Molecular Neurobiology (2019) 56:736–747
https://doi.org/10.1007/s12035-018-1125-6

Neuroprotective Effects of Dehydroepiandrosterone Sulfate Through Inhibiting Expression of Matrix Metalloproteinase-9 from Bradykinin-Challenged Astroglia

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Received: 13 January 2018 / Accepted: 11 May 2018 / Published online: 23 May 2018

Abstract

Dehydroepiandrosterone sulfate (DHEAS), one of the most important neuroactive steroids, is produced in the adrenals and the brain. DHEAS is believed to play a critical role in modulating different forms of cellular control, including processes associated with human neural systems. Its production rate and level in serum, adrenals, and brain gradually decrease with advancing age. The decline of DHEAS level was associated with age-related neuronal dysfunction and degeneration, most probably because the steroids protect the central nervous system (CNS) neurons against neurotoxic challenges. Moreover, increasing studies show that matrix metalloproteinases (MMPs), MMP-9 especially, are upregulated by proinflammatory mediators in the CNS disorders. The increased MMP-9 as an inflammatory biomarker of several CNS disorders that may participate in the CNS inflammation and neurodegeneration. Herein, we investigate the effects of DHEAS on brain inflammation by the model we have defined of bradykinin (BK)-induced MMP-9 expression in rat brain astrocyte (RBA) and its mechanism. The results showed that DHEAS significantly reduce MMP-9 induced by BK. Pretreatment with DHEAS can inhibit BK-stimulated phosphorylation of c-Src and PYK2. Moreover, DHEAS attenuated BK-stimulated NADPH oxidase (Nox)-derived reactive oxygen species (ROS) production, suggesting that DHEAS has an antioxidative effect. We further demonstrated that DHEAS blocked activation of ERK1/2, Akt, and c-Fos/AP-1 by BK. Finally, DHEAS decreased MMP-9-related events including RBA migration and neuronal apoptosis. The results will provide new insights into the anti-inflammatory action of DHEAS, supporting that DHEAS may have a neuroprotective effect in the improvement of the CNS disorders by reducing neuroinflammation.

Keywords Dehydroepiandrosterone sulfate · Neuroprotection · Anti-inflammation · Bradykinin · Matrix metalloproteinase-9 · Brain astrocytes

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Introduction

Dehydroepiandrosterone sulfate (DHEAS) is the sulfated metabolite of DHEA and one of the most important natural and neuroactive steroids. It is produced in the adrenals and the brain. In human plasma, DHEA concentrations average 10 nmol/L, while DHEAS concentrations reach 10 μmol/L in young adults 20–35 years of age [1]. The production rate and levels of DHEA and DHEAS in serum, adrenals, and brain decrease gradually with advancing age. At age 80, DHEAS levels are only about 20% those at age 25, unlike the age-independent plasma levels of cortisol [2]. The decline of its level was associated with age-related neuronal dysfunction and degeneration, most probably because these steroids protect central nervous system (CNS) neurons against noxious agents [3]. Moreover, DHEAS is the major circulating steroid in human which is known for its clinical effects on hypertension, memory disorders, and cancer

prevention [4]. In the CNS, DHEAS plays an important regulatory role in brain functions. Moreover, increasing evidence shows that DHEAS also has profound psychotropic effects, such as anxiolytic effects in animal experiments [5]. These findings suggest that DHEAS may act as endogenous neuroprotective factors. Here, we investigate and evaluate the effects of DHEAS on anti-brain inflammation and neuroprotection.

The primary role of steroids is to modulate genetic transcription through binding to their intracellular receptors, which consequently elicit different physiological responses. Many reports have indicated that some neurosteroids such as DHEAS may act on metabotropic sigma receptors like sigma-1 receptor, which coupling to Gi protein [6, 7]. Although DHEAS has well characterized effects on memory and cognitive performances [8], little is known about the underlying molecular mechanisms. Moreover, DHEAS is able to increase the immunoendocrine production of neuroprotective growth factors (e.g., IGF and VEGF), which is reduced in AD subjects, suggesting a new approach in the treatment of dementia [9]. DHEAS can prevent the toxicity of free radical and serum free culture insults by suppressing the generation of lipid peroxide and increasing the activities of antioxidant enzymes [10]. The neuroprotective effects of DHEAS may be depending on PI3K/Akt and ERK/MAPK signaling pathways [11, 12]. In brain glial cells, DHEAS can inhibit TNF production in brain astrocytic and microglial cells [13]. While DHEAS can function to protect neural system, the molecular mechanisms underlying the neuroprotective effects of DHEAS remain undefined.

