

Reactive Oxygen Species-Dependent c-Fos/Activator Protein 1 Induction Upregulates Heme Oxygenase-1 Expression by Bradykinin in Brain Astrocytes

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Abstract

Heme oxygenase-1 (HO-1) plays a crucial role in tissue pathological changes such as brain injuries. Our previous studies have demonstrated that bradykinin (BK) induces the expression of several inflammatory proteins, including matrix metalloproteinase-9 and COX-2, *via* mitogen-activated protein kinases and nuclear factor- κ B (NF- κ B) in rat brain astrocytes (RBA-1). However, the molecular mechanisms underlying BK-induced HO-1 expression in RBA-1 cells remain poorly defined. Here we demonstrated that BK induced HO-1 expression and enzymatic activity *via* a B₂ BK receptor-activated reactive oxygen species (ROS)-dependent signaling pathway. NADPH oxidase (Nox)-dependent ROS generation led to activation of extracellular signal-regulated kinase 1/2 (ERK1/2) and c-Jun-N-terminal kinase (JNK) and then activated the downstream molecules NF- κ B and c-Jun, respectively. The c-Fos, an activator protein 1 (AP-1) subunit, was upregulated by activation of NF- κ B and c-Jun, which bound to HO-1 promoter and thereby turned on transcription of *HO-1* gene. The rat *HO-1* promoter containing a putative AP-1 cis-binding site was identified as a crucial domain linking to BK action. Taken together, these results suggested that in RBA-1 cells, activation of ERK/NF- κ B and JNK/c-Jun cascades by a Nox/ROS-dependent event enhancing c-Fos/AP-1 activity is essential for HO-1 upregulation and activation induced by BK. Moreover, ROS-dependent NF-E2-related factor 2 activation also contributes to HO-1 induction by BK in astrocytes. *Antioxid. Redox Signal.* 13, 1829–1844.

Introduction

HEME OXYGENASE (HO) is the rate-limiting enzyme that metabolizes heme molecules to biliverdin, ferrous iron, and carbon monoxide and is involved in both physiological processes and pathological changes in the central nervous system (CNS) (34, 47, 48). Three gene products of HO, including HO-1, HO-2, and HO-3, have been characterized (35, 41). In normal mature rat brain, the HO-2 isozyme is responsible for most of HO constitutive activity and widely distributed throughout the mammalian neuraxis; the HO-1, a stress protein, expression is low and restricted to small groups of scattered neurons and neuroglia (15, 41). Several studies have shown that brain HO-1 expression is markedly induced by diverse factors including glutathione depletion, subarachnoid hemorrhage, trauma, and ischemia (15, 18). Moreover, the HO-1 gene is induced by noxious stimuli (*e.g.*, β -amyloid, hydrogen peroxide [H₂O₂], and cytokines) in brain diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), and other

neurological conditions (12). In AD and mild cognitive impairment, oxidative stress is measured as the percentage of astrocytes expressing HO-1 in postmortem temporal cortex and hippocampus after dual HO-1/glial fibrillary acidic protein (GFAP) immunohistochemistry (43). In PD brain, overexpression of HO-1 in nigral astroglia and accompanying iron liberation may facilitate the bioactivation of dopamine to neurotoxic free radical intermediates and predispose nearby neuronal constituents to oxidative damage (46). Recently, HO-1 expression has been reported to act as an important protective endogenous mechanism against many biological stresses in several pathologic conditions such as inflammatory processes, atherosclerosis, ischemia-reperfusion systems, or brain disorders (16, 47). These results suggest that regulating astrocytic HO-1 expression may play a critical role in the pathogenesis of brain disorders.

Reactive oxygen species (ROS) are produced by various enzymatic reactions and chemical processes or directly inhaled, including O₂⁻, OH[•], and H₂O₂. The ROS are

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essential for many physiological functions and the killing of invading microorganisms (10, 49). However, several lines of evidence have suggested the pathogenesis of human diseases related to increased oxidative stress (10, 49). It has been shown that ROS can damage DNA, lipids, proteins, and carbohydrates, leading to impairment of cellular functions and enhancement of inflammatory reactions (19, 28, 49). In the brain, the physiological role of ROS (along with O_2^- and NO) also extends to the control of vascular tone, which is tightly modulated by metabolic activity within neurons (14, 19). Under pathological conditions, increasing ROS production by several stimuli such as bradykinin (BK) can regulate expression of several inflammatory genes during brain injury (5, 6, 19). Recently, increasing evidence attributes the cellular damage in neurodegenerative disorders such as AD to oxidative stress, which leads to generation of free radicals being responsible for brain inflammatory disorders, causing deleterious effects during CNS pathogenesis (6, 19, 32). Moreover, many reports have shown that ROS are major signaling molecules that mediate microglial activation induced by several inflammatory mediators, including A β and lipopolysaccharide (LPS) (30, 39). Although the effects of BK associated with ROS have been reported in cerebral arteriolar effects and cardiac and renal diseases (5, 9), BK-induced astrocytic responses through the generation of ROS are not well characterized.

BK and related peptides are elevated during brain trauma, stroke, and neurogenic inflammation (29). Among brain cells, astrocytes possess a G protein-coupled receptor B₂ BK receptor (B₂BKR), which couples to a G_q protein to activate phospholipase C β , phosphoinositide breakdown, protein kinase C (PKC), and intracellular Ca²⁺ mobilization in several cell types including astrocytes (7, 21). These signaling pathways contribute to several cellular responses including proliferation, migration, or gene expression through activation of different mitogen-activated protein kinase (MAPK) cascades such as extracellular signal-regulated kinase 1/2 (ERK1/2) (11, 23). Several studies have demonstrated that induction of HO-1 is mediated through activation of several signal molecules including PI3K/Akt and MAPKs (8, 40, 42). Moreover, a variety of putative regulatory elements, including antioxidant response elements (AREs), NF-E2-related factor 2 (Nrf2), activator protein 1 (AP-1), and nuclear factor- κ B (NF- κ B) sites, in the 5' region of HO-1 promoters have been reported in various animal species (2, 3). Recently, BK has been shown to regulate expression of several genes through different transcription factors including AP-1 (53) and NF- κ B (24).

Therefore, BK may play a potential role in the regulation of specific gene expression, such as HO-1, and thereby prevent inflammatory responses. However, the mechanisms of intracellular signaling pathways involved in BK-induced HO-1 expression in rat brain astrocytes (RBA-1) are unclear. Here, we found that BK induces HO-1 gene expression *via* a ROS-dependent pathway in RBA-1. This upregulation is mediated *via* an AP-1 element of the rat HO-1 promoter that is a target of the c-Fos/AP-1 subunit. Two independent pathways, c-Jun-N-terminal kinase (JNK)/c-Jun and ERK/NF- κ B pathways, are involved in c-Fos/AP-1 induction, which both require the generation of ROS, and in B₂BKR-dependent induction of HO-1 gene expression and activity in RBA-1 cells.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM)/Ham's nutrient mixture F-12 (F-12) medium and fetal bovine serum were purchased from Invitrogen. Hybond C membrane and enhanced chemiluminescence (ECL) Western blotting detection system were obtained from GE Healthcare Bio-sciences. HO-1 antibody was from Stressgen (No. SPA895). Phospho-(Thr²⁰²/Tyr²⁰⁴)-ERK1/2, phospho-(Thr¹⁸⁰/Tyr¹⁸²)-p38 MAPK, phospho-(Thr¹⁸³/Tyr¹⁸⁵)-JNK1/2, and phospho-p65 antibody kits were from Cell Signaling. NADPH oxidase 1 (Nox1), Nox2, Nox4, phospho-c-Jun, and c-Fos antibodies were from Santa Cruz. All primary antibodies were diluted at 1:1000 in phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA) (Calbiochem). D-Arg-[Hyp³, Thi⁵, D-Tic⁷, Oic⁸]-BK (Hoe140), PD98059, SB202190, SP600125, Bay11-7082, curcumin, and tanshinone IIA (TSIIA) were from Biomol. The B₂BKR, Nox1, Nox2, Nox 4, and Nrf2 siRNA were from Invitrogen. Bicinchoninic acid (BCA) protein assay reagent was from Pierce. BK, N-acetyl-cysteine (NAC), enzymes, and other chemicals were from Sigma.

Cell cultures and treatment

A RBA-1 cell line was used throughout this study. This cell line originated from a primary astrocyte culture of neonatal rat cerebrum and was naturally developed through successive cell passages (27). Cells were cultured and treated as previously described (24). Primary astrocyte cultures were prepared from the cortex of 6-day-old Sprague-Dawley rat pups as previously described (23) with modifications. The purity of primary astrocyte cultures was assessed with the astrocyte-specific marker, GFAP, showing nearly 95% GFAP-positive astrocytes (Fig. 1E, upper part). Cells were plated onto 12-well culture plates and made quiescent at confluence by incubation in serum-free DMEM/F-12 for 24 h. Growth-arrested cells were incubated with or without different concentrations (1 nM to 1 μ M) of BK at 37°C for the indicated time intervals. When the inhibitors were used, cells were pretreated with the inhibitor for 1 h before exposure to BK.

