Exp Biol Med (Maywood) OnlineFirst, published on January 19, 2015 as doi:10.1177/1535370214567612

Original Research

Effective suppression of nitric oxide production by HX106N through transcriptional control of heme oxygenase-1

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Abstract

Heme oxygenase-1 (HO-1) has been suggested to be a key neuroprotective enzyme because of its widespread distribution in the brain as well as its strong antioxidative effects. HX106N, a water-soluble botanical formulation, has previously been demonstrated to prevent amyloid β-induced memory impairment and oxidative stress in mice by upregulating HO-1 levels. In this study, the underlying molecular mechanisms of HX106N-induced HO-1 expression were investigated using BV-2 cells, a murine microglial cell line, and primary microglia. Treatment with HX106N induced the expression of HO-1 at the transcriptional level through the stress-responsive element-containing enhancer present in the *ho-1* promoter. Nuclear factor E2-related factor 2 (Nrf2) was activated in cells treated with HX106N. The results from knockdown assay showed that small interfering RNA of Nrf2 attenuated HX106N-mediated HO-1 expression. Pharmacological inhibitors of p38 and JNK mitogen-activated protein kinases suppressed the HX106N-mediated induction of HO-1. The NF-κB signaling pathway was activated by HX106N and played a role in HX106N-induced HO-1 expression. Furthermore, HO-1 and one of its by-products during the enzymatic degradation of heme, CO, were found to be involved in HX106N-mediated suppression of NO production. Taken together, these data indicate that HX106N exerts potent antioxidative effects by increasing the expression of HO-1 through multiple signaling pathways, leading to the suppression of NO production.

Keywords: HX106N, heme oxygenase-1, nuclear factor E2-related factor 2, NF-κB, nitric oxide

Experimental Biology and Medicine **2015; 0: 1–11. DOI: 10.1177/1535370214567612**

Introduction

The level of heme oxygenase-1 (HO-1) has been reported to influence the state and progress of various neurological diseases, including neurodegenerative diseases and ischemic brain diseases.[1–4] HO-1, one of major isoforms of heme oxygenase in the brain, is a rate-limiting enzyme responsible for catalyzing the degradation of heme to biliverdin, carbon monoxide (CO), and ferrous iron.[5] The expression of HO-1 can be induced specifically in microglia and astrocytes in response to a wide range of stress stimuli. Numerous studies have demonstrated that the induction of HO-1 plays a key role in cellular defense responses through the anti-inflammatory and antioxidative activities of its enzymatic by-products as well as through the HO-1 protein itself.[6–10] In the nervous system, the neuroprotective role of HO-1 has been well documented. Transgenic mice that overexpress HO-1 in the brain are resistant to ischemic brain damage,[11] while HO-1 overexpression protects neurons from various oxidative stimuli, such as glutamate and hydrogen peroxide.[12,13] Owing to its antioxidative activities and widespread expression in the brain, HO-1 has been proposed as a therapeutic target for several nervous system disorders.[14]

The expression of HO-1 is regulated by complex cooperative interactions between various signaling kinases and transcription factors. Mitogen-activated protein kinases (MAPKs) have been reported to play a major role in HO-1 expression, and other kinases, including phosphatidylinositol 3-kinase (PI3K) and protein kinases A and C, might also be involved.[15] Multiple *cis*-acting elements in the *ho-1* promoter play critical roles in transcriptional regulation. In particular, the stress-responsive element (StRE), a 10-bp sequence motif in the E1 and E2 enhancers, has been identified as a dominant *cis*-element for the induction of *ho-1* gene expression.[16,17] Activating protein-1 (AP-1), composed of homo- and heterodimers from the Jun and Fos families,

and nuclear factor E2-related factor 2 (Nrf2) are StRE-binding activating transcription factors for the *ho-1* gene.[16,18] Two potential binding sequences for NF-κB were also identified within the immediate 5′ flanking region of the human *ho-1* gene, but it is currently unclear if these sequences are functional.[19] The consensus NF-κB binding sequence has not been observed in other mammalian *ho-1* genes.[19] However, accumulating evidence indicates that NF-κB contributes to HO-1 expression. Several agents, including curcumin, resveratrol, and docosahexaenoic acid, have been reported to induce HO-1 expression through NF-κB.[20–22]

HX106N is a botanical formulation that ameliorates memory impairment in amyloid β peptide-treated mice and generates strong antioxidative and neuroprotective activities, most likely by upregulating HO-1 expression.[23] In the present study, we investigated the molecular mechanisms underlying HX106N-mediated HO-1 expression using BV-2 cells and primary microglial cultures. HX106N induced the expression of *ho-1* RNA by activating various transcription factors and signaling molecules such as Nrf2, NF-κB, JNK, and p38 MAPK, all of which are known to be involved in HO-1 expression. HX106N-mediated suppression of nitric oxide (NO) production was inhibited by HO-1-specific inhibitors and small interfering RNA (siRNA). Our data suggest that the inhibitory effects of HX106N on NO production most likely resulted from the induction of the *ho-1* gene by multiple signaling pathways, which might contribute to the antioxidative activities of HX106N.

Materials and methods

Cell culture and reagents

BV-2 cells (a gift from Dr Eui-Ju Choi, Korea University, Korea) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) at 37°C under 5% CO_2. Primary cortical microglia were isolated from Institute Cancer Research (ICR) mice on postnatal day 1–3 (P1–3) with a mild trypsinization method as described previously.[24] LPS (*Escherichia coli*, 0111:B4), SB203580, SP600125, BAY 11-7802, Zinc (II) protoporphyrin-IX (ZnPP), hemoglobin (Hb), and carbon monoxide releasing molecule 3 (CORM-3) were purchased from Sigma (St Louis, MO, USA). PD98059 was purchased from Calbiochem (La Jolla, CA, USA).

Preparation of HX106N

HX106N was prepared from four plants as described previously.[23] The following plants were used: *Dimocarpus longan* Lour. (*Longanae Fructus*), *Liriope platyphylla* Wang et Tang. (*Liriopes Radix*), *Salvia miltiorrhiza* Bunge (*Salvia miltiorrhiza Radix*), and *Gastrodia elata* Blume (*Gastrodiae Rhizoma*). To prepare HX106N in a consistent manner, a cell-based bioassay was also used as reported by Lee *et al*.[23]

Western blot analysis

After treatment, cells were washed with cold PBS and lysed with phosphosafe extraction buffer (Novagen, Madison, WI, USA). The lysates were then separated by 10% SDS-PAGE and electrophoretically transferred to PVDF membranes. The membranes were incubated with primary antibodies specific for HO-1 (1:4000, Stressgen, Victoria, Canada), Nrf2 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA), c-Jun (1:500, Santa Cruz), p-JNK (1:1000, Cell Signaling, Beverly, MA), JNK (1:1000), p-p38 (1:1000, Cell Signaling), p38 (1:1000, Santa Cruz), p-IKKα/β (1:1:1000, Cell Signaling), IKKα (1:500, Santa Cruz), IκBα (1:500, Santa Cruz), p65 (1:500, Santa Cruz), iNOS (1:1000, BD Biosciences, Franklin Lakes, NJ), Arginase-1 (1:1000, Cell signaling), Arginase-2 (1:500, Santa Cruz), β-actin (1:5000, Sigma), or TFIIB (1:500, Santa Cruz). The membranes were then incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Sigma) antibodies. The bands were visualized by enhanced chemiluminescence (Millipore, Billerica, MA, USA) using X-ray film, and the band density was quantitated using Image J software.

Northern blot analysis

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The RNA was separated with 1% agarose-formaldehyde gel electrophoresis and transferred to a nylon membrane (Hybond-N, Amersham, Buckinghamshire, UK). The membrane was hybridized with ^{32}P-labeled mouse HO-1 and GAPDH probes overnight at 68°C. The blot was then washed and exposed to autoradiography film. The band density was measured using Image J software. Specific probes for mouse HO-1 and GAPDH were labeled by random priming using the Klenow fragment of DNA polymerase I (Stratagene, La Jolla, CA, USA) and [α-^{32}P]dCTP (Perkin Elmer, Norwalk, CT, USA). The PCR primer sequences were as follows: HO-1 (forward, TAC ACA TCC AAG CCG AGA AT; reverse, GTT CCT CTG TCA GCA TCA CC) and GAPDH (forward, ACG GCA AAT TCA ACG GCA CAG; reverse, GGC GGC ACG TCA GAT CC).

