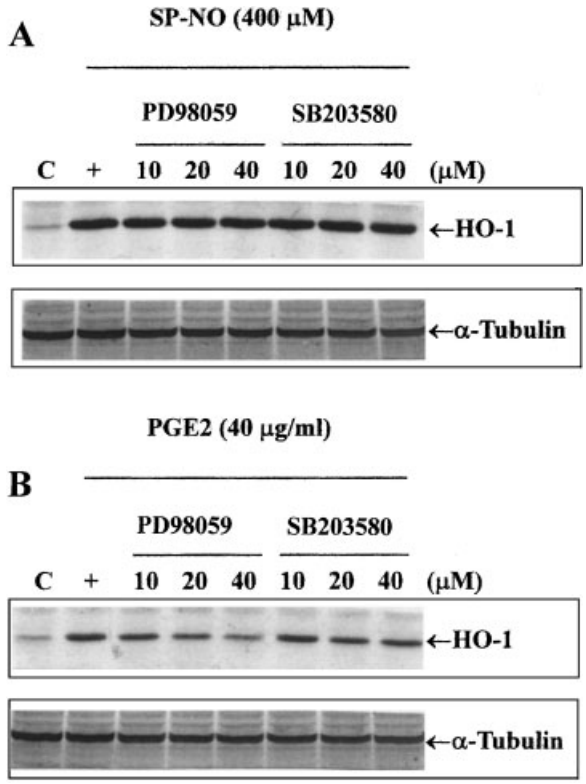

Fig. 5. A Specific MAPK inhibitor PD98059, but not a specific p38 kinase inhibitor SB203580, inhibited PGE_2 (not SP-NO)-induced HO-1 protein expression. RAW264.7 macrophages were treated with different doses of PD98050 or SB203580 for 1 h followed by SP-NO (**A**; 400 μM) or PGE_2 (**B**; 20 μg/ml) treatment for a further 12 h. Expression of HO-1 protein was analyzed by Western blotting using a specific antibody. α-Tubulin was used as an internal control to identify the equal amount of protein loaded in each lane.

oxidative stress, the precise mechanism of HO-1 remains elusive. HO-1 can convert heme into products including bilirubin, ferritin, and CO. Both bilirubin and ferritin can function as potent anti-oxidative molecules in vivo and in vitro to elicit cytoprevention [Yamaguchi et al., 1996]. CO may be involved in neuronal transmission and modulation of vasomotor tone [Ingi and Ronnett, 1995]. Otterbein et al. [2000] reported that CO performed anti-inflammatory effects through activation of MAPK cascade and indicated that CO may have an important protective function in inflammatory disease states. In this study, we demonstrated that NO and PGE_2 induced HO-1 gene expression in RAW 264.7 macrophages. The results of current study provided an interesting phenomenon indicating a cross-talk between inflammatory molecules NO and PGE_2 on HO-1 gene expression. The physiological function of HO-1 protein induced by NO and PGE_2 is still undefined and deserved for further study.

NO and PGE_2 are two pleiotropic mediators produced at inflammatory site by inducible enzymes, iNOS and COX-2, respectively. A variety