Total RNA extraction and reverse transcription-polymerase chain reaction analysis

Total RNA was extracted from RBA-1 cells as previously described (24). The cDNA obtained from 0.5 μ g total RNA was used as a template for polymerase chain reaction (PCR) amplification. Oligonucleotide primers were designed based on GenBank entries for rat HO-1 and β -actin. The following primers were used for amplification reaction: for HO-1 (8), forward primer 5'-CACGCATATACCCGCTACCT-3' and reverse primer 5'-TCTGTCACCCTGTGCTTGAC-3'; for c-Fos, forward primer 5'-AGACGAAGGAAGACGTGTAAGCACTGCAGCT-3' and reverse primer 5'-AAGGAGAATCCGAAGGGAAAGGAATAAGATG-3'; for β -actin, forward primer 5'-GAACCCTAAGGCCAACCCTG-3' and reverse primer 5'-TGGCATAGAGGTCTTTACGG-3'. The amplification was performed in 30 cycles at 55°C for 30 s, 72°C for 1 min, and 94°C for 30 s. PCR fragments were analyzed on 2% agarose 1 \times TAE gel containing ethidium bromide and their size was compared with a molecular weight marker. Amplification of β -actin, a

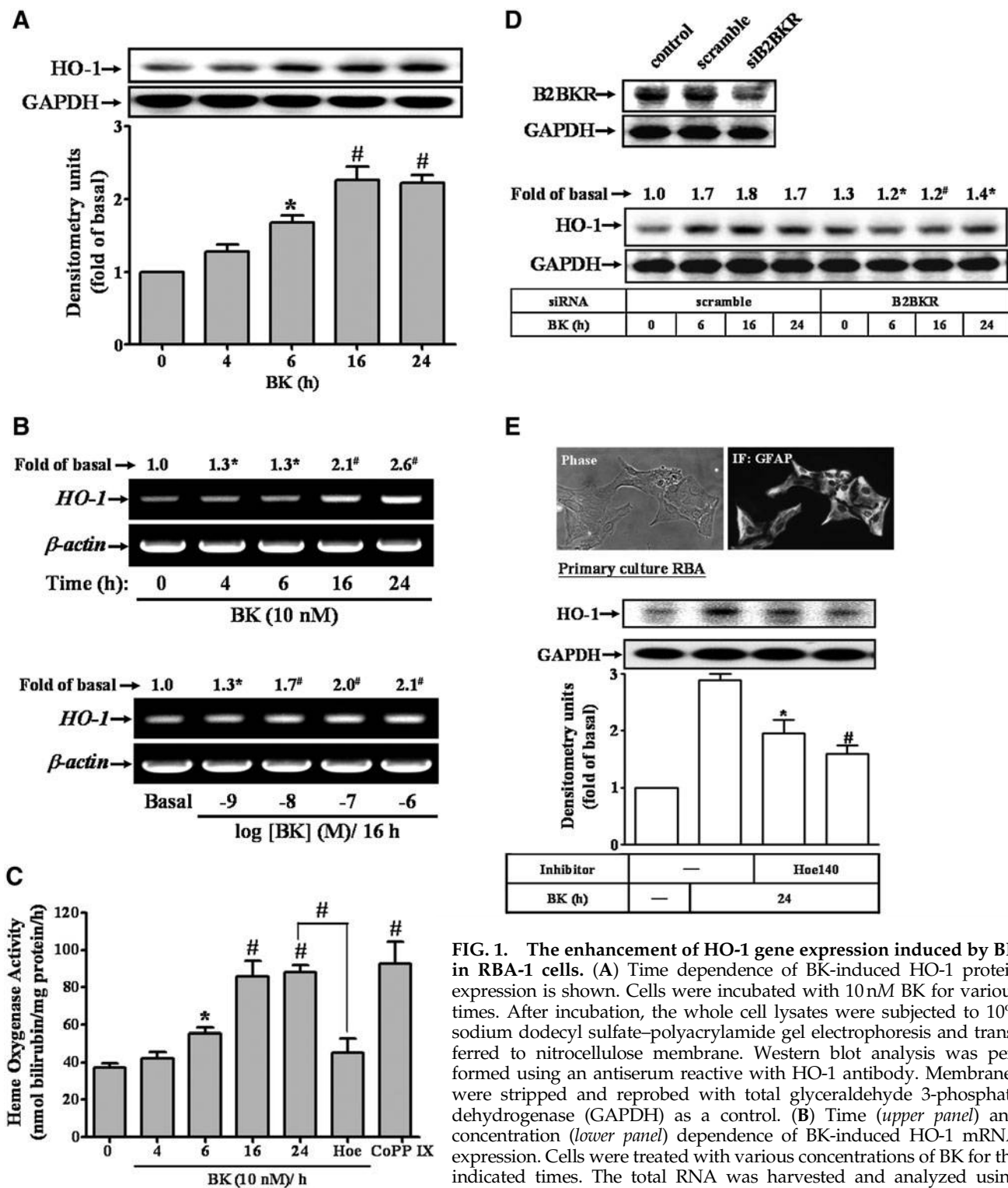
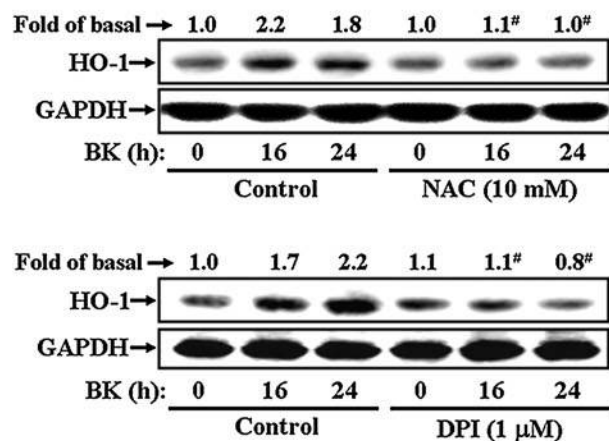
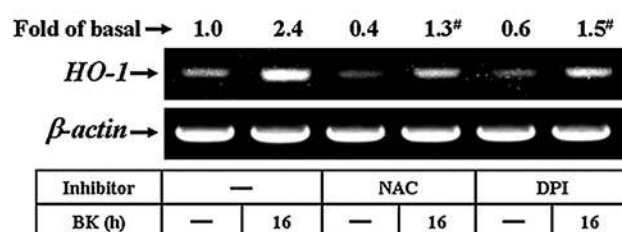
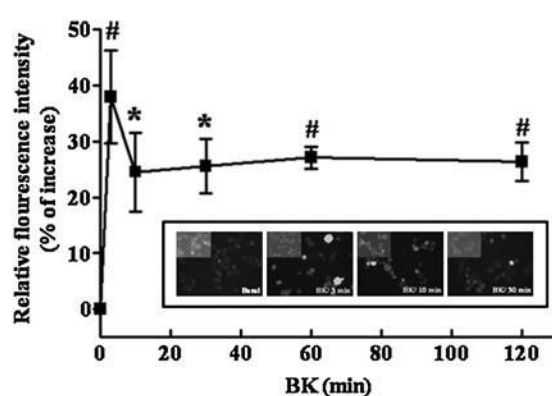
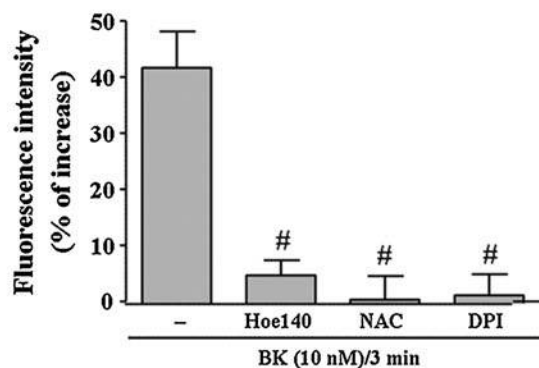
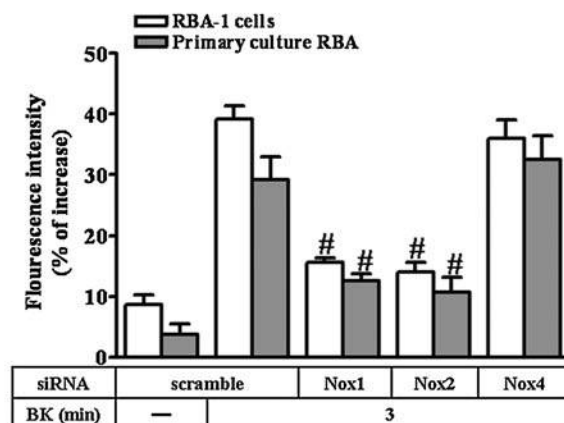
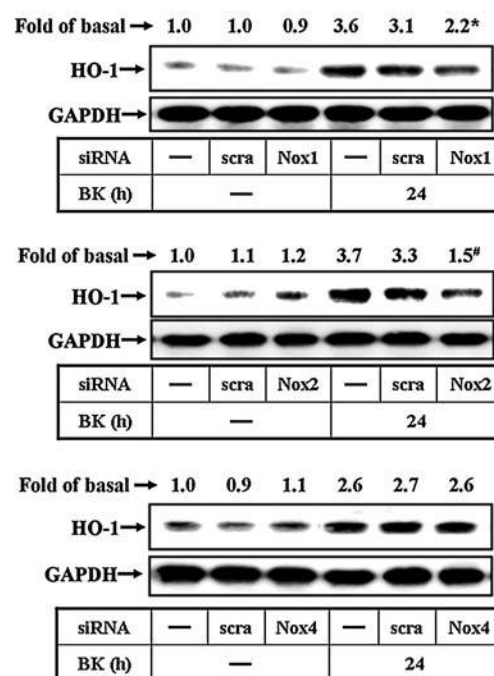
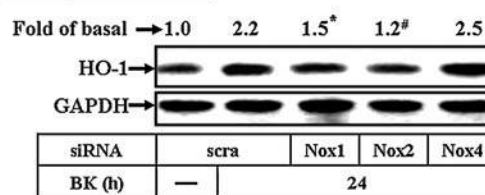


FIG. 1. The enhancement of HO-1 gene expression induced by BK in RBA-1 cells. (A) Time dependence of BK-induced HO-1 protein expression is shown. Cells were incubated with 10 nM BK for various times. After incubation, the whole cell lysates were subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. Western blot analysis was performed using an antiserum reactive with HO-1 antibody. Membranes were stripped and reprobed with total glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a control. (B) Time (upper panel) and concentration (lower panel) dependence of BK-induced HO-1 mRNA expression. Cells were treated with various concentrations of BK for the indicated times. The total RNA was harvested and analyzed using reverse transcription–polymerase chain reaction as described under Materials and Methods section. (C) Time dependence of BK-enhanced HO enzymatic activity is shown. Cells were incubated with 10 nM BK for the indicated times. Then the cells were treated with or without Hoe140 (10 μ M) for 1 h before exposure to 10 nM BK for 24 h. Cells were treated with 1 μ M CoPP IX for 24 h as a positive control. The cell lysates were collected and the HO enzymatic activity was analyzed as described under Materials and Methods section. (D) Cells were transfected with B₂BKR or scramble (as a control) siRNA and then treated with BK for the indicated times. The B₂BKR and HO-1 protein were analyzed using Western blotting. (E) Rat primary astrocytes were isolated and stained using anti-GFAP antibody (upper part) and cells were pretreated with or without Hoe140 (Hoe, 10 μ M) before exposure to BK for 24 h. The protein was analyzed using Western blotting as described under Materials and Methods section. Data are expressed as mean \pm standard error of the mean of three independent experiments (bar graph). * p < 0.05 and [#] p < 0.01, when compared with the respective values of cells stimulated with vehicle (A–C) or BK (C–E) only. The figure represents one of three similar experiments. BK, bradykinin; B₂BKR, B₂ BK receptor; CoPP IX, cobalt protoporphyrin IX; GFAP, glial fibrillary acidic protein; HO-1, heme oxygenase-1; Hoe140, D-Arg-[Hyp³, Thi⁵, D-Tic⁷, Oic⁸]-BK; RBA-1, rat brain astrocytes.