Luciferase reporter plasmid assay

Reporter plasmids containing the 15 kb *ho-1* promoter (pHO15-Luc), E1 (pE1-Luc), and E1M (pE1M-Luc) sequences were provided by Dr Eun-Hye Joe (Ajou University School of Medicine, Suwon, Korea). Reporter plasmid with five copies of an NF-κB response element (pNF-κB-Luc) was purchased from Promega (Madison, WI, USA). These plasmids encode luciferase as a reporter gene. BV-2 cells were transiently transfected with various reporter plasmids (3 μg) and a β-galactosidase plasmid (1 μg, Invitrogen) using FuGENE6 according to the manufacturer's protocols (Roche, Indianapolis, IN, USA). Twenty-four hours after transfection, the cells were treated with HX106N in the presence or absence of pharmacological inhibitors and incubated for another 9 h. Cell lysates were prepared, and a luciferase activity assay was performed using the Luciferase Reporter kit according to the manufacturer's protocol (Promega) with a microplate luminometer (MicroLumat Plus LB96V, Berthold, Germany). Luciferase activity was normalized to β-gal activity.

Electrophoretic mobility shift assay (EMSA)

BV-2 cells were treated with HX106N for 3 h and the cells were harvested. The cells were incubated with 200 μL of lysis buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, and protease inhibitor cocktail [Roche]) for 20 min. The same volume of lysis buffer B (lysis buffer A containing 0.5% Nonidet-P40) was added and incubated for an additional 20 min followed by centrifugation. The pellets were washed with 400 μL of lysis buffer A. After centrifugation, the pellets were resuspended in 20 μL of lysis buffer C (10 mM HEPES [pH 7.9], 400 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, and protease inhibitor cocktail [Roche]) and incubated on ice for 1 h. A centrifugation step generated the nuclear extracts. The binding reaction was performed in binding buffer (Novagen) containing 0.01 unit of poly(dI-dC), 2 μg of salmon sperm DNA, 0.05 pmol [^{32}P]-labeled DNA probe, and 10 μg of nuclear extracts. After incubation for 30 min on ice, the reaction was analyzed on non-denaturing 6% polyacrylamide gels that were electrophoresed using 0.5X TBE buffer (44.5 mM Tris base, 22.3 mM boric acid, and 1 mM EDTA [pH 8.0]). The gels were exposed to autoradiography film at –80°C. Oligonucleotide sequences designed for probe preparation were as follows (the binding motif is underlined): AP-1 (GCTTGATGACTCAGCCGGAA), ARE (TGGGGAACCTGTGCTGAGTCACTGGAG), and NF-κB (AGTTGAGGGGACTTTCCCAGGC). The specificity of the retarded complexes was confirmed by competition with 100-fold excess unlabeled oligonucleotide. For supershift/interference assays, the nuclear extracts were preincubated with 4 μg of anti-Nrf2, c-Jun, or p65 antibody (Santa Cruz) for 30 min on ice before the addition of the probe.

Transfection

The siRNA specific for Nrf2 (siNrf2), c-Jun (sic-Jun), IKKα (siIKKα), HO-1 (siHO-1), and a non-specific control siRNA (siCon; SN-1001) were synthesized by Bioneer (Daejeon, Korea). The siRNA was transfected into BV-2 cells using the Lipofectamine2000 reagent according to the manufacturer's protocol (Invitrogen). After 24 h, the cells were used for assays.

Measurement of NO

The level of NO present in culture supernatants was measured using a NO assay kit according to the manufacturer's instruction (R&D Systems, Minneapolis, MN, USA).

Statistics

The data are presented as the mean ± SD. Significant differences between the experimental groups were analyzed using Student's *t*-test. P-values less than 0.05 were considered significant.

Results

Effects of HX106N on the expression of HO-1

HO-1 is known to play critical roles in the maintenance of cellular homeostasis under stressful conditions.[15] Our previous study showed that HX106N has antioxidative activity, which coincided with a marked increase in the expression level of HO-1. However, the underlying molecular mechanism remained to be elucidated. In this study, we investigated the mechanism by which HX106N regulates HO-1 expression using BV-2 cells, a murine microglial cell line. First, BV-2 cells were treated with 1 mg/mL HX106N for various time periods, and whole cell lysates were prepared for the detection of HO-1 by Western blot analysis. The amount of HO-1 protein increased 6 h after treatment with HX106N, remained at a high level for 24 h, and then returned to the basal level after 48 h (Figure 1(a)). The effect on HO-1 was dose dependent, as shown in Figure 1(b). When the cells were treated with HX106N at a 1 mg/mL concentration for 24 h, the protein level of HO-1 was increased 11-fold (Figure 1(b)). To test whether this

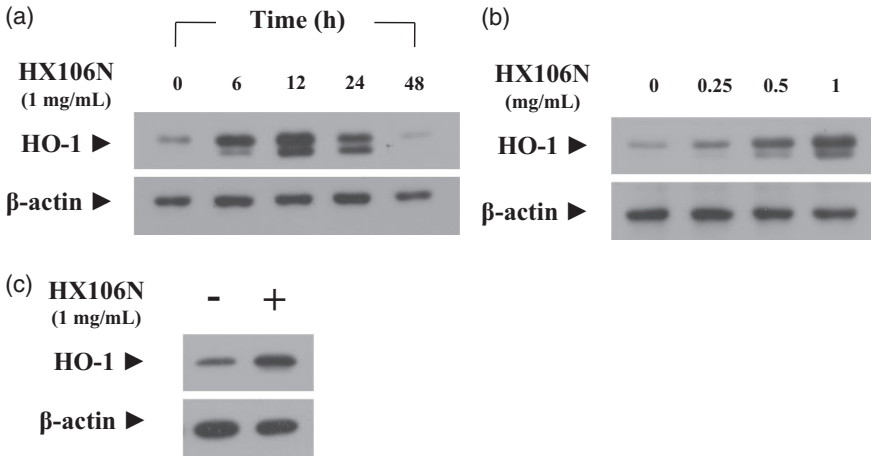

Figure 1 Effects of HX106N on the expression of HO-1 in BV-2 cells and primary microglia. (a) BV-2 cells were treated with 1 mg/mL HX106N for the indicated time periods. Whole cell lysates were prepared and subjected to Western blot analysis using antibodies specific for HO-1 and β-actin. β-actin was used as a loading control. (b) Cells were treated with various concentrations of HX106N for 24 h, and HO-1 and β-actin were detected in whole cell lysates by Western blot analysis. (c) Primary microglial cultures were obtained from the cortex of P1–P2 mouse pups as described in ''Materials and methods'' section and treated with 1 mg/mL HX106N for 24 h. The levels of HO-1 and β-actin in cell lysates were determined by Western blot analysis

result was restricted to a particular cell type, primary microglia were isolated from the cortex of P1–P2 mice and treated with 1 mg/mL HX106N for 24 h. When measured by Western blot analysis, treatment with HX106N increased HO-1 expression threefold compared to the control level (Figure 1(c)). These data indicated that HX106N could effectively induce HO-1 expression in cells of microglial origin.

Effects of HX106N on ho-1 transcription

Expression of the *ho-1* gene is known to be regulated mainly at the transcription level.[15] To investigate the effect of HX106N on *ho-1* transcription, BV-2 cells were treated with various concentrations of HX106N for 12 h, and total RNAs were prepared followed by Northern blot hybridization. The basal RNA level of HO-1 was very low but increased substantially after treatment with HX106N in a dose-dependent manner, resulting in an approximately 15-fold increase at a 1 mg/mL concentration (Figure 2(a)). When the RNA level of HO-1 was measured at earlier time points, 3, 6, and 9 h after treatment with 1 mg/mL HX106N, it was increased by 2.9-, 6.5-, and 10.0-fold, compared to untreated cells, respectively (data not shown). These results indicated that HX106N regulated the expression of HO-1 at the RNA level.

The effect of HX106N on the *ho-1* promoter was examined using a luciferase reporter plasmid, pHO15-Luc, containing the 15 kb full-length *ho-1* promoter sequence. BV-2 cells were transfected with pHO15-Luc and treated with various concentrations of HX106N for 9 h. Treatment with HX106N significantly increased the level of luciferase activity in a dose-dependent manner, and a 27-fold increase was observed at the 1 mg/mL concentration (Figure 2(b)).