Matrix metalloproteinases (MMPs), a large family of zinc-dependent endopeptidases, are a crucial molecule for the turnover of extracellular matrix (ECM) and pathophysiological processes [14]. In the CNS, MMPs, MMP-9 especially, have been demonstrated to participate in morphogenesis, wounding healing, and neurite outgrowth [15]. Expression of MMP-9 has been up-regulated by various brain injuries, which may contribute to the pathogenesis of brain diseases [16]. Moreover, cytokines and lipopolysaccharide (LPS) have been indicated to enhance MMP-9 expression and activity in culture rat brain astrocytes [17, 18]. These studies demonstrated that MMP-9 may be involved in brain inflammation and injury. Our previous studies have demonstrated that several pro-inflammatory mediators like bradykinin (BK) can induce MMP-9 expression in brain astrocytes [19]. Here, we used the model in RBA cells to evaluate the effects of DHEAS on BK-induced MMP-9 expression and MMP-9-related events such as cell migration and neuronal cell death.

Reactive oxygen species (ROS) are produced by various enzymatic and chemical processes or directly inhaled. The ROS at low level have physiological roles as signaling molecules in various cellular and developmental processes [20, 21] and killing of invading microorganisms [22]. In contrast, recent report indicated that oxidative stress plays an important role in the progression of various diseases [22]. Moreover, ROS has been shown to interact with cellular components such as DNA that lead to cellular dysfunctions and inflammatory responses [21, 23]. Under pathological conditions, many proinflammatory factors (e.g., bradykinin) induce expression of several inflammatory genes during brain injury by enhancing ROS production [21, 24]. Recently, increasing evidence attributes the neurodegenerative diseases like Alzheimer's disease (AD) to oxidative stress (production of free radicals) that leads to brain inflammation during CNS pathogenesis [21, 24, 25]. Moreover, ROS also exert as a signaling factor mediated microglial activation induced by several proinflammatory factors [26]. The effects of BK associated with ROS generation have been indicated in several organ disorders [27]. Our recent study showed that BK induces ROS-mediated MMP-9 responses in rat brain astrocytes [28].

Based on these reports and our previous studies in the brain inflammatory responses by BK [29, 30], the experiments were performed to investigate the effects and molecular mechanisms of DHEAS on BK-induced MMP-9 expression in brain astrocytes (RBA cells). In the study, we found that DHEAS decreased BK-induced MMP-9 expression. Subsequently, BK-stimulated phosphorylation of protein kinases (e.g., c-Src and PYK2) also been inhibited by DHEAS. Moreover, DHEAS reduced BK-stimulated Nox/ROS-dependent activation of MAPKs (e.g., ERK1/2) cascade in RBA cells. Finally, we further demonstrated that DHEAS also blocked MMP-9-related events including RBA migration and neuronal apoptosis by BK. These results suggested that DHEAS may have an anti-oxidative, anti-inflammatory, and neuroprotective action in the CNS.

Experimental Procedures

Materials

Dulbecco's modified Eagle's medium (DMEM)/F-12 medium, fetal bovine serum (FBS), and TRIzol were from Invitrogen (Carlsbad, CA). Hybond C membrane and enhanced chemiluminescence (ECL) Western blot detection system were from GE Healthcare Biosciences (Buckinghamshire, UK). Phospho-c-Src (Tyr^{416}) (#6943) antibodies were from Cell Signaling (Danver, MA). Phospho-PYK2 (Tyr^{402}) (sc-101790), Phospho-ERK1/2 (Tyr^{204}) (sc-7383), Phospho-Akt (Ser^{473}) (sc-81433), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were from Santa Cruz (Santa Cruz, CA). Bicinchoninic acid (BCA) protein assay reagent was from Pierce (Rockford, IL). DHEAS, BK, enzymes, and other chemicals were from Sigma (St. Louis, MO). SDS-PAGE reagents were from MDBio, Inc. (Taipei, Taiwan).