A**B****C****D****E****F****Primary culture RBA**

relatively invariant internal reference RNA, was performed in parallel, and cDNA amounts were standardized to equivalent β -actin mRNA levels. These primer sets specifically recognize only the genes of interest as indicated by amplification of a single band of the expected size (209 bp for *HO-1*, 600 bp for *c-Fos*, and 514 bp for β -actin) and direct sequence analysis of the PCR product.

Preparation of cell extracts and Western blotting analysis

Growth-arrested RBA-1 cells were incubated with or without BK at 37°C for the indicated times. The cell lysates were collected and the protein concentration was determined by using the BCA reagents according to the instructions of the manufacturer. Samples from these cell lysates (30 μ g protein) were denatured and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis using a 10% (w/v) running gel. The phosphorylation of MAPKs was identified and quantified using Western blotting as previously described (24). The immunoreactive bands detected using ECL reagents were developed by Hyperfilm-ECL.

Determination of HO activity

HO activity was determined as previously described (8) with some modifications. Briefly, cells were incubated with BK, washed twice with PBS, gently scraped off the dish, and centrifuged (1000 *g*, 10 min, 4°C). The cell pellet was suspended in MgCl₂ (2 mM) phosphate (100 mM) buffer (pH 7.4), frozen at –70°C, thawed three times, and finally sonicated on ice before centrifugation at 18,000 *g* for 10 min at 4°C. The supernatant (400 μ l) was added to a reaction mixture (200 μ l final volume, pH 7.4) containing NADPH (0.8 mM), glucose-6-phosphate (2 mM), glucose-6-phosphate-1-dehydrogenase (0.2 U), 2 mg of rat liver cytosol as a source of biliverdin reductase, PBS (100 mM), and the substrate hemin (10 μ M). The reaction was conducted for 1 h at 37°C in the dark and terminated by addition of 1 ml chloroform. The extracted bilirubin was measured by the difference in absorption between 464 and 530 nm (extinction coefficient = 40 mM^{–1} cm^{–1}). HO activity was expressed as picomoles of bilirubin per milligram of protein per hour.

Measurement of intracellular ROS generation

The peroxide-sensitive fluorescent probe 2',7'-dichloro-fluorescein diacetate (DCF-DA) was used to assess the generation of intracellular ROS (31) with minor modifications. RBA-1 cells in monolayers were incubated with RPMI-1640 supplemented with 5 μ M DCF-DA for 45 min at 37°C. The supernatant was removed and replaced with fresh RPMI-1640 media before stimulation with BK (10 nM). Relative fluorescence intensity was recorded over time (3–120 min) by using a fluorescent plate reader (Thermo; Appliskan) at an excitation wavelength of 485 nm and emission was measured at a wavelength of 530 nm. Fluorescent images were also obtained by using fluorescence microscopy (Axiovert 200M; Zeiss).

Plasmid construction, transfection, and luciferase reporter gene assays

The plasmids encoding short hairpin RNA of c-Fos (c-Fos shRNA) was kindly provided by Dr. C.P. Tseng (Chang Gung University, Taiwan). The rat *HO-1* promoter (accession No. J02722.1; –766 to +20) was constructed (sense primer: GGTACCCAGGAAGTCACAGTGTGGCC; antisense primer: CCCGAGCTCGTCGAGCTGTGGCGCTCCAT; 63°C) and cloned to the pGL3-basic vector containing the luciferase reporter system. All plasmids were prepared by using Qiagen plasmid DNA preparation kits. RBA-1 cells were transfected with plasmid using the DNA PLUS-Lipofectamine reagent. Transfection with pGal encoding for β -galactosidase was used for control of transfection efficiencies. To assess promoter activity, cells were collected and disrupted by sonication in lysis buffer (25 mM Tris phosphate [pH 7.8], 2 mM ethylenediaminetetraacetic acid, 1% Triton X-100, and 10% glycerol). After centrifugation, aliquots of the supernatants were tested for luciferase activity using a luciferase assay system (Promega). Firefly luciferase activities were standardized for β -galactosidase activity.

Immunofluorescence staining

Growth-arrested cells were treated with 10 nM BK, washed twice with ice-cold PBS, fixed with 4% (w/v) paraformaldehyde in PBS for 30 min, and then permeabilized with 0.3% Triton X-100 in PBS for 15 min. The staining was performed by incubating with 10% normal goat serum in PBS for 30 min,

FIG. 2. ROS is a fundamental element during BK-induced HO-1 expression in RBA-1 cells. (A) Cells were pretreated with NAC (10 mM, upper panel) or DPI (1 μ M, lower panel) for 1 h before exposure to 10 nM BK for 24 h. Cells were harvested for Western blotting analysis of HO-1 expression as described in Figure 1A. (B) Cells were pretreated with NAC (10 mM) or DPI (1 μ M) for 1 h before exposure to 10 nM BK for 16 h. The total RNA was collected and analyzed using reverse transcription–polymerase chain reaction. (C) Time dependence of BK-stimulated intracellular ROS generation is shown. RBA-1 cells were incubated with the peroxide-sensitive fluorescent probe DCF-DA (5 μ M) for 45 min, followed by stimulation with 10 nM BK for the indicated times. (D) After cells were loaded with DCF-DA, cells were pretreated with Hoe140 (10 μ M), NAC (10 mM), or DPI (1 μ M) for 1 h, followed by stimulation with 10 nM BK for 3 min. (E) RBA-1 cells and primary culture astrocytes were transfected with distinct Nox or scramble (as a control) siRNA and loaded with DCF-DA, followed by stimulation with 10 nM BK for 3 min. (F) After distinct Nox siRNA transfection, cells were incubated with BK for 24 h. The HO-1 expression was analyzed using Western blotting. The fluorescence images (inset panel) and intensity of cells were determined as described under Materials and Methods section. Data are expressed as mean \pm standard error of the mean of three independent experiments. **p* < 0.05 and #*p* < 0.01, when compared with the respective values of cells stimulated with vehicle (C) and BK (A, B, and D) alone. The figure represents one of three individual experiments. BK, bradykinin; DCF-DA, 2',7'-dichlorofluorescein diacetate; DPI, diphenyleneiodonium; HO-1, heme oxygenase-1; Hoe140, D-Arg-[Hyp³, Thi⁵, D-Tic⁷, Oic⁸]-BK; NAC, N-acetyl-cysteine; Nox, NADPH oxidase; RBA-1, rat brain astrocytes; ROS, reactive oxygen species.

followed by incubating with an anti-c-Fos polyclonal antibody (1:200 dilution) for 1 h in PBS with 1% BSA, washing three times with PBS, incubating for 1 h with fluorescein isothiocyanate-conjugated goat anti-rabbit antibody (1:200 dilution) in PBS with 1% BSA, washing three times with PBS, and finally mounting with aqueous mounting medium. The images were observed under a fluorescence microscope (Axiovert 200M; Zeiss).

Chromatin immunoprecipitation assay

The primers were designed by referencing nucleotide sequence of the rat HO gene as previously described (36). Briefly, RBA-1 cells were crosslinked with 1% formaldehyde for 10 min at 37°C and washed thrice with ice-cold PBS containing 1 mM phenylmethylsulfonyl fluoride and 1% aprotinin. Soluble chromatin was prepared using a chromatin immunoprecipitation (ChIP) assay kit (Upstate) according to the manufacturer's recommendations and immunoprecipitated without (control) or with anti-c-Fos antibody (sc-7202; Santa Cruz) and normal goat immunoglobulin G. Following washes and elution, precipitates were heated overnight at 65°C to reverse cross-linking of DNA and protein. DNA fragments were purified using phenol-chloroform extraction and ethanol precipitation. The purified DNA was subjected to PCR amplification using the primers specific for the region (−460 to −2) containing a putative AP-1-binding site present in the HO-1 promoter region (sense primer: 5'-CAGTCTAGGCAGGTG TTAGT-3'; antisense primer: 5'-AGCGAGCAGCTGCCCCG GCC-3'). PCR fragments were analyzed on 2% agarose 1× TAE gel containing ethidium bromide and the size (458 bp) was compared with a molecular weight marker.

Analysis of data

Data were estimated using a GraphPad Prism Program (GraphPad). Quantitative data were analyzed using analysis of variance followed by Tukey's honestly significant difference tests between individual groups. Data were expressed as mean ± standard error of the mean. A *p*-value of <0.05 was considered significant.

Results

BK induces HO-1 expression and HO activity

To investigate the effects of BK on HO-1 gene expression and activity, BK-induced HO-1 protein expression in a time-dependent manner was determined using Western blot (Fig. 1A). There was a significant increase within 6 h, which sustained up to 24 h. Next, BK-induced HO-1 mRNA expression in a time-dependent (Fig. 1B, upper panel) and concentration-dependent (Fig. 1B, lower panel) manner was detected using reverse transcription (RT)-PCR. To further determine whether BK-induced HO-1 expression accompanied with an increase in HO activity, the HO enzymatic activity was assessed by measuring the heme metabolite bilirubin in the cell lysates. The data showed that BK induced HO enzymatic activity in a time-dependent manner and a significant increase occurred at 6 h and sustained up to 24 h (Fig. 1C). Cells treated with cobalt protoporphyrin IX (a HO-1 inducer) for 24 h significantly induced HO activity (as a positive control). Moreover, pretreatment with Hoe140 (10 μM), a selective B₂BKR antagonist, markedly attenuated BK-induced

HO activity, suggesting that BK induces HO activity *via* a B₂BKR in RBA-1 cells. Therefore, we further confirmed the involvement of B₂BKR in BK-induced HO-1 expression, using cells transfected with a B₂BKR siRNA. As shown in Figure 1D, transfection with B₂BKR siRNA significantly knocked down the B₂BKR protein expression and attenuated BK-induced HO-1 expression in RBA-1 cells.

To demonstrate whether BK-induced HO-1 expression also occurred in primary cultured astrocytes, as shown in Figure 1E, we first identified the primary cultured astrocytes by GFAP immunofluorescent staining, showing nearly 95% GFAP-positive astrocytes (upper panel). BK also induced HO-1 protein expression, which was inhibited by pretreatment with Hoe140 (lower panel). These results indicated that BK-induced responses in rat primary cultured astrocytes are similar to those of RBA-1 cells. Thus, the following experiments were performed using RBA-1 cells, which can be applied as a model throughout this study.