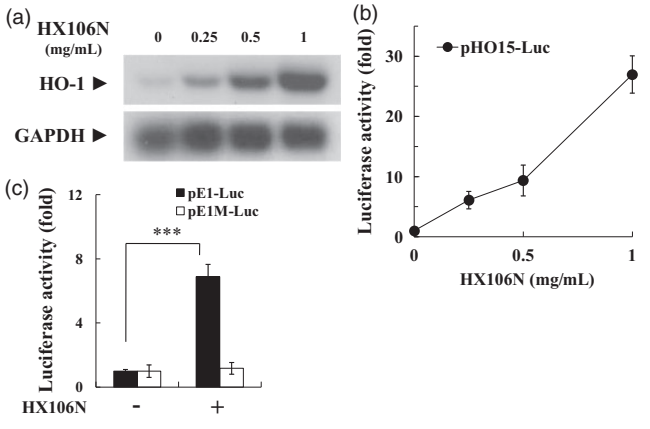

Figure 2 Effects of HX106N on the transcription of *ho-1*. (a) BV-2 cells were treated with various concentrations of HX106N for 12 h, and the total RNA was extracted, followed by Northern blot analysis. GAPDH was used as a loading control. (b) BV-2 cells were transfected with a luciferase reporter plasmid containing the full-length mouse *ho-1* promoter, pHO15-Luc. Twenty-four hours later, the cells were treated with various concentrations of HX106N for 9 h and subjected to a luciferase assay as described in the ''Materials and methods'' section. (c) BV-2 cells were transfected with luciferase plasmids containing the original (pE1-Luc) or mutant (pE1M-Luc) sequence of E1. The cells were treated with 1 mg/mL HX106N for 9 h, and the level of luciferase activity was measured. The values are presented as the mean ± SD of triplicate samples from one of three independent experiments. ***$P < 0.001$ compared to the untreated group

The *ho-1* promoter contains multiple copies of the StRE in the region called E1 and E2, which have been shown to be the primary loci responsible for *ho-1* transcriptional activation in response to various agents.[15,25,26] To test whether these enhancers are also involved in HX106N-mediated *ho-1* transcription, BV-2 cells were transfected with the luciferase reporter plasmids containing the wild-type (pE1-Luc) or mutant (pE1M-Luc) E1 enhancer sequence. Treatment with 1 mg/mL HX106N resulted in a 6.9-fold increase in the level of luciferase activity over that of cells transfected with pE1-Luc, while no significant change was found in cells transfected with pE1M-luc (Figure 2(c)). These data indicated that HX106N regulated the transcription of *ho-1* through the StRE-containing enhancer present in the *ho-1* promoter.

Effects of HX106N on Nrf2 and AP-1 activation

Because the StRE is structurally and functionally similar to the antioxidant response element (ARE) and AP-1 binding site, Nrf2 and AP-1 have been proposed to be the major transcription factors regulating the expression of *ho-1* in response to various stimuli.[16,18,27,28] Therefore, the effect of HX106N on the activation of Nrf2 and AP-1 was examined by assessing the nuclear accumulation of Nrf2 and c-Jun, a major component of the AP-1 family. BV-2 cells were treated with 1 mg/mL HX106N, and the nuclear proteins were extracted at various time points, followed by Western blot analysis to determine the levels of these two transcription factors. The levels of Nrf2 and c-Jun remained very low or undetectable at all of the time points examined in untreated cells, but a marked increase was observed after 1 h of HX106N treatment and was maintained throughout the experimental time course (Figure 3(a)).

To verify these results, a gel retardation assay was performed using ARE and AP-1 oligonucleotide probes. BV-2 cells were treated with 1 mg/mL HX106N for 3 h, and nuclear extracts were prepared, followed by EMSA. Treatment with HX106N significantly increased the amount of DNA–protein complex for both probes (Figure 3(b) and (c)). These complexes were specific because competition with cold oligonucleotide probes decreased the signal. Supershift/interference assays were carried out to test the binding of Nrf2 or c-Jun to ARE or AP-1 oligonucleotides, respectively. In the presence of Nrf2 or c-Jun antibodies, the formation of DNA-protein complex was inhibited (Figure 3(b) and (c)).

To examine the roles of Nrf2 and AP-1 in the HX106N-induced HO-1 expression, BV-2 cells were transfected with siRNA specific for Nrf2 (siNrf2), c-Jun (sic-Jun), or a non-specific siRNA (siControl). Twenty-four hours later, the cells were treated with 1 mg/mL HX106N for 6 h, and whole cell lysates were prepared followed by Western blot analysis. When compared to the case of siControl, siNrf2 and si-cJun transfection downregulated the protein level of Nrf2 and c-Jun by 54 and 62%, respectively (Figure 3(d)). The level of HO-1 was greatly increased by HX106N treatment in the cells transfected with siControl, while it was reduced by 40% in those with siNrf2 (Figure 3(d)). Sic-Jun transfection affected the basal level of HO-1, but

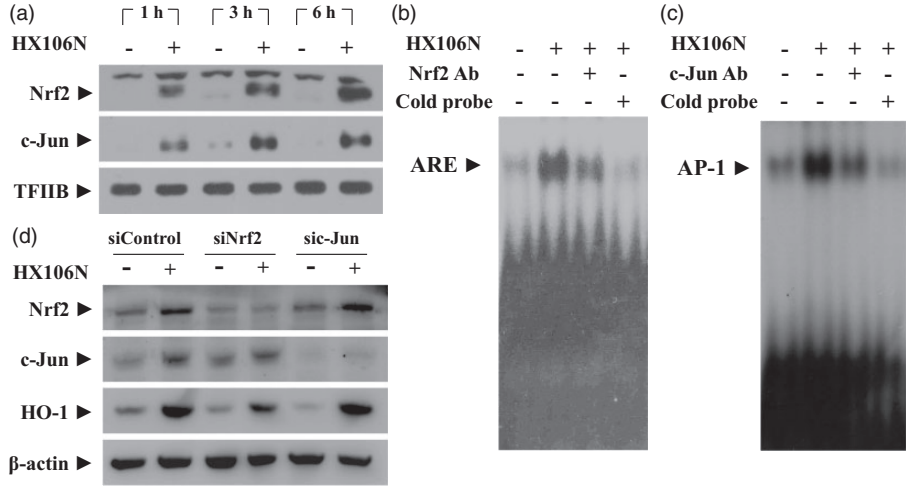

Figure 3 Effects of HX106N on Nrf2 and AP-1 transcription factors. (a) BV-2 cells were treated with 1 mg/mL HX106N for the indicated time periods, and nuclear protein was extracted to determine the level of Nrf2, c-Jun, and TFIIB by Western blot analysis. TFIIB was used as a loading control for the nuclear extracts. (b, c) Cells were treated with 1 mg/mL HX106N for 3 h, and nuclear extracts were prepared. EMSA was performed using [^{32}P]-labeled nucleotide sequences for ARE or AP-1. Competition analysis was performed to demonstrate the specificity of the DNA–protein complex using unlabeled cold oligonucleotides. For supershift/interference assays, the nuclear extracts were premixed with 4 μg of Nrf2 or c-Jun antibody for 30 min, and the EMSA assays were performed. (d) Cells were transfected with siRNA specific for Nrf2 or c-Jun and then treated with 1 mg/mL HX106N for 6 h. Whole cell lysates were prepared followed by Western blot analysis to determine the protein level of Nrf2, c-Jun, and HO-1. β-actin was used as a loading control

not HX106N-induced HO-1 expression (Figure 3(d)). Taken together, these data indicated that Nrf2 played a role in the HX106N-mediated induction of HO-1, although both Nrf2 and AP-1 transcription factors were activated by HX106N.