Cell Cultures and Treatments

The rat brain astrocytic cell line (RBA, CTX TNA2) was purchased from BCRC (Hsinchu, Taiwan) and used throughout this study. Cells were plated onto 12-well culture plates and made quiescent at confluence by incubation in serum-free DMEM/F-12 for 24 h, and then incubated with BK (10 nM) at 37 °C for the indicated time intervals. When DHEAS (0.01–1 mM) or the inhibitors were used, cells were pretreated with DHEAS or the inhibitor for 1 h before exposure to BK. Treatment of RBA with DHEAS (1 mM) alone had no significant effect on cell viability determined by an XTT assay (data not shown). The SK-N-SH cells, a human neuroblastoma cell line, were purchased from American Type Culture Collection (Manassas, VA) and cultured in DMEM/F-12 supplemented with 10% FBS [31].

MMP Gelatin Zymography

Growth-arrested cells were incubated with BK for the indicated time intervals. After treatment, the cultured media were collected and analyzed by gelatin zymography [29]. Gelatinolytic activity was manifested as horizontal white bands on a blue background. Because cleaved MMPs were not reliably detectable, only pro-form zymogens were quantified.

Total RNA Extraction and Real-Time PCR Analysis

Total RNA was extracted from RBA cells [29]. The cDNA obtained from 0.5 μg total RNA was used as a template for PCR amplification. Oligonucleotide primers were designed on the basis of GenBank entries for rat MMP-9 and GAPDH. The primers were as follows:

MMP-9: 5′-AGTTTGGTGTCGCGGAGCAC-3′ (sense).

5′-TACATGAGCGCTTCCGGCAC-3′ (antisense).

GAPDH: 5′-(AACTTTGGCATCGTGGAAGG)-3′ (sense).

5′-(GTGGATGCAGGGATGATGTTC)-3′ (anti-sense).

The amplification was performed in 30 cycles at 55 °C, 30 s; 72 °C, 1 min; 94 °C, 30 s. PCR fragments were analyzed on 2% agarose 1× TAE gel containing ethidium bromide and their size was compared with a molecular weight markers. Amplification of β-actin, a relatively invariant internal reference RNA, was performed in parallel, and cDNA amounts were standardized to equivalent β-actin mRNA levels. The image densitometry analysis was quantified by an UN-SCAN-IT gel 6.1 software (Orem, UT).

Preparation of Cell Extracts and Western Blot Analysis

Growth-arrested cells were incubated with BK at 37 °C for the indicated time intervals. The cells were washed with ice-cold phosphate-buffered saline (PBS), scraped, and collected by centrifugation at 45,000×*g* for 1 h at 4 °C to yield the whole cell extract, as previously described [30]. Samples were analyzed by Western blot, transferred to nitrocellulose membrane, and then incubated overnight using a 1:1000 dilution (100 ng/ml) of anti-phospho-c-Src, phospho-PYK2, phospho-ERK1/2, phospho-Akt, or GAPDH antibody. Membranes were washed four times with TTBS for 5 min each, incubated with a 1:2000 dilution of anti-rabbit horseradish peroxidase antibody for 1 h. The immunoreactive bands were detected by ECL reagents and captured by a UVP BioSpectrum 500 Imaging System (Upland, CA). The image densitometry analysis was quantified by an UN-SCAN-IT gel 6.1 software (Orem, UT).

Measurement of Intracellular ROS Generation

The peroxide-sensitive fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCF-DA) was used to assess the generation of intracellular ROS [32] with minor modifications. RBA cells on monolayers were incubated with 5 μM DCF-DA in RPMI-1640 at 37 °C for 45 min. The supernatant was removed and replaced with fresh RPMI-1640 medium before exposure to DHEAS or BK (10 nM). Relative fluorescence intensity was recorded at the indicated time 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.

Determination of NADPH Oxidase Activity by Chemiluminescence Assay

The