BK induces HO-1 expression through Nox-derived ROS generation

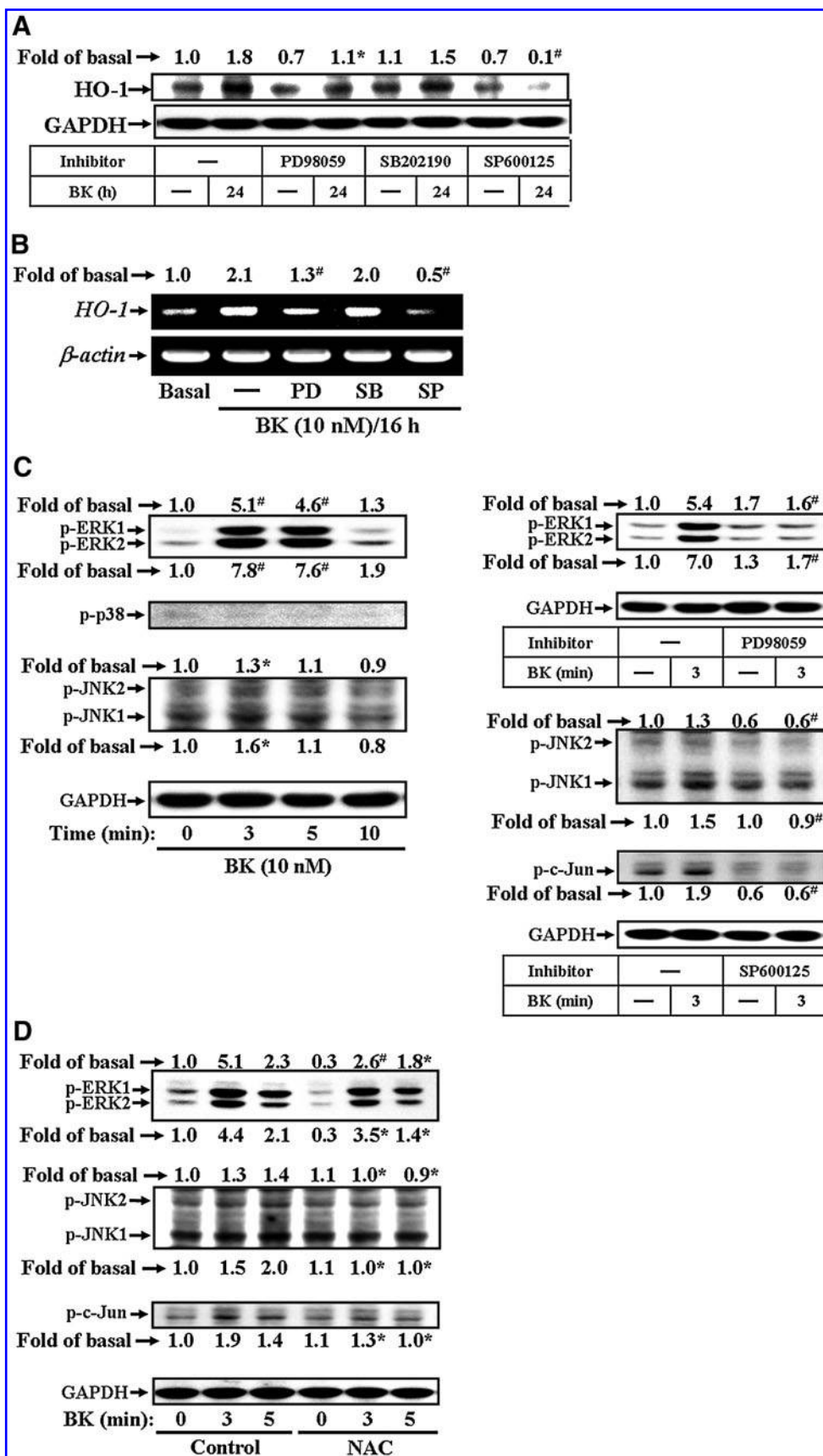
Several studies have demonstrated that ROS contributes to HO-1 expression in various cell types (42). To determine whether ROS participated in HO-1 induction, a ROS scavenger NAC and Nox (a major source of ROS) activity inhibitor diphenyleneiodonium (DPI) were used. Pretreatment with NAC (10 mM) or DPI (1 μM) attenuated the BK-induced HO-1 protein and mRNA expression (Fig. 2A, B), suggesting that Nox-derived ROS may play a potential role in BK-induced HO-1 expression in RBA-1 cells. To explore whether BK induced ROS generation, the cells were loaded with DCF-DA (a ROS indicator) and then stimulated with BK (10 nM) for the indicated time intervals. Our data showed that BK increased the ROS generation in a time-dependent manner with a maximal response within 3 min and sustained over 120 min (Fig. 2C). The results were further supported by the data of fluorescence images obtained using a fluorescent microscopy (Fig. 2C, inset panel). As shown in Figure 2D, BK-induced ROS generation was markedly attenuated by pretreatment with Hoe140, NAC, or DPI, suggesting that BK-stimulated ROS generation led to HO-1 expression *via* a B₂BKR-dependent activation of Nox in RBA-1 cells.

To further determine which Nox isotype was involved in these responses, a variety of Nox siRNA were used. As shown in Figure 2E, transfection with Nox1 or Nox2 siRNA, but not Nox4, knocked down the expression of respective Nox protein and significantly attenuated BK-induced ROS generation and HO-1 expression (Fig. 2F) in both RBA-1 cells and primary culture astrocytes. These results suggested that BK-induced HO-1 expression is mediated through Nox-dependent ROS generation in brain astrocytes.

Involvement of ROS-dependent ERK1/2 and JNK1/2 pathways in the induction of HO-1 by BK

HO-1 expression is regulated *via* the activation of MAPKs in many cell types (8, 42). Recently, we have demonstrated that MAPKs participate in the expression of several inflammatory genes in RBA-1 cells (22, 21). Therefore, we further determined whether MAPKs also involve in BK-induced HO-1 expression in RBA-1 cells. First, we found that pretreatment with the inhibitors of MEK1/2 (PD98059, 30 μM) and JNK

FIG. 3. Reactive oxygen species-dependent MAPKs activation are involved in BK-induced HO-1 expression in rat brain astrocytes cells. (A) Cells were treated with or without PD98059 (30 μ M), SB202190 (10 μ M), or SP600125 (1 μ M) for 1 h before exposure to 10 nM BK for 24 h. Cells were harvested for Western blotting analysis of HO-1 expression as described in Figure 1A. (B) Similarly, the total RNA was collected and analyzed using reverse transcription-polymerase chain reaction as described under Materials and Methods section. (C) Time dependence of BK-stimulated MAPKs phosphorylation is shown. Cells were treated with 10 nM BK for the indicated times (left panel). The cells were pretreated with or without PD98059 (30 μ M) or SP600125 (1 μ M) for 1 h before exposure to 10 nM BK for 3 min (right panel). (D) Cells were pretreated with or without NAC (10 mM) for 1 h, followed by stimulation with 10 nM BK for the indicated times. Cells were harvested for Western blotting analysis of phospho-ERK, phospho-p38 MAPK, phospho-JNK, or phospho-c-Jun, respectively as described under Materials and Methods section. Data are expressed as mean of three independent experiments. * $p < 0.05$ and # $p < 0.01$, when compared with the respective values of cells stimulated with vehicle (C) and BK (A–D) only. The figure represents one of three similar experiments. BK, bradykinin; ERK, extracellular signal-regulated kinase; HO-1, heme oxygenase-1; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NAC, N-acetyl-cysteine.



(SP600125, 1 μ M) but not p38 MAPK (SB202190, 10 μ M) significantly blocked BK-induced HO-1 protein and mRNA expression (Fig. 3A, B), indicating that ERK1/2 and JNK1/2 were involved in BK-induced HO-1 expression in RBA-1 cells.

To determine whether phosphorylation of these MAPKs was necessary for BK-induced HO-1 expression, activation of these kinases was assayed using Western blot with an antibody specific for the phosphorylated form of ERK1/2, p38 MAPK, or JNK1/2. As shown in Figure 3C, BK stimulated phosphorylation of ERK1/2 and JNK1/2 in a time-dependent manner, but failed to stimulate p38 MAPK phosphorylation. Moreover, phosphorylation of ERK1/2 and JNK1/2 as well as c-Jun stimulated by BK was blocked by pretreatment with

PD98059 and SP600125, respectively. Next, we examined whether BK-stimulated phosphorylation of ERK1/2 and JNK1/2 was mediated through ROS generation. As shown in Figure 3D, pretreatment with NAC significantly attenuated BK-stimulated phosphorylation of ERK1/2, JNK1/2, and c-Jun. These results indicated that ROS plays a critical role in BK-stimulated ERK1/2 and JNK1/2 phosphorylation, leading to HO-1 expression in RBA-1 cells.

BK induces HO-1 expression via ROS/ERK cascade and NF- κ B activation

To investigate whether NF- κ B was involved in BK-induced HO-1 expression, an NF- κ B inhibitor Bay11-7082 (38) was used. Pretreatment with Bay11-7082 (1 μ M) inhibited BK-induced HO-1 protein and mRNA expression (Fig. 4A, B). It has been shown that phosphorylation and translocation of NF- κ B, such as p65 subunit, is required for induction of NF- κ B-targeted genes (50). To determine whether p65 was phosphorylated and translocated into nucleus stimulated by BK in RBA-1 cells, the whole cell lysate and nuclear fraction were prepared and analyzed using Western blotting. As shown in Figure 4C and D, BK stimulated a rapid phosphorylation and translocation of p65 in RBA-1 cells, which was significantly blocked by pretreatment with Bay11-7082, PD98059, or NAC, but not with SP600125. NF- κ B activation by BK was further confirmed by reporter gene activity assay. RBA-1 cells were transfected with an NF- κ B luciferase reporter gene (p κ B-Luc). As shown in Figure 4E, BK-stimulated NF- κ B transcription activity was inhibited by pretreatment with Bay11-7082, PD98059, or NAC, but not SP600125. These results demonstrated that NF- κ B was involved in HO-1 ex-