Effect of HX106N on MAPKs activation

MAPKs have been implicated as one of the major regulators of HO-1 expression.[29–31] To test whether MAPK signaling has any effect on HX106N-mediated HO-1 expression, pharmacological MAPK inhibitors (i.e. JNK: SP600125; p38 MAPK: SB203580; ERK: PD98059) were applied to the cells. When BV-2 cells were treated with different concentrations of SP600125 or SB203580 in the presence of HX106N, the induction of HO-1 protein by HX106N was significantly reduced by 63 and 93%, respectively, in a dose-dependent manner at a 20 μM concentration (Figure 4(a)). The HX106N-mediated increase of *ho-1* RNA was also attenuated by both inhibitors (data not shown). Treatment with PD98059 had little effect on the level of HO-1 expression, suggesting that JNK and p38 MAPK but not ERK were involved in HX106N-induced HO-1 expression (Figure 4(b)).

To test the effects of HX106N on the activation of the JNK and p38 pathways, BV-2 cells were treated with 1 mg/mL HX106N, and total proteins were prepared at various time points followed by Western blot analysis. A marked increase in the phosphorylated JNK and p38 levels was observed after 15 min of HX106N treatment, which subsequently decreased over time (Figure 4(c)). No significant change in the level of total JNK and p38 was observed (Figure 4(c)). These data indicated that HX106N activated JNK and p38 signaling pathways.

Because MAPKs have been implicated in upstream signaling pathways leading to Nrf2 activation, the JNK and p38 signaling pathways were investigated for involvement in HX106N-mediated Nrf2 activation. BV-2 cells were treated with 20 μM SP600125 or SB203580 in the presence of 1 mg/mL HX106N for 6 h, and the nuclear extracts were isolated and analyzed by Western blot analysis for Nrf2. As shown in Figure 4(d), HX106N-mediated nuclear accumulation of Nrf2 was not affected by treatment with SP600125 or SB203580.

To confirm the above results, the effect of specific inhibitors of JNK and p38 MAPK (SP600125 and SB203580, respectively) on Nrf2-mediated transcriptional activation was examined using a reporter plasmid containing StRE, pE1-Luc. BV-2 cells transfected with pE1-Luc were treated with respective inhibitors and HX106N for 9 h, and the luciferase activity was measured. Neither SP600125 nor SB203580 affected HX106N-mediated increase of luciferase activity (Figure 4(e)). These data suggested that JNK and p38 MAPK did not act as an upstream kinase in HX106N-mediated activation of Nrf2.

Effect of HX106N on NF-κB activation

It has been reported that NF-κB is important for HO-1 expression in response to diverse stimuli.[15,19] To test whether the inhibition of NF-κB activation has any effect on HX106N-induced HO-1 expression, BV-2 cells were treated with different concentrations of a pharmacological inhibitor for IκB kinase (IKK), BAY 11-7082, in the presence of 1 mg/mL HX106N for 24 h. Whole cell lysates were prepared and analyzed to measure the protein level of HO-1 by Western blot analysis. The expression of HO-1 protein was highly increased in the cells treated with HX106N, whereas treatment with BAY 11-7082 decreased the expression by 43 and 70% at 2.5 and 5 μM, respectively (Figure 5(a)). The increased RNA level of HO-1 was also reduced by 2.5 μM of BAY 11-7082 to 62% (data not shown). To confirm the above data, siRNA specific for IKKα (siIKKα) was

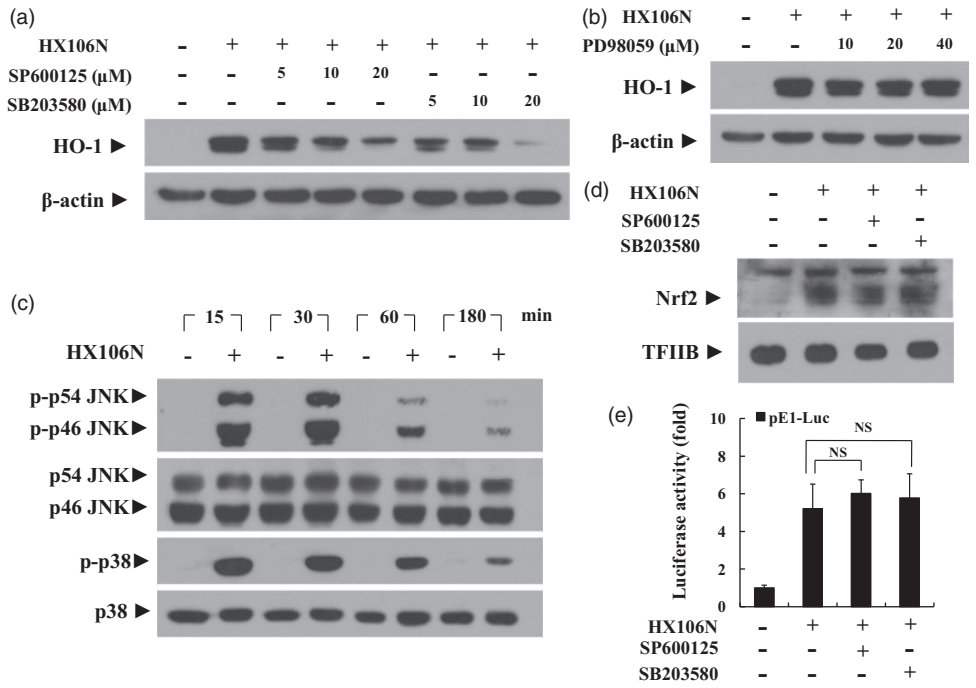

Figure 4 Effects of HX106N on the MAPK signaling pathway. (a) BV-2 cells were treated with 1 mg/mL HX106N and various concentrations of SP600125 or SB203580, specific inhibitors for JNK or p38 MAPK, respectively. Twenty-four hours later, the total protein was extracted and subjected to Western blot analysis for the detection of HO-1 and β-actin. β-actin was used as a loading control. (b) The same experiment was performed using an ERK inhibitor, PD98059. (c) Cells were treated with 1 mg/mL HX106N, and whole cell lysates were prepared at the indicated time points. The level of total and phosphorylated forms of JNK and p38 was determined by Western blot analysis. (d) Cells were treated with 20 μM SP600125 or SB203580 in the presence of 1 mg/mL HX106N. After 6 h, nuclear proteins were extracted to measure the level of Nrf2 by Western blot analysis. TFIIB was used as a loading control for nuclear extracts. (e) Cells transfected with pE1-Luc were treated with 1 mg/mL HX106N and 20 μM of SP600125 or SB203580. After 9 h, cell lysates were prepared and subjected to a luciferase assay. The values are presented as the mean ± SD of triplicate samples from one of three independent experiments. NS = non-significant compared to the HX106N-treated group

transfected to BV-2 cells. The cells were treated with 1 mg/mL HX106N for 6 h, and total proteins were extracted to measure the levels of IKKα and HO-1 using Western blot analysis. As shown in Figure 5(b), the protein level of IKKα was greatly reduced by siIKKα transfection. In these siIKKα-transfected cells, HX106N-mediated HO-1 induction was decreased by 54%, as compared to siControl-transfected cells (Figure 5(b)). These results demonstrated that the activation of NF-κB might contribute to HX106N-mediated HO-1 expression.

To examine the effect of HX106N on the NF-κB signaling pathway, BV-2 cells were treated with 1 mg/mL HX106N, and total proteins were prepared at various time points for the detection of total and phosphorylated forms of IKK and IκB. As shown in Figure 5(c), IKK phosphorylation was detected 15 min after HX106N treatment and returned to the basal level within 3 h. Consistent with the above results, a significant decrease in the level of IκB was observed between 15 and 30 min of treatment with HX106N (Figure 5(c)).

The effect of HX106N on NF-κB activation was assessed by measuring the nuclear accumulation of the NF-κB p65 subunit. Nuclear extracts were prepared from BV-2 cells treated with 1 mg/mL HX106N for the indicated time periods in Figure 5(d) and subjected to Western blot analysis using an antibody specific for p65. The nuclear level of p65 was very low in the untreated cells but was strongly increased at all time points (1–6 h) (Figure 5(d)).

To confirm these observations with a gel retardation assay using an NF-κB oligonucleotide probe, BV-2 cells were treated with 1 mg/mL HX106N for 3 h, and the nuclear protein was extracted, followed by EMSA. The level of DNA–protein complex was very low in untreated cells but was dramatically increased by treatment with HX106N (Figure 5(e)). In the supershift/interference assay, addition of an antibody against p65 diminished the upper complex, indicating that the band was indeed the p65 subunit bound to the NF-κB oligonucleotide (Figure 5(e)). These results suggested that HX106N induced the activation of NF-κB.