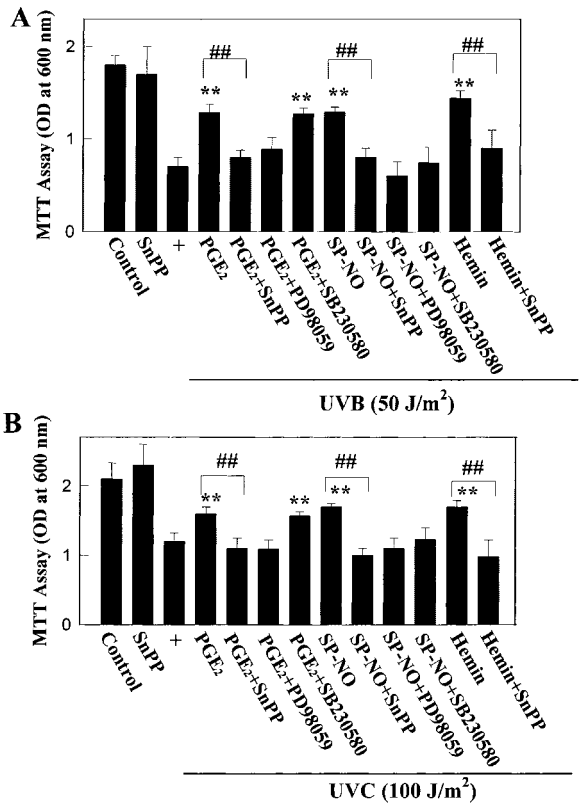

Fig. 6. Preventive effects of PGE_2 and SP-NO on UVB- or UVC-irradiation-induced cell death in RAW264.7 macrophages. (**A**, **B**) Cells were treated with PGE_2 (20 μg/ml) or SP-NO (400 μM) in the presence or absence of HO inhibitor SnPP (20 μM) for 6 h followed by UVB (50 J/m^2) or UVC (100 J/m^2) irradiation for a further 24 h. Further, cells were treated with different concentration of PD98059 or SB203580, for 1 h followed by UVB- or UVC-irradiation for a further 24 h. Viability of cells under different treatment were measured using MTT assay as described in Materials and Methods. Each value presented here was described as mean ± SE of three independent experiments. $^{**}P < 0.01$ significantly different from the UVC or UVB treated group, as analyzed by Student's *t*-test. $^{\#\#}P < 0.01$ significantly different from the PGE_2 or SP-NO treated group, as analyzed by Student's *t*-test.

of cells including endothelium, macrophages, chondrocytes upregulate both iNOS and COX-2 and produce NO and PGE_2 simultaneously in response to cytokines and other activators [Nusing and Barsig, 1999; Ianaro et al., 2000]. A complex relationship is emerging with regard to cross-talk between NO and COX pathways [Chen et al., 1999a; Mei et al., 2000; Bogdan, 2001]. NO has been reported to stimulate COX activity [Levin et al., 1981; Salvemini et al., 1993], but others reported that NO attenuated PGE_2 production in LPS-stimulated peritoneal macrophages [Amin et al., 1997; Habib et al., 1997]. Patel et al. [1999] reported that low concentration of NO attenuates PGE_2 production in response to LPS due to decrease expression of cytosolic COX-2 proteins, and Clancy et al. [2000] reported that induction of prostaglandin production by NO was through activating COX-1, but inhibiting COX-2. It appeared that a complex regulatory mechanism appeared in NO and PGE_2 productions. In this study, treatment of macrophages with NO donors SNP and SP-NO stimulated PGE_2 production in the presence or absence of LPS/IFN-γ through increase COX-2 protein in RAW264.7 macrophages. However, exogenous PGE_2 exhibited no effect on COX-2, iNOS proteins, and NO production. We proposed that exogenous NO participated in the induction of PGE_2 production during inflammation through increasing COX-2 gene expression.

Induction of HO activity has been demonstrated to prevent cells from extracellular stimulant-induced cytotoxic effects. The cytoprotection by HO might be attributable to its augmentation of iron efflux, induction of SOD and reduced glutathione, reflecting a role for HO in regulating cell viability [Yang et al., 1999; Frankel et al., 2000]. In this study, NO donors SNP and SP-NO, and PGE_2 induced HO-1 gene expression and protected cells from UV irradiation induced cell death. Hemin, a well-known HO-1 inducer, exhibited significant protection on UV-induced cell death. The preventive effects of SP-NO and PGE_2 on UV-induced cell death was blocked by HO inhibitor SnPP. These results suggested that inflammatory molecules NO and PGE_2 might be protective through HO-1 induction. Interestingly, PD98059 and SB203580 abolished the preventive effects of SP-NO on UV-induced cell death, however both of them showed no effect on HO-1 protein expression induced by SP-NO. Mechanism of PD98059 and SB203580 reversed SP-NO-prevented cell death induced by UV irradiation is still undefined. Activation of MAPK cascades including MAPK, p38 kinase and JNK has been demonstrated in UV-irradiated cells [Price et al., 1996]. Eom et al. [2001] reported that SB203580 showed to prevent cells from UV-induced cell death. Results of this study indicated that proteins except HO-1 located at the downstream of MAPK and p38 kinase cascades might be involved in the prevention of cells from UV-irradiation-induced cell death under SP-NO treatment.

In conclusion, the results of this study provided first molecular evidences to demonstrate that inflammatory products NO and PGE_2 stimulate HO-1 gene expression synergistically, and are directly involved in LPS/IFN-γ-induced HO-1 gene expression. Induction of HO-1 by NO and PGE_2 participated in the prevention of cells from UV-irradiation-induced cell death. We proposed that induction of HO-1 by NO and PGE_2 play critical physiological functions including anti-apoptosis, anti-oxidative, and anti-carcinogenic effects, and protected cells from deleterious damages.

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