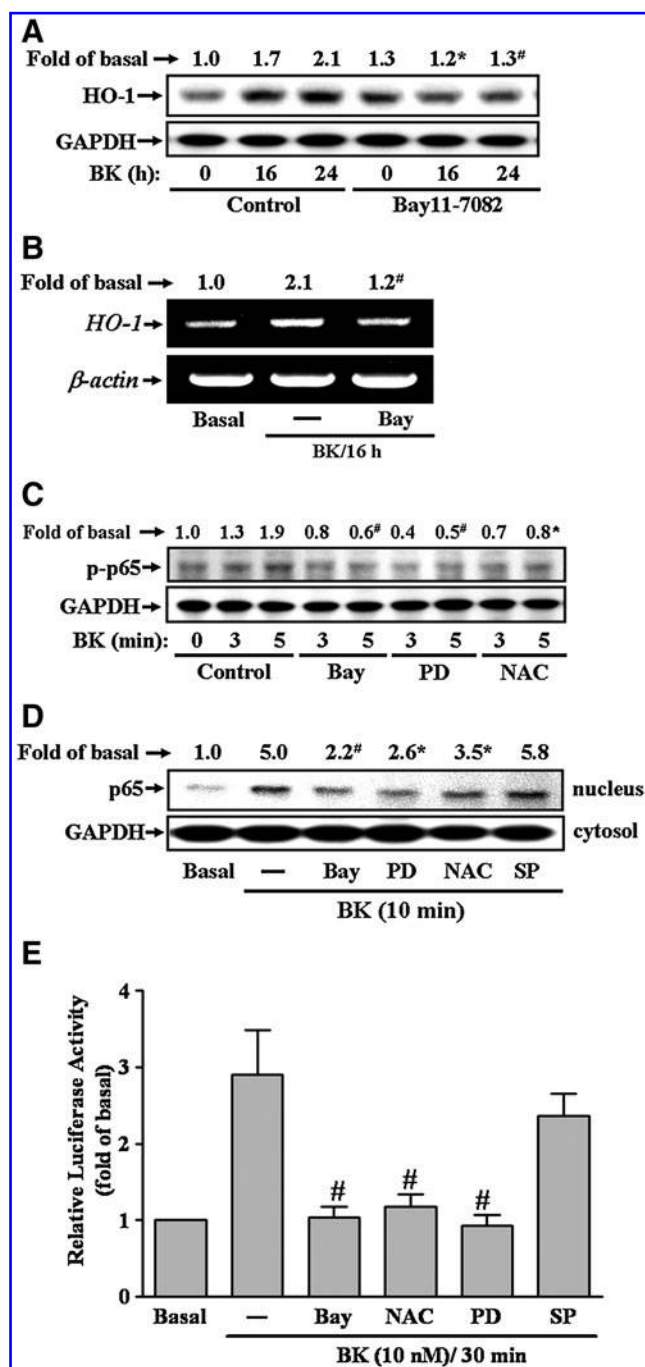


FIG. 4. Effect of Bay11-7082 on BK-induced HO-1 expression in RBA-1 cells. (A) Cells were pretreated with Bay11-7082 (1 μ M) for 1 h before exposure to 10 nM BK for 24 h. Cells were harvested for Western blotting analysis of HO-1 expression as described in Figure 1A. (B) The total RNA was collected and analyzed using reverse transcription-polymerase chain reaction. (C) RBA-1 cells were pretreated with Bay11-7082 (Bay, 1 μ M), PD98059 (PD, 30 μ M), or NAC (10 mM) for 1 h and then incubated with BK (10 nM) for the indicated times. (D) Cells were pretreated with Bay11-7082 (Bay, 1 μ M), PD98059 (PD, 30 μ M), NAC (10 mM), or SP600125 (SP, 1 μ M) for 1 h and then incubated with BK (10 nM) for 10 min. The cell lysates (C) and cell fractions (D) were prepared and Western blotted using an anti-phosphop65 (C) or anti-p65 (D) antibody, respectively. (E) RBA-1 cells were transiently cotransfected with 0.9 μ g of p κ B-Luciferase and 0.1 μ g of pGal encoding for β -galactosidase for 24 h. Cells were pretreated with Bay11-7082 (Bay, 1 μ M), PD98059 (PD, 30 μ M), NAC (10 μ M), or SP600125 (SP, 1 μ M) for 1 h and then incubated with BK (10 nM) for 30 min before measuring the luciferase and β -galactosidase activities. The values for beetle luciferase were related to values for β -galactosidase and are depicted as relative luciferase activities. Data are expressed as mean \pm standard error of the mean of at least three independent experiments. * p < 0.05 and # p < 0.01, when compared with cells stimulated by BK. BK, bradykinin; HO-1, heme oxygenase-1; RBA-1, rat brain astrocytes.

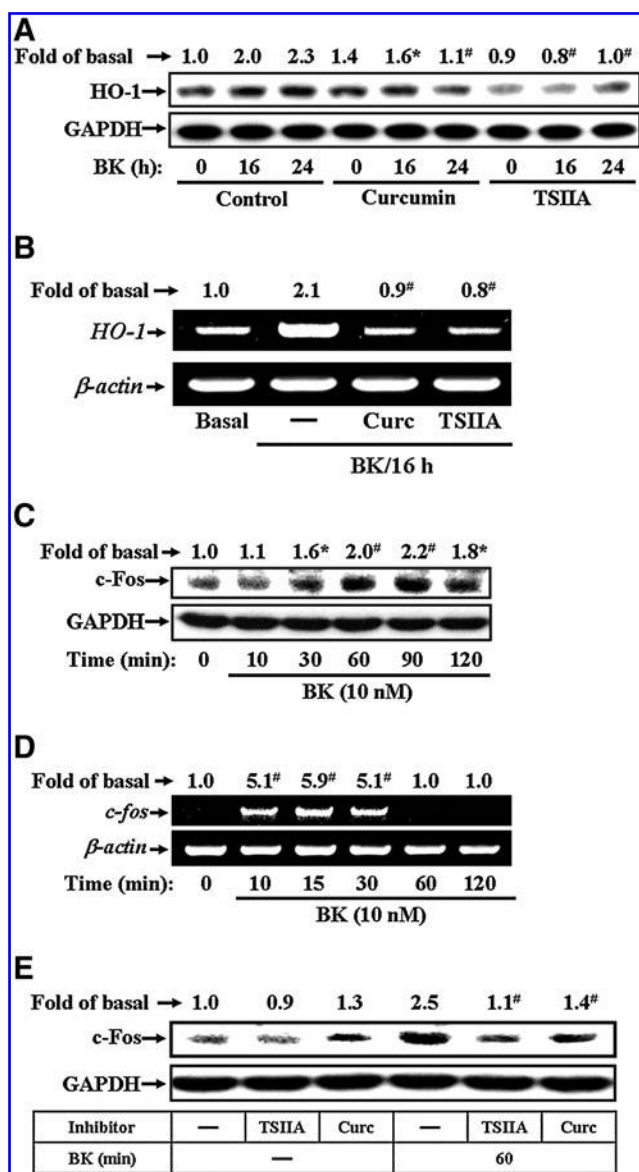


FIG. 5. Upregulation of activator protein 1 is a critical event in BK-induced HO-1 expression. (A) Cells were pretreated with or without curcumin (10 μ M) or TSIIA (10 μ M) for 1 h before exposure to 10 nM BK for 24 h. Cells were harvested for Western blotting analysis of HO-1 expression as described in Figure 1A. (B) The total RNA was collected and analyzed using RT-PCR as described under Materials and Methods section. (C) Time dependence of BK-induced c-Fos protein and mRNA (D) expression is shown. Cells were incubated with 10 nM BK for the indicated times. (E) Cells were pretreated with or without curcumin (10 μ M) or TSIIA (10 μ M) for 1 h and then treated with BK (10 nM) for 60 min. After incubation, the cell lysates (C and E) and total RNA (D) were collected and analyzed using Western blotting and RT-PCR, respectively. Data are expressed as mean of three independent experiments. * p < 0.05 and # p < 0.01, when compared with the respective values of cells stimulated with vehicle (C and D) and BK (A, B, and E) only. The figure represents one of three similar experiments. BK, bradykinin; HO-1, heme oxygenase-1; RT-PCR, reverse transcription-polymerase chain reaction; TSIIA, tanshinone IIA.

pression mediated through ROS generation followed by the activation of ERK in RBA-1 cells.

The induction of transcription factor c-Fos/AP-1 is required for BK-induced HO-1 expression

To determine whether BK-induced HO-1 expression was mediated through transcription factor AP-1, two AP-1 inhibitors, that is, curcumin and TSIIA, were used. As shown in Figure 5A and B, pretreatment with AP-1 inhibitor curcumin or TSIIA attenuated BK-induced HO-1 protein and mRNA expression, suggesting that AP-1 is required for BK-induced HO-1 expression in RBA-1. To further investigate whether BK stimulated the expression of AP-1 (containing immediate early gene c-Fos and c-Jun subunits), the levels of protein and mRNA of c-Fos and c-Jun were determined using Western blot and RT-PCR, respectively. BK induced c-Fos protein (Fig. 5C) and mRNA (Fig. 5D) expression in a time-dependent manner, but not c-Jun (data not shown). The c-Fos induction by BK reached a peak within 60 min (protein) and 15 min (mRNA), respectively. BK-induced c-Fos protein expression was attenuated by pretreatment with curcumin or TSIIA (Fig. 5E). These results suggested that BK-induced HO-1 expression is mediated through upregulation of transcription factor AP-1, particularly c-Fos subunit, in RBA-1 cells.

BK-induced c-Fos/AP-1 expression is mediated through ROS-dependent ERK/NF- κ B and JNK/c-Jun cascades

To further investigate whether ERK/NF- κ B and JNK/c-Jun pathways were involved in BK-induced AP-1 expression, as shown in Figure 6A, BK-induced c-Fos/AP-1 expression was inhibited by pretreatment with Hoe140, NAC, PD98059, SP600125, curcumin, or Bay11-7082. BK-induced c-Fos/AP-1 expression was further supported by the results obtained using immunofluorescence staining against a c-Fos antibody. The result showed that BK-stimulated expression and accumulation (in nucleus) of c-Fos/AP-1 was attenuated by pretreatment with NAC in RBA-1 cells (Fig. 6B). The AP-1 activation by BK was further confirmed by reporter gene activity assay. The AP-1 transcription activity was enhanced by BK (Fig. 6C, inset panel), which was significantly inhibited by pretreatment with Hoe140, NAC, PD98059, SP600125, curcumin, TSIIA, or Bay11-7082 (Fig. 6C). These results suggested that BK-induced AP-1 expression and activation is mediated through ROS-dependent ERK/NF- κ B and JNK/c-Jun pathways. To ensure that c-Fos/AP-1 was essential for BK-induced HO-1 expression, transfection of cells with c-Fos shRNA downregulated the expression of total c-Fos protein and markedly abolished BK-induced HO-1 expression (Fig. 6D), indicating that upregulation of c-Fos/AP-1 was required for BK-induced HO-1 expression in RBA-1 cells.