To test whether JNK and p38 MAPK were involved in HX106N-mediated NF-κB activation, BV-2 cells were treated with 20 μM SP600125 or SB203580 in the presence of 1 mg/mL HX106N. Six hours later, nuclear proteins were prepared, and Western blot analysis was performed to determine the level of p65. Treatment with both inhibitors had little effects on the HX106N-induced nuclear accumulation of p65 (Figure 5(f)).

It was also investigated whether SP600125 or SB203580 inhibitors have any effect on NF-κB-mediated transcriptional activity. BV-2 cells were transfected with a luciferase plasmid containing five copies of an NF-κB response element (pNF-κB-Luc) and treated with each inhibitor in the presence of 1 mg/mL HX106N for 9 h. The level of luciferase activity was elevated by 3.4-fold after HX106N treatment, while little changes were observed in the cells

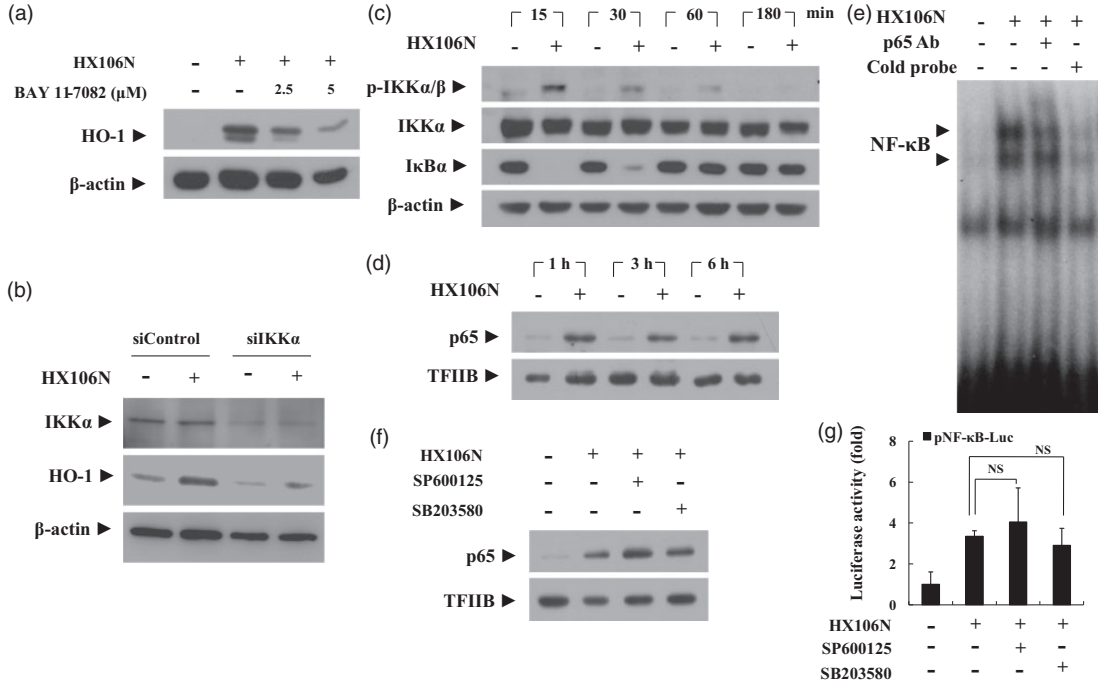

Figure 5 Effects of HX106N on the NF-κB signaling pathway. (a) BV-2 cells were treated with 1 mg/mL HX106N and 2.5 or 5 μM BAY11-7802, an IKK inhibitor, for 24 h. Whole cell lysates were harvested and subjected to Western blot analysis for the detection of HO-1 and β-actin. β-actin was used as a loading control. (b) The siRNAs specific for IKKα and IKKβ were transfected to BV-2 cells, and the cells were treated with 1 mg/mL HX106N. After 6 h, whole proteins were prepared and the protein level of IKKα, HO-1, and β-actin was measured by Western blot hybridization. (c) The total protein was prepared from cells treated with 1 mg/mL HX106N at the indicated time points. The level of phospho-IKKα/β, IKKα, IκBα, and β-actin was measured by Western blot analysis. (d) Cells were treated with 1 mg/mL HX106N for the indicated time periods, and nuclear proteins were extracted to determine the level of p65 and TFIIB by Western blot analysis. TFIIB was used as a loading control for the nuclear extracts. (e) Cells were treated with 1 mg/mL HX106N for 3 h. Nuclear extracts were prepared, and EMSA was conducted using a [^{32}P]-labeled NF-κB oligonucleotide as a probe. To perform the supershift/interference assays, an antibody specific for p65 was preincubated with the nuclear extracts for 30 min before adding the probe. (f) Cells were treated with 20 μM SP600125 or SB203580 in the presence of 1 mg/mL HX106N for 6 h. Nuclear proteins were analyzed by Western blot analysis using specific antibodies for p65 and TFIIB. (g) Cells were transfected with a luciferase reporter plasmid containing NF-κB response element (pNF-κB-Luc). Twenty-four hours later, the cells were treated with 1 mg/mL HX106N and 20 μM of SP600125 or SB203580 for 9 h, followed by a luciferase assay. The values are presented as the mean ± SD of triplicate samples from one of three independent experiments. NS = non-significant compared to the HX106N-treated group

treated with SP600125 or SB203580 (Figure 5(g)). These data suggested that both of JNK and p38 were not associated with NF-κB activation.

Effect of HX106N on LPS-induced NO production

Excessive NO production by iNOS in activated microglia is reported to be a key mediator of oxidative damage in various neurodegenerative diseases.[32] HX106N has previously been shown to inhibit NO production through regulation of iNOS expression. As HO-1 has been reported to regulate the inflammatory response, including NO production,[33,34] it was investigated if HO-1 affected HX106N-mediated NO suppression. BV-2 cells were incubated with 100 ng/mL LPS for 24 h in the presence or absence of 1 mg/mL HX106N, and the culture supernatants were analyzed with the Griess assay to measure the amount of nitrite, a stable end product of NO. The nitrite level increased up to 28.2 μM in the supernatant from LPS-stimulated cells but was significantly decreased to 7.0 μM after treatment with HX106N (Figure 6(a)). When 100 nM ZnPP, a pharmacological inhibitor of HO-1, was added to the cells, the nitrite level increased to 15.3 μM (Figure 6(a)). Treatment with ZnPP did not affect HX106N-induced RNA expression of HO-1 (Figure 6(b)).

To test whether ZnPP has any effect on HX106N-suppressed iNOS expression, whole cell lysates were extracted from the same experiments and subjected to Western blot analysis for the detection of iNOS. As shown in Figure 6(c), treatment with HX106N significantly decreased LPS-induced iNOS expression by 83%, and this inhibition was not affected by ZnPP. These data suggested that HO-1 might not contribute to HX106N-mediated iNOS suppression.

We confirmed the above observation by knockdown of HO-1 using a specific siRNA. BV-2 cells were transfected with siRNA specific for HO-1 (siHO-1) or a non-specific siRNA (siControl) and treated with 1 mg/mL HX106N for 24 h. The Western blot analysis showed that siHO-1 significantly decreased HX106N-induced HO-1 expression, by 58%, whereas siControl had no effect (Figure 6(d)).

SiRNA-transfected cells were stimulated with 100 ng/mL LPS in the presence or absence of 1 mg/mL HX106N for 24 h, and the level of nitrite and iNOS expression was measured in the culture supernatant and cellular proteins, respectively. As shown in Figure 6(e), LPS-induced nitrite production in siHO-1-transfected cells increased by 1.3-fold compared to siControl-transfected cells, although the increase was not statistically significant.

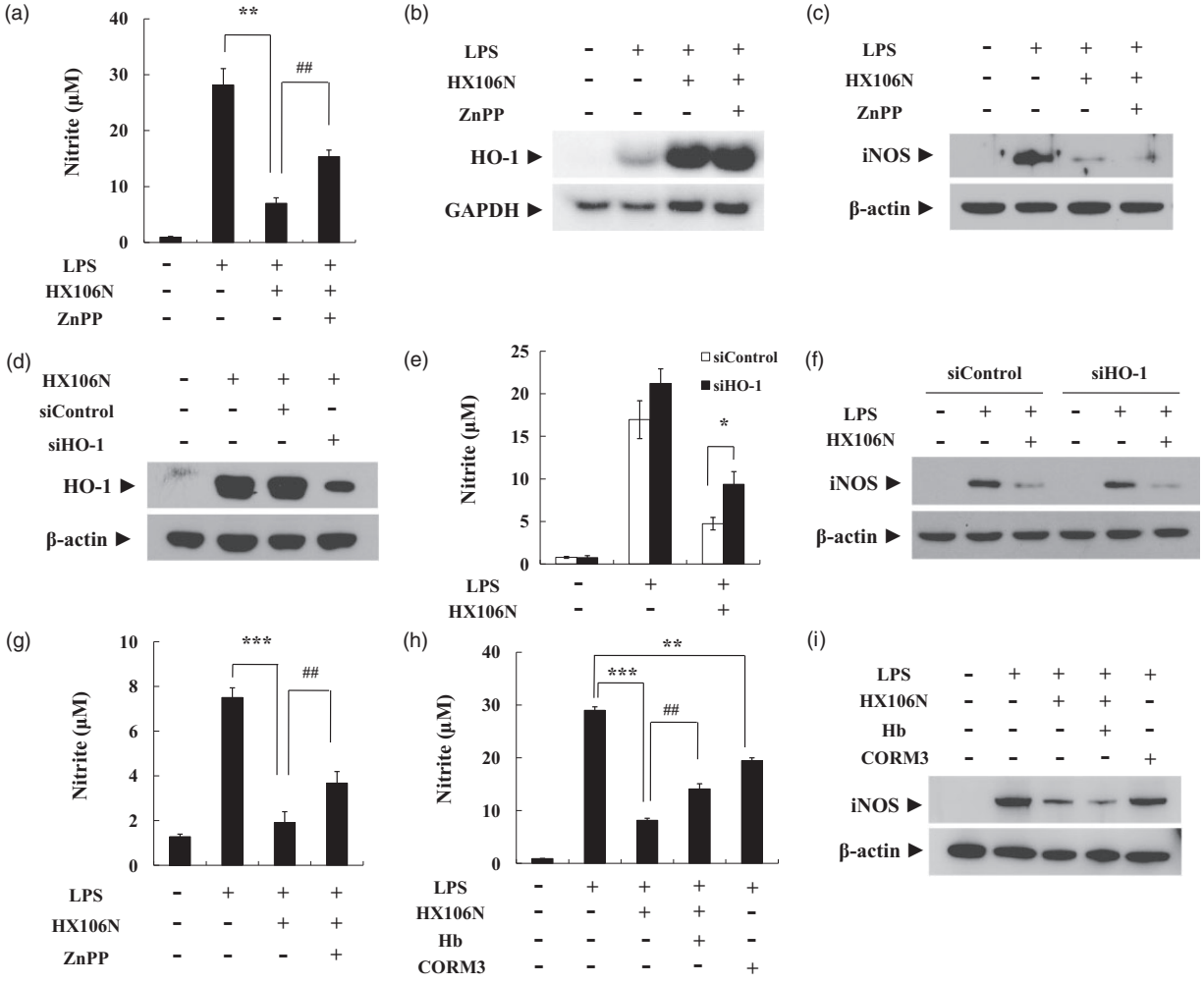

Figure 6 Effects of HX106N on NO production in LPS-stimulated BV-2 cells and primary microglia. (a, c) BV-2 cells were stimulated with 100 ng/mL LPS in the presence or absence of 1 mg/mL HX106N and 100 nM ZnPP, an HO-1 inhibitor. After 24 h, the culture supernatant was subjected to the Griess assay to examine the level of nitrite, and whole cell lysates were harvested for Western blot analysis to detect iNOS and β-actin. β-actin was used as a loading control. The values for nitrite are presented as the mean ± SD of triplicate samples of a representative experiment. $^{**}P < 0.01$ compared to the LPS-treated group;$^{\#\#}P < 0.01$ compared to the LPS and HX106N-treated group. (b) Total RNAs were isolated from the cells incubated with the above reagents for 12 h and subjected to Northern blot analysis to detect *ho-1* RNA. GAPDH was used as a loading control. (d) Cells transfected with siHO-1 or siControl were treated with 1 mg/mL HX106N for 24 h. The total protein was prepared for Western blot analysis using antibodies against HO-1 and β-actin. (e, f) Cells transfected with siHO-1 or siControl were stimulated with 100 ng/mL LPS in the presence or absence of 1 mg/mL HX106N. After 24 h, the culture supernatant and total proteins were harvested for the Griess assay and Western blot analysis. The values are presented as the mean ± SD of triplicate samples of a representative experiment. $^{**}P < 0.01$ compared to the siControl-transfected group. (g) Primary microglial cells were stimulated with 100 ng/mL LPS in the presence or absence of 1 mg/mL HX106N and 100 nM ZnPP. After 48 h, the culture supernatant was prepared and analyzed by Griess assay. The values are presented as the mean ± SD of triplicate samples of a representative experiment. $^{***}P < 0.001$ compared to the LPS-treated group;$^{\#\#}P < 0.01$ compared to the LPS and HX106N-treated group. (h, i) Cells were incubated with 100 ng/mL LPS in the presence or absence of 1 mg/mL HX106N, 1 μM Hb, and 100 μM CORM-3. Twenty-four hours later, the levels of nitrite and iNOS were determined by the Griess assay from the supernatant and Western blot analysis using the total proteins, respectively. The values for nitrite are presented as the mean ± SD of triplicate samples of a representative experiment. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to the LPS-treated group;$^{\#\#}P < 0.01$ compared to the LPS and HX106N-treated group

When cells were treated with HX106N, the level of nitrite in siHO-1-transfected cells was twofold higher than that in siControl-transfected cells (Figure 6(e)). These results confirmed the involvement of HO-1 in HX106N-mediated NO suppression. HX106N-mediated suppression of iNOS expression was not affected by siHO-1 transfection, consistent with the results from ZnPP treatment (Figure 6(f)).

Primary cultured microglia were also used to test the effects of HX106N on NO production. The cells were stimulated with 100 ng/mL LPS in the presence or absence of 1 mg/mL HX106N and 100 nM ZnPP for 48 h and the culture supernatant was harvested to detect the nitrite level. Consistent with the results from the BV-2 cells, treatment with HX106N effectively suppressed the increase in the level of nitrite in LPS-stimulated primary microglia, and this suppression was antagonized by ZnPP treatment (Figure 6(g)). Taken together, HO-1 appears to be involved in the HX106N-mediated inhibition of NO production but not in the control of iNOS expression.

CO, an enzymatic by-product of HO-1 activity, has been known to suppress NO production without affecting the iNOS protein level.[35,36] Therefore, it was tested whether CO was involved in the HX106N-mediated downregulation of NO. BV-2 cells were incubated with 100 ng/mL LPS in

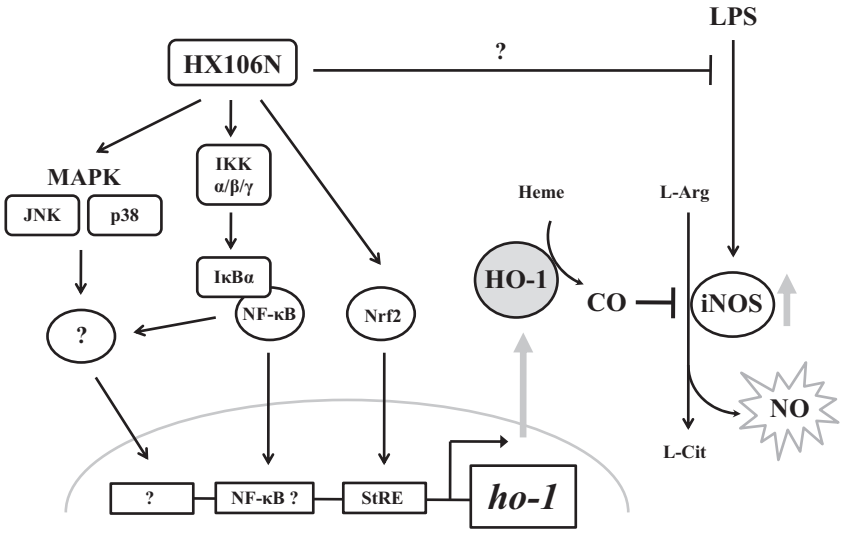

Figure 7 A proposed model for transcriptional regulation of the *ho-1* gene by HX106N and the HO-1 induction-mediated NO suppression.

the presence or absence of 1 mg/mL HX106N, 1 μM Hb, a CO scavenger, and 100 μM CORM-3, a CO donor, for 24 h. The supernatant and cell lysates were harvested and used to measure the level of nitrite and iNOS protein, respectively. As expected, Hb attenuated the inhibitory effect of HX106N on LPS-induced NO production, while CORM-3 significantly suppressed the production of NO (Figure 6(h)). As shown in Figure 6(i), the decreased level of iNOS protein by HX106N was not affected by Hb, while CORM-3 also had little effect on LPS-induced iNOS expression. These data indicated that CO is a critical factor for HX106N-mediated NO suppression, but with no effect on iNOS expression.