BK-induced recruitment of c-Fos/AP-1 enhances the HO-1 promoter transcriptional activity and HO activity

To investigate whether c-Fos bound to the HO-1 promoter region, a ChIP-PCR assay was performed. Chromatin was immunoprecipitated with an anti-c-Fos antibody and the HO-1 promoter region (−460 to −2) was amplified using PCR. As shown in Figure 7A, BK stimulated *in vivo* binding of c-Fos to the HO-1 promoter in a time-dependent manner with a

maximal response within 30 min, which was attenuated by pretreatment with Hoe140 or NAC. The data suggested that BK-stimulated binding of c-Fos to *HO-1* promoter is mediated through a B₂BKR/ROS-dependent pathway. To further examine whether BK induced *HO-1* gene regulation *via* initiating its promoter activity, the *HO-1* promoter was constructed and its activity was evaluated using a promoter-luciferase activity assay. The *HO-1* promoter was constructed into a pGL3-basic vector containing a luciferase reporter system (as illustrated in Fig. 7B, upper part), which contains several putative recognition elements for a variety of transcriptional factors. To determine the effect of BK on the *HO-1* promoter activity, as shown in Figure 7B, BK increased the *HO-1* promoter activity in a time-dependent manner (inset panel) with a maximal response within 6 h, which was inhibited by pretreatment with Hoe140, NAC, DPI, PD98059, SP600125,

Bay11-7082, curcumin, or TSIIA. Finally, we determined whether these signaling pathways were involved in BK-induced *HO-1* enzymatic activity. As shown in Figure 7C, BK-enhanced *HO-1* enzymatic activity was significantly abolished by pretreatment with NAC, PD98059, SP600125, Bay11-7082, curcumin, or TSIIA. These results confirmed that BK induced *HO-1* promoter activity and *HO-1* enzymatic activity *via* enhancing c-Fos/AP-1 binding to the *HO-1* promoter. The induction of the *HO-1* promoter activity was mediated through B₂BKR/ROS-dependent ERK/NF- κ B and JNK/c-Jun pathways converging to c-Fos/AP-1 upregulation in RBA-1 cells.

BK-induced *HO-1* expression via ROS-dependent Nrf2 signaling

In addition, Nrf2 has been reported to mediate the expression of a wide array of ROS-dependent genes. Thus, we also demonstrated whether ROS-dependent Nrf2 signaling was involved in BK-induced *HO-1* expression. As shown in Figure 8A, BK stimulated Nrf2 translocation from cytoplasm into nucleus in a time-dependent manner, with a significant response within 5–10 min (upper panel). Pretreatment with NAC significantly inhibited Nrf2 translocation stimulated by BK (Fig. 8A, lower panel), suggesting that BK stimulated a ROS-dependent Nrf2 translocation in RBA-1 cells. To further

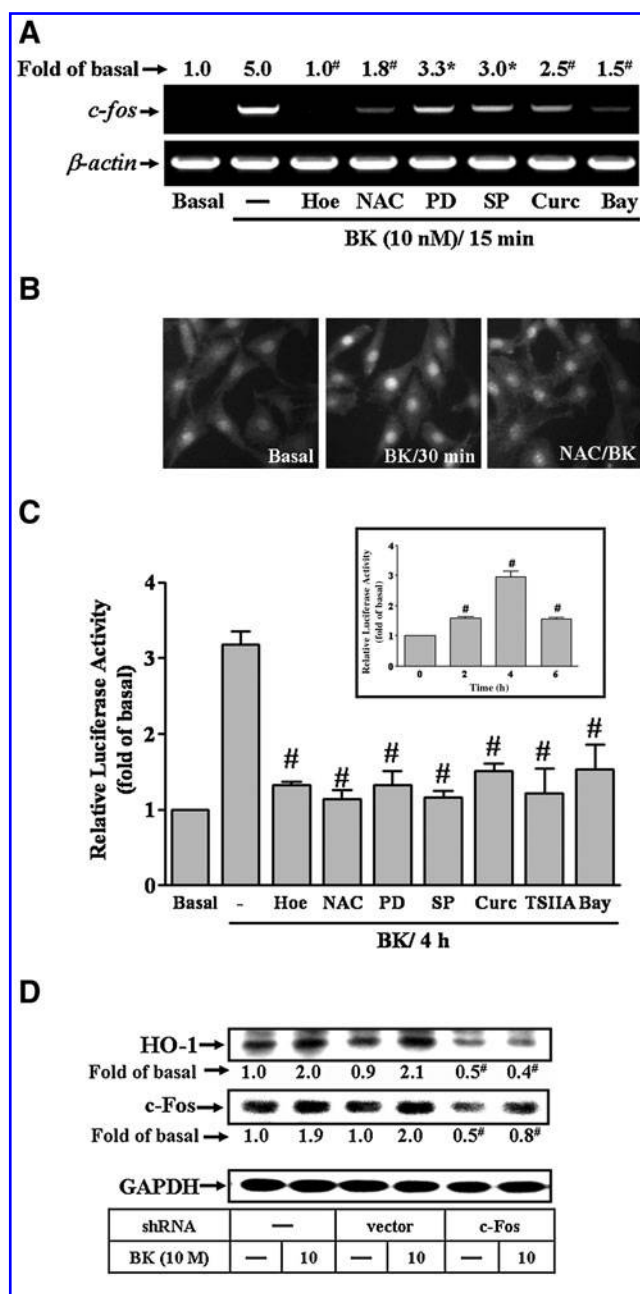


FIG. 6. Involvement of signaling molecules in BK-induced c-Fos/AP-1 expression in RBA-1 cells. (A) Cells were treated with or without Hoe140 (Hoe, 10 μ M), NAC (10 mM), PD98059 (PD, 30 μ M), SP600125 (SP, 1 μ M), curcumin (Curc, 10 μ M), or Bay11-7082 (Bay, 1 μ M) for 1 h before exposure to 10 nM BK for 15 min. The total RNA was collected and analyzed using reverse transcription-polymerase chain reaction as described under Materials and Methods section. (B) BK-stimulated c-Fos expression and nuclear accumulation were determined using immunofluorescence staining. Cells were pretreated with 10 mM NAC for 1 h and then stimulated with 10 nM BK for 30 min. Cells were fixed and labeled with anti-c-Fos antibody and a fluorescein isothiocyanate-conjugated secondary antibody. Individual cells were imaged as described in Materials and Methods section. (C) Time dependence of BK-stimulated AP-1-luciferase reporter gene expression (inset panel) is shown. To determine the involvement of signaling molecules in BK-induced AP-1 promoter transcription activity, cells were transiently co-transfected with AP-1-luciferase plasmid and a β -galactosidase plasmid and pretreated with Hoe140 (Hoe, 10 μ M), NAC (10 mM), PD98059 (PD, 30 μ M), SP600125 (SP, 1 μ M), curcumin (Curc, 10 μ M), TSIIA (10 μ M), or Bay11-7082 (Bay, 1 μ M) for 1 h before exposure to 10 nM BK for 4 h. AP-1 promoter activity was measured and normalized to β -galactosidase activity. (D) RBA-1 cells were transfected with c-Fos shRNA plasmid encoding c-Fos shRNA or empty vector (as a control) and then stimulated with BK (10 nM) for indicated times. After incubation, the cell lysates were collected and assayed using Western blot as described in Materials and Methods section. Data are represented as mean \pm standard error of the mean of three independent experiments. * p < 0.05 and [#] p < 0.01, when compared with the cells exposed with vehicle (inset panel) or BK alone. The figure represents one of three similar experiments. AP-1, activator protein 1; BK, bradykinin; Hoe140, D-Arg-[Hyp³, Thi⁵, D-Tic⁷, Oic⁸]-BK; NAC, N-acetyl-cysteine; RBA-1, rat brain astrocytes; shRNA, short hairpin RNA; TSIIA, tanshinone IIA.

demonstrate the role of Nrf2 in BK-induced HO-1 expression, cells were transfected with Nrf2 siRNA and then exposed to BK for the indicated time intervals. We found that transfection with Nrf2 siRNA knocked down the expression of total Nrf2 protein and attenuated BK-induced HO-1 expression in RBA-1 cells (Fig. 8B, upper panel) and primary culture astrocytes (Fig. 8B, lower panel). These results indicated that ROS-dependent Nrf2 signaling may be involved in BK-induced HO-1 expression in RBA-1 cells.

Discussion

The inducible HO-1 is upregulated in response to xenobiotics, oxidative stress, cellular injury, and diseases (41, 51). The role of HO-1 expression in the regulation of inflammation

has emerged over the past decade, besides its well-known function as an indicator of oxidative stress (3, 51). HO-1 contributes to a wide range of biological activities in different brain diseases, such as stroke and AD (47, 51). Previous studies on brains from AD patients demonstrate colocalization of HO-1 protein in GFAP-expressing astrocytes and in neurons of the hippocampus (44). Moreover, expression and activity of HO-1 have been shown to be elevated in various neuroinflammatory and neurodegenerative disorders (47, 51), indicating that HO-1 may be a critical molecule in the pathophysiological processes in these diseases. Moreover, BK and related peptides are simultaneously produced and released following injury to the human CNS (29). Despite an obviously important role of BK or related peptides in brain diseases, the processes by which BK was implicated in astrocytic functions are not completely understood. Thus, we used a well-established rat astroglial cell line RBA-1 cells (27) to investigate the mechanisms underlying BK-induced HO-1 expression and its activity. Our results suggest that in RBA-1 cells, activation of ROS-dependent JNK/c-Jun and ERK/NF- κ B pathways leading to induction of c-Fos/AP-1, mediated through a B₂BKR, is essential for BK-induced HO-1 gene expression and its activity.

ROS concentration-dependently exerts a key role in the normal physiological functions and the inflammatory

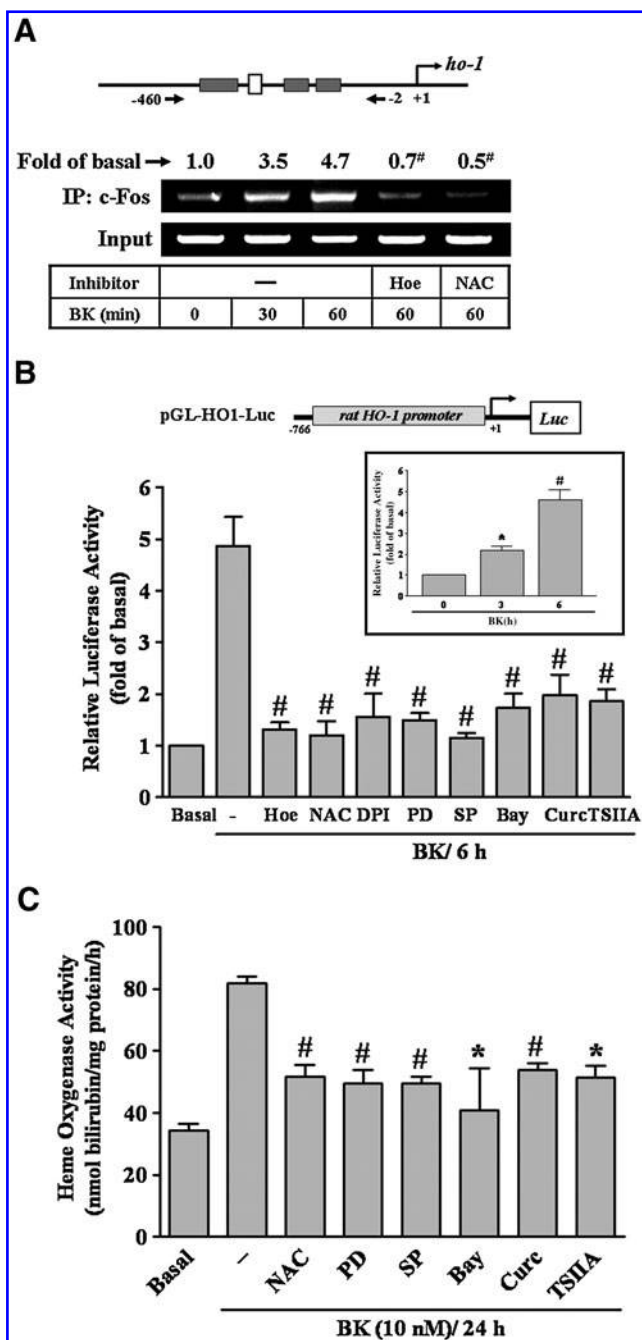


FIG. 7. BK induces *HO-1* promoter transcription activity and *HO-1* enzymatic activity in RBA-1 cells. (A) Schematic illustration of transcriptional factor binding sites on the *HO-1* promoter. Time dependence of BK-stimulated recruitment of c-Fos/activator protein 1 to the *HO-1* promoter is shown. Cells were treated with 10 nM BK for 30 and 60 min. Pretreatment with Hoe140 (10 μ M, Hoe) or NAC (10 mM) inhibited the recruitment of c-Fos to the *HO-1* promoter induced by BK for 60 min. Chromatin immunoprecipitation assays were performed using an anti-c-Fos antibody. Precipitated *HO-1* promoter region (-460 to -2) was amplified by polymerase chain reaction primer pairs indicated by the arrows; 1% of the chromatin was assayed to verify equal loading (Input). (B) Schematic representation of a 5'-promoter portion of the rat *HO-1* gene fused to the pGL-luciferase reporter gene (pGL-HO1-Luc). The translational start site (+1) of the luciferase reporter gene is indicated by an arrow. RBA-1 cells were transiently cotransfected with pGL-HO1-Luc and pGal coding for β -galactosidase. After transfection, cells were treated with BK for various times (inset panel) or in the absence or presence of Hoe140 (Hoe, 10 μ M), NAC (10 mM), DPI (1 μ M), PD98059 (PD, 30 μ M), SP600125 (SP, 1 μ M), Bay11-7082 (Bay, 1 μ M), curcumin (Curc, 10 μ M), or TSIIA (10 μ M) for 6 h before being harvested for measuring the luciferase and β -galactosidase activities as described in Materials and Methods section. (C) Cells were treated with or without NAC (10 mM), PD98059 (PD, 30 mM), SP600125 (SP, 1 μ M), Bay11-7082 (Bay, 1 μ M), curcumin (Curc, 10 μ M), or TSIIA (10 μ M) for 1 h before exposure to 10 nM BK for 24 h. The cell lysates were collected and the HO enzymatic activity was analyzed as described under Materials and Methods section. Data are expressed as mean \pm standard error of the mean of three independent experiments. * p < 0.05 and [#] p < 0.01, when compared with the respective values of cells stimulated with vehicle (inset panel) and BK only. BK, bradykinin; HO-1, heme oxygenase-1; Hoe140, D-Arg-[Hyp³, Thi⁵, D-Tic⁷, Oic⁸]-BK; NAC, N-acetyl-cysteine; RBA-1, rat brain astrocytes; TSIIA, tanshinone IIA.

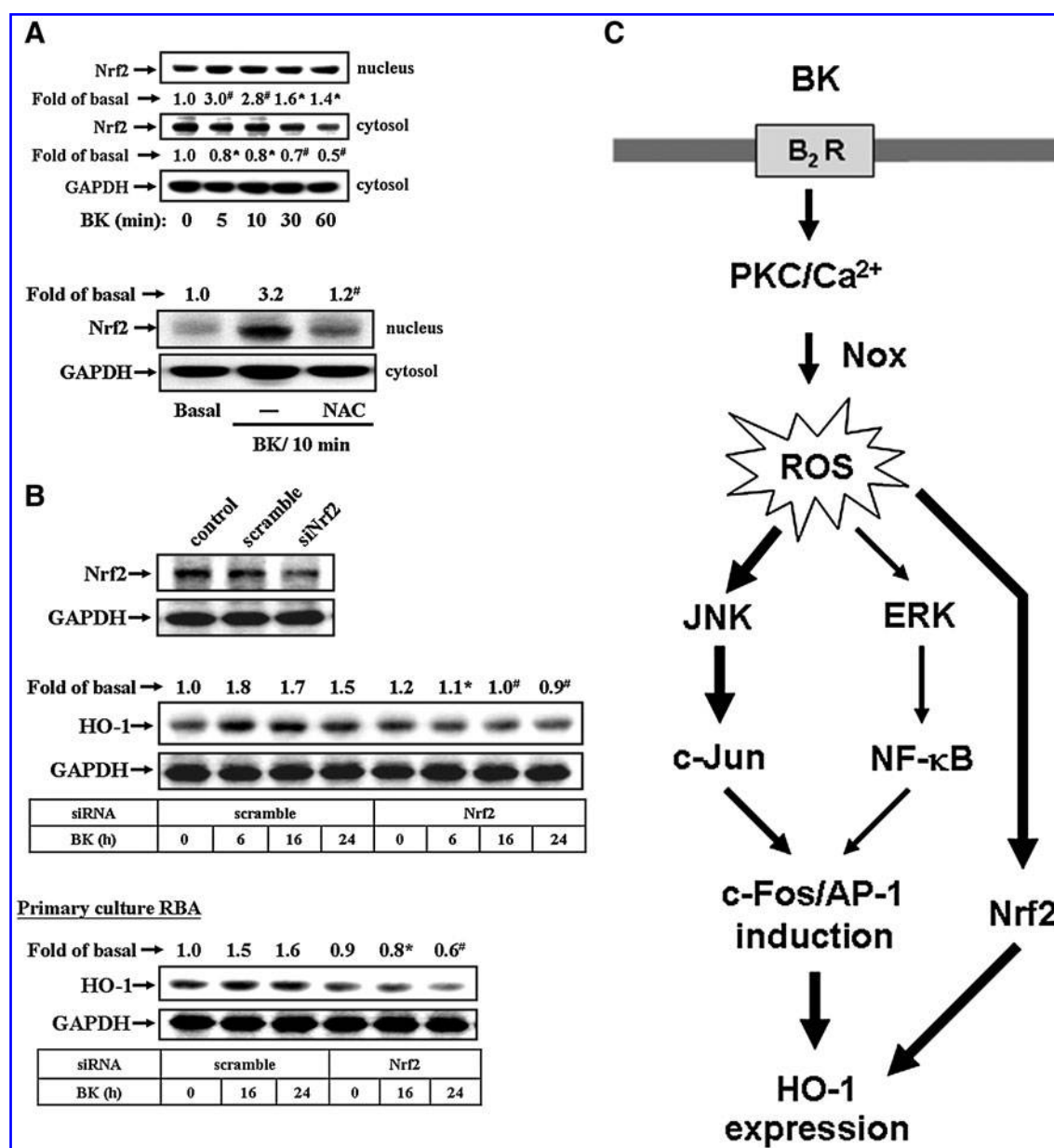


FIG. 8. ROS-dependent Nrf2 signaling is involved in BK-induced HO-1 expression. (A) Time dependence of BK-stimulated Nrf2 translocation into nucleus is shown. RBA-1 cells were treated with 10 nM BK for the indicated times (*upper panel*). Cells were pretreated with NAC (10 mM) for 1 h before exposure to 10 nM BK for 10 min (*lower panel*). (B) RBA-1 cells (*upper panel*) and primary culture RBA (*lower panel*) were transfected with Nrf2 or scramble (as a control) siRNA and then treated with BK (10 nM) for the indicated times. The Nrf2 (*upper panel*) and HO-1 (*lower panel*) protein were analyzed using Western blotting. Data are expressed as mean of three independent experiments. * $p < 0.05$ and [#] $p < 0.01$, when compared with the respective values of cells stimulated with vehicle (A, *upper panel*) and BK (A, *lower panel* and B) alone. The figure represents one of three individual experiments. (C) Schematic pathway for BK-induced ROS-dependent c-Fos/AP-1 upregulation and HO-1 expression in RBA-1 cells. Each *solid line* and *arrow* represents a step in an activating pathway. BK binds to B₂BKR and activates ERK (minor part) and JNK (major part) through Nox/ROS-dependent pathway to induce nuclear translocation and activation of NF-κB and c-Jun phosphorylation, respectively. Activation of NF-κB and c-Jun lead to sequential upregulation and activation of c-Fos/AP-1 and then induced HO-1 gene expression and its activity in RBA-1 cells. Moreover, the ROS-dependent Nrf2 signaling is also involved in these responses. AP-1, activator protein 1; BK, bradykinin; B₂BKR, B₂ BK receptor; ERK, extracellular signal-regulated kinase; HO-1, heme oxygenase-1; JNK, c-Jun N-terminal kinase; NAC, N-acetyl-cysteine; NF-κB, nuclear factor-κB; Nox, NADPH oxidase; Nrf2, NF-E2-related factor 2; RBA-1, rat brain astrocytes; ROS, reactive oxygen species.

responses (17, 28, 49). In the brain, ROS also extends to the control of vascular tone, which is tightly modulated by metabolic activity within neurons (14). Moreover, increasing oxidative stress (*i.e.*, ROS production) by diverse stimuli can regulate the expression of inflammatory genes such as COX-2 linked to pathogenesis of human CNS disorders (5, 17, 25, 49). Recently, increasing evidence attributes the cellular damage in neurodegenerative disorders such as AD to oxidative stress, which is due to generation of free radicals implicated in brain inflammatory disorders (6, 19, 32). The effects of BK on ROS generation have been reported in cerebral arterioles and cardiac and renal diseases (5, 9, 13). In this study, we found that BK-induced HO-1 expression was mediated through Nox-dependent ROS generation, because pretreatment with ROS scavenger NAC or Nox activity inhibitor DPI attenuated BK-induced responses (Fig. 2A, B). The involvement of Nox-dependent ROS in BK-induced HO-1 expression was further confirmed by ROS generation, which was also inhibited by pretreatment with Hoe140, NAC, or DPI (Fig. 2C, D), suggesting that B₂BKR/Nox-ROS is involved in BK-induced HO-1 expression in RBA-1 cells. Consistently, many reports have also shown that ROS are a major signaling factor that mediates microglial activation induced by inflammatory mediators, including A β and LPS (30, 39). Herein we are the first group to establish that intracellular ROS generation contributes to upregulation of HO-1 induced by BK in brain astrocytes.

Moreover, Nox are considered to be a major source of ROS in several physiological and pathological processes (10, 25). To date, five Nox isotypes have been discovered, including Nox1–5 (25). Several reports have shown that Nox1, Nox2, and Nox4 are expressed in brain cells (10, 25), suggesting that these Nox isotypes may be crucial to ROS generation in the brain. However, the role of these enzymes in the upregulation of brain astrocytic HO-1 remains unclear. First, our data have demonstrated that Nox activity is involved in these responses by pretreatment with a Nox inhibitor DPI (Fig. 2). Next, the data of knockdown distinct Nox by transfection with their respective siRNA indicated that BK-induced ROS generation and HO-1 expression were mediated through activation of Nox, including Nox1 and Nox2, in RBA-1 cells and primary culture astrocytes (Fig. 2E, F). These results are consistent with previous studies showing that Nox is expressed in astrocytes and contributes to ROS generation (1, 33). Additionally, we also suggested that BK-stimulated Nox/ROS signaling might be mediated through PKC or Ca²⁺ signals, confirmed by pretreatment with pan-PKC inhibitor (GF109203X) or intracellular Ca²⁺ chelator BAPTA/AM (data not shown). The regulatory mechanisms will be investigated in detail in our future research.

Abnormal MAPK regulation might be implicated in CNS inflammation and injury (4, 26). Moreover, BK has been reported to act as an important inflammatory mediator through activation of MAPK cascades in different cell types (21, 29, 53). In the present study, we demonstrated that ERK1/2 and JNK1/2, but not p38 MAPK, are essentially required for HO-1 induction by BK, because BK-induced responses were attenuated by pretreatment with respective MAPK selective pharmacological inhibitors including MEK1/2 (PD98059) or JNK (SP600125) (Fig. 3A, B). The involvement of these two kinases in BK-induced HO-1 expression was further confirmed by BK-stimulated phosphorylation of ERK1/2 and

JNK1/2 (Fig. 3C). These results are consistent with the HO-1 expression mediated through ERK1/2 and JNK1/2 induced by ARE in HepG2 cells (52) and heme-mediated neuronal injury (4). Moreover, pretreatment with ROS scavenger NAC significantly attenuated the phosphorylation of ERK1/2 and JNK/c-Jun stimulated by BK (Fig. 3D), suggesting that increased ROS are crucial for BK-stimulated ERK1/2 and JNK1/2 phosphorylation in RBA-1 cells. These results are consistent with the reports showing that ROS-dependent activation of MAPKs, including ERK1/2 and JNK1/2, is involved in the regulation of cellular functions by diverse stimuli in several cell types (8, 37). The distinct groups of MAPKs are activated by a ROS-dependent manner, which is cell type-specific and dependent on the external stimuli.

The progressive increase of oxidative stress during injuries not only causes oxidative damage to cellular macromolecules, but also modulates the pattern of gene expression through functional alterations of transcription factors. Here we focus on the roles of transcription factors (*i.e.*, NF- κ B and AP-1), which are well known to be modulated during oxidative stress associated with inflammatory diseases (45). Moreover, several reports have shown that BK-induced gene expression is mediated through several transcription factors such as NF- κ B (23) and AP-1 (53). However, the mechanistic connection between the HO-1 expression induced by BK and the ROS-dependent pathway has not been established in RBA-1 cells. Here, we demonstrated that NF- κ B was essential for BK-induced HO-1 expression, which was inhibited by pretreatment with an NF- κ B inhibitor Bay11-7082 (Fig. 4A, B). Moreover, the involvement of NF- κ B in BK-induced responses was further ascertained by NF- κ B phosphorylation, translocation into nucleus, and promoter activity determined using Western blot, cell fraction isolation, and promoter reporter analyses (Fig. 4). These responses were significantly attenuated by pretreatment with the inhibitor of NF- κ B (Bay11-7082), MEK1/2 (PD98059), or a ROS scavenger (NAC), but not JNK inhibitor (SP600125). These results suggested that the ROS/ERK cascade, but not JNK, is involved in NF- κ B signaling, which contributes to BK-induced HO-1 expression in RBA-1 cells. In addition, AP-1 was required for BK-induced HO-1 expression, which was attenuated by pretreatment with two AP-1 inhibitors curcumin or TSA (Fig. 5A, B). These results are consistent with the finding that AP-1 plays a key role in the HO-1 upregulation by arsenite in murine embryonic fibroblasts (20).

The involvement of AP-1 in these responses was further supported by the results indicating that BK induced c-Fos, an AP-1 subunit, expression and activation *via* ROS-dependent ERK/NF- κ B and JNK/c-Jun pathways, as determined using RT-PCR and promoter reporter analyses (Fig. 6). Moreover, the role of c-Fos/AP-1 in BK-induced HO-1 expression was confirmed by transfection with c-Fos shRNA to knock down c-Fos protein and to attenuate BK-induced HO-1 expression (Fig. 6D). Several lines of evidence have shown that the 5' region of HO-1 promoter contains a variety of putative regulatory elements, including AP-1 and NF- κ B sites, in several animal species (2, 3). Thus, we also demonstrated that BK-stimulated c-Fos/AP-1 was recruited to an HO-1 promoter by a ChIP-PCR assay, which was reduced by pretreatment with Hoe140 or NAC, suggesting that BK induces c-Fos/AP-1 recruitment *via* a ROS-dependent manner (Fig. 7A). Next, we found that BK stimulated HO-1 promoter activity, which was

attenuated by Hoe140, NAC, PD98059, SP600125, Bay11-7082, curcumin, or TSIIA (Fig. 7B), indicating that BK induced HO-1 promoter activity *via* a ROS-dependent pathway. Similarly, the HO enzymatic activity is stimulated by BK through the same pathway (Fig. 7C). These results are consistent with the reports indicating that angiotensin II regulates a variety of gene expression including HO-1 through a ROS-dependent activation of NF- κ B in the heart (13), and alpha-lipoic acid induces HO-1 expression through the production of ROS and subsequent activation of the p44/42 MAPK pathway and AP-1 in vascular smooth muscle cells (8). In addition to AP-1, a well-known redox-related transcription factor Nrf2 has been shown to contribute to the regulation of several antioxidant and cytoprotective genes including HO-1 (2, 3). In this study, we first demonstrated that BK can stimulate Nrf2 translocation *via* a ROS-dependent manner (Fig. 8A), which subsequently results in HO-1 upregulation in brain astrocytes (Fig. 8B). These findings are the first evidence to demonstrate that BK induced HO-1 expression *via* a ROS-dependent Nrf2 pathway in astrocytes.

In conclusion, we have demonstrated that BK directly induces HO-1 expression *via* B₂BKR and ROS-dependent activation of JNK/c-Jun and ERK/NF- κ B, linking to upregulation of c-Fos/AP-1, which results in the promotion of HO enzymatic activity in RBA-1 cells. Moreover, ROS-dependent Nrf2 signaling also plays a crucial role in BK-mediated HO-1 expression in astrocytes. On the basis of the observations from literatures and our findings, Figure 8C depicts a model for the molecular mechanisms underlying BK-induced HO-1 expression and activity in RBA-1 cells. These findings concerning BK-induced HO-1 expression and activity in brain astrocytes imply that BK might play a critical role in the modulation of brain injuries and diseases. Pharmacological approaches suggest that targeting HO-1 and their upstream signaling components may provide useful therapeutic strategies for brain injury, tumor, and inflammatory diseases.

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Author Disclosure Statement

No competing financial interests exist.

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Abbreviations Used

AD = Alzheimer's disease
AP-1 = activator protein 1
ARE = antioxidant response element
B₂BKR = B₂ BK receptor
BCA = bicinchoninic acid
BK = bradykinin
BSA = bovine serum albumin
c-Fos shRNA = short hairpin RNA of c-Fos
ChIP = chromatin immunoprecipitation
CNS = central nervous system
CoPP IX = cobalt protoporphyrin IX
DCF-DA = 2',7'-dichlorodihydrofluorescein diacetate
DMEM/F-12 = Dulbecco's modified Eagle's medium/
Ham's nutrient mixture F-12
DPI = diphenyleneiodonium
ECL = enhanced chemiluminescence
ERK = extracellular signal-regulated kinase
GAPDH = glyceraldehyde 3-phosphate dehydrogenase
GFAP = glial fibrillary acid protein
H₂O₂ = hydrogen peroxide
HO-1 = heme oxygenase-1
Hoe140 = D-Arg-[Hyp³, Thi⁵, D-Tic⁷, Oic⁸]-BK
JNK = c-Jun N-terminal kinase
LPS = lipopolysaccharide
MAPK = mitogen-activated protein kinase
NAC = N-acetyl cysteine
NF- κ B = nuclear factor- κ B
Nox = NADPH oxidase
Nrf2 = NF-E2-related factor 2
PBS = phosphate-buffered saline
PD = Parkinson's disease
PKC = protein kinase C
RBA-1 = rat brain astrocytes
ROS = reactive oxygen species
RT-PCR = reverse transcription-polymerase chain
reaction
TSIIA = tanshinone IIA

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