Because arginase, an enzyme that converts L-arginine into L -ornithine and urea, is known to reciprocally regulate NOS activity,[37] it was also investigated whether HX106N has any effect on the level of arginase expression. The expression of both arginase-1 and ariginase-2 was not affected by treatment with HX106N (data not shown).

Discussion

In this report, we demonstrated that HX106N increased the expression of HO-1 and suppressed the production of NO. Various cellular factors appeared to be involved in HX106N-mediated induction of the *ho-1* gene, including Nrf2, NF-κB, JNK, and p38 MAPK (Figure 7).

Nrf2 plays a crucial role in the increased expression of HO-1 by HX106N as evidenced by reduction of HO-1 induction when Nrf2-specific siRNA was used. Nrf2 is normally present in the cytoplasm in a combined form with Keap1 (Kelch-like ECH-associated protein 1) but when Nrf2 and Keap1 dissociate by various stimulations, Nrf2 translocates to the nucleus to activate the expression of a wide range of antioxidant genes.[38,39] We investigated which signal transduction pathway is used for activation of Nrf2, but had no success thus far. Virtually all kinases previously known to phosphorylate and activate Nrf2 did not seem to be involved in HX106N-mediated induction of HO-1 in the experiments involving the pharmacological inhibitors of MAPKs, PI3K, protein kinase C (PKC), and casein kinase 2 (CK2) (data not shown). Some chemopreventive agents directly oxidize or chemically modify the cysteine thiols of Keap1, leading to the dissociation of Keap1 and subsequent release of Nrf2.[40,41] Therefore, it is possible that HX106N may contain compound(s) that directly modulate the Nrf2-Keap1 interaction without involving an upstream signaling pathway.

The expression of HO-1 is also reported to be regulated by NF-κB.[20–22] The activation of this transcription factor was found to be important in HX106N-mediated upregulation of HO-1, as shown in the experiments involving a pharmacological inhibitor or siRNA specific for IKK. Since the functional NF-κB binding site has not been clearly identified in the *ho-1* gene,[19] further molecular studies to find the actual binding site for NF-κB that control HX106N-induced *ho-1* gene expression would be of interest.

A variety of kinases have been identified to phosphorylate and activate transcription factors involved in regulation of HO-1 expression. We demonstrated that JNK and p38 MAPK played a role(s) in HX106N-mediated HO-1 induction using a pharmacological inhibitor (SP600125 and SB203580) but could not find the actual transcription factor acting as their downstream mediator. It is likely that other transcription factors participate in HO-1 induction by HX106N, since the *ho-1* promoter contains a variety of *cis*-acting elements, including c-AMP-responsive element (CRE), STAT, and a heat shock element.[15] Both STAT3 and HSF-1 are regulated by JNK,[42,43] while the activation of CRE-binding protein by p38 MAPK was demonstrated to be involved in HO-1 expression induced by raloxifene, an estrogen receptor modulator.[44] In the case of p38 MAPK, it is also possible that p38 MAPK contributes to HX106N-induced HO-1 expression without direct effect on transcription factors, because this protein is known to regulate the phosphorylation of TATA-binding protein.[45]

HX106N effectively suppressed the production of NO, and HO-1 played an important role in this process through CO generation. However, HO-1 and CO were not involved in HX106N-mediated suppression of iNOS expression. CO is known to inhibit iNOS activity, but not its expression, by interacting with the heme iron moiety of the enzyme.[35,36] Therefore, it is possible that CO released from HO-1-catalyzed reactions may inhibit iNOS activity, resulting in the regulation of NO production by HX106N.

Our data indicate that HX106N exhibits antioxidative activity through the induction of the *ho-1* gene in microglial cells. Although the active compound(s) in HX106N remain to be identified, we were able to make HX106N containing consistent activities using cell-based bioassays.[23] HX106N prepared as such could reproducibly improve the cognitive functions in the amnesia animal models.[23,46] Taken together, further in-depth investigations are warranted to test HX106N as a therapeutic agent for various neurodegenerative diseases.

AUTHORS' CONTRIBUTIONS

DSL, JK, J-GJ, and SK participated in the design and interpretation of the studies and the analysis of the data. DSL, BNK, SL, and JL performed the experiments. DSL, JK, J-GJ, and SK wrote and reviewed the manuscript.

ACKNOWLEDGEMENTS

This work was supported by grants from Korea's Forest Service (Forest Science & Technology Projects; S111112L060140), the Ministry of Knowledge Economy (Regional Innovation System support program, Korea Institute for Advancement of Technology; B0009639), and the Ministry of Education, Science and Technology (Basic Science Research Program, National Research Foundation of Korea; 2012R1A1A2008018). ViroMed Co. Ltd was a participating company for the first two grants.

CONFLICT OF INTEREST

DSL and JGJ are the employee of ViroMed, Co., Ltd.
SK holds stocks of ViroMed, Co. Ltd. (11%)
One patent pending (PCT/KR2012/002330)

REFERENCES

1. Schipper HM, Stopa EG. Expression of heme oxygenase-1 in the senescent and Alzheimer-diseased brain. *Ann Neurol* 1995;**37**:758–68
2. Castellani R, Smith MA, Richey PL, Perry G. Glycoxidation and oxidative stress in Parkinson disease and diffuse Lewy body disease. *Brain Res* 1996;**737**:195–200
3. Calingasan NY, Chen J, Kiaei M, Beal MF. Beta-amyloid 42 accumulation in the lumbar spinal cord motor neurons of amyotrophic lateral sclerosis patients. *Neurobiol Dis* 2005;**19**:340–7
4. Beschorner R, Adjodah D, Schwab JM, Mittelbronn M, Pedal I, Mattern R, Schluesener HJ, Meyermann R. Long-term expression of heme oxygenase-1 (HO-1, HSP-32) following focal cerebral infarctions and traumatic brain injury in humans. *Acta Neuropathol* 2000;**100**:377–84
5. Tenhunen R, Marver HS, Schmid R. Enzymatic conversion of heme to bilirubin by microsomal heme oxygenase. *Proc Natl Acad Sci USA* 1968;**61**:748–55
6. Stocker R, Yamamoto Y, Mcdonagh AF, Glazer AN, Ames BN. Bilirubin is an antioxidant of possible physiological importance. *Science* 1987;**235**:1043–6
7. Dore S, Takahashi M, Ferris CD, Zakhary R, Hester LD, Guastella D, Snyder SH. Bilirubin, formed by activation of heme oxygenase-2, protects neurons against oxidative stress injury. *Proc Natl Acad Sci USA* 1999;**96**:2445–50
8. Otterbein LE, Bach FH, Alam J, Soares M, Lu HT, Wysk M, Davis RJ, Flavell RA, Choi AMK. Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. *Nat Med* 2000;**6**:422–8
9. Balla G, Jacob HS, Balla J, Rosenberg M, Nath K, Apple F, Eaton JW, Vercellotti GM. Ferritin: a cytoprotective antioxidant strategem of endothelium. *J Biol Chem* 1992;**267**:18148–53
10. Choi J, Lee J, Kim SH, Kim J, Kim S. PG201 downregulates the production of nitrite by upregulating heme oxygenase-1 expression through the control of phosphatidylinositol 3-kinase and NF-E2-related factor 2. *Nitric Oxide* 2013;**33**:42–55
11. Panahian N, Yoshiura M, Maines MD. Overexpression of heme oxygenase-1 is neuroprotective in a model of permanent middle cerebral artery occlusion in transgenic mice. *J Neurochem* 1999;**72**:1187–203
12. Chen K, Gunter K, Maines MD. Neurons overexpressing heme oxygenase-1 resist oxidative stress-mediated cell death. *J Neurochem* 2000;**75**:304–13
13. Le W, Xie W, Appel SH. Protective role of heme oxygenase-1 in oxidative stress-induced neuronal injury. *J Neurosci Res* 1999;**56**:652–8
14. Cuadrado A, Rojo AI. Heme oxygenase-1 as a therapeutic target in neurodegenerative diseases and brain infections. *Curr Pharm Design* 2008;**14**:429–42
15. Ryter SW, Alam J, Choi AM. Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications. *Physiol Rev* 2006;**86**:583–650
16. Alam J, Stewart D, Touchard C, Boinapally S, Choi AM, Cook JL. Nrf2, a Cap'n'Collar transcription factor, regulates induction of the heme oxygenase-1 gene. *J Biol Chem* 1999;**274**:26071–8
17. Alam J, Cook JL. Transcriptional regulation of the heme oxygenase-1 gene via the stress response element pathway. *Curr Pharm Design* 2003;**9**:2499–511
18. Alam J, Den Z. Distal AP-1 binding sites mediate basal level enhancement and TPA induction of the mouse heme oxygenase-1 gene. *J Biol Chem* 1992;**267**:21894–900
19. Alam J, Cook JL. How many transcription factors does it take to turn on the heme oxygenase-1 gene? *Am J Resp Cell Mol* 2007;**36**:166–74
20. Lu DY, Tsao YY, Leung YM, Su KP. Docosahexaenoic acid suppresses neuroinflammatory responses and induces heme oxygenase-1 expression in BV-2 microglia: implications of antidepressant effects for omega-3 fatty acids. *Neuropsychopharmacol* 2010;**35**:2238–48
21. Hill-Kapturczak N, Thamilselvan V, Liu FY, Nick HS, Agarwal A. Mechanism of heme oxygenase-1 gene induction by curcumin in human renal proximal tubule cells. *Am J Physiol-Renal* 2001;**281**:F851–F9
22. Juan SH, Cheng TH, Lin HC, Chu YL, Lee WS. Mechanism of concentration-dependent induction of heme oxygenase-1 by resveratrol in human aortic smooth muscle cells. *Biochem Pharmacol* 2005;**69**:41–8
23. Lee DS, Choi J, Kim SH, Kim S. Ameliorating effects of HX106N, a water-soluble botanical formulation, on Abeta25-35-induced memory impairment and oxidative stress in mice. *Biol Pharm Bull* 2014;**37**:954–60
24. Saura J, Tusell JM, Serratosa J. High-yield isolation of murine microglia by mild trypsinization. *Glia* 2003;**44**:183–9
25. Alam J, Cai J, Smith A. Isolation and characterization of the mouse heme oxygenase-1 gene. Distal 5′ sequences are required for induction by heme or heavy metals. *J Biol Chem* 1994;**269**:1001–9
26. Alam J, Camhi S, Choi AM. Identification of a second region upstream of the mouse heme oxygenase-1 gene that functions as a basal level and inducer-dependent transcription enhancer. *J Biol Chem* 1995;**270**:11977–84
27. Balogun E, Hoque M, Gong P, Killeen E, Green CJ, Foresti R, Alam J, Motterlini R. Curcumin activates the haem oxygenase-1 gene via regulation of Nrf2 and the antioxidant-responsive element. *Biochem J* 2003;**371**:887–95
28. Camhi SL, Alam J, Otterbein L, Sylvester SL, Choi AM. Induction of heme oxygenase-1 gene expression by lipopolysaccharide is mediated by AP-1 activation. *Am J Respir Cell Mol Biol* 1995;**13**:387–98

29. Drechsler Y, Dolganiuc A, Norkina O, Romics L, Li W, Kodys K, Bach FH, Mandrekar P, Szabo G. Heme oxygenase-1 mediates the anti-inflammatory effects of acute alcohol on IL-10 induction involving p38 MAPK activation in monocytes. *J Immunol* 2006;**177**:2592–600
30. Immenschuh S, Ramadori G. Gene regulation of heme oxygenase-1 as a therapeutic target. *Biochem Pharmacol* 2000;**60**:1121–8
31. Ryter SW, Xi S, Hartsfield CL, Choi AM. Mitogen activated protein kinase (MAPK) pathway regulates heme oxygenase-1 gene expression by hypoxia in vascular cells. *Antioxid Redox Signal* 2002;**4**:587–92
32. Brown GC, Bal-Price A. Inflammatory neurodegeneration mediated by nitric oxide, glutamate, and mitochondria. *Mol Neurobiol* 2003;**27**:325–55
33. Wang WW, Smith DL, Zucker SD. Bilirubin inhibits iNOS expression and NO production in response to endotoxin in rats. *Hepatology* 2004;**40**:424–33
34. Srisook K, Cha YN. Super-induction of HO-1 in macrophages stimulated with lipopolysaccharide by prior depletion of glutathione decreases iNOS expression and NO production. *Nitric Oxide* 2005;**12**:70–9
35. Sawle P, Foresti R, Mann BE, Johnson TR, Green CJ, Motterlini R. Carbon monoxide-releasing molecules (CO-RMs) attenuate the inflammatory response elicited by lipopolysaccharide in RAW264.7 murine macrophages. *Brit J Pharmacol* 2005;**145**:800–10
36. White KA, Marletta MA. Nitric-oxide synthase is a cytochrome-P-450 type hemoprotein. *Biochemistry-Us* 1992;**31**:6627–31
37. Steppan J, Ryoo S, Schuleri KH, Gregg C, Hasan RK, White AR, Bugaj LJ, Khan M, Santhanam L, Nyhan D, Shoukas AA, Hare JM, Berkowitz DE. Arginase modulates myocardial contractility by a nitric oxide synthase 1-dependent mechanism. *Proc Natl Acad Sci USA* 2006;**103**:4759–64
38. Trachootham D, Lu W, Ogasawara MA, Nilsa RD, Huang P. Redox regulation of cell survival. *Antioxid Redox Signal* 2008;**10**:1343–74
39. Surh YJ, Kundu JK, Na HK. Nrf2 as a master redox switch in turning on the cellular signaling involved in the induction of cytoprotective genes by some chemopreventive phytochemicals. *Planta Med* 2008;**74**:1526–39
40. Na HK, Surh YJ. Transcriptional regulation via cysteine thiol modification: a novel molecular strategy for chemoprevention and cytoprotection. *Mol Carcinogen* 2006;**45**:368–80
41. Dinkova-Kostova AT, Talalay P. Direct and indirect antioxidant properties of inducers of cytoprotective proteins. *Mol Nutr Food Res* 2008;**52**:S128–38
42. Park J, Liu AY. JNK phosphorylates the HSF1 transcriptional activation domain: role of JNK in the regulation of the heat shock response. *J Cell Biochem* 2001;**82**:326–38
43. Lee PJ, Camhi SL, Chin BY, Alam J, Choi AM. AP-1 and STAT mediate hyperoxia-induced gene transcription of heme oxygenase-1. *Am J Physiol Lung Cell Mol Physiol* 2000;**279**:L175–82
44. Lee SA, Kim EY, Jeon WK, Woo CH, Choe J, Han S, Kim BC. The inhibitory effect of raloxifene on lipopolysaccharide-induced nitric oxide production in RAW264.7 cells is mediated through a ROS/p38 MAPK/CREB pathway to the up-regulation of heme oxygenase-1 independent of estrogen receptor. *Biochimie* 2011;**93**:168–74
45. Carter AB, Knudtson KL, Monick MM, Hunninghake GW. The p38 mitogen-activated protein kinase is required for NF-kappaB-dependent gene expression. The role of TATA-binding protein (TBP). *J Biol Chem* 1999;**274**:30858–63
46. Lee DS, Jeoung JG, Kim S. Effects of HX106N, a water-soluble botanical formulation on scopolamine-induced memory impairment in mice. *Korean J Food Nutr* 2014;**27**:673–77

(Received April 29, 2014, Accepted November 25, 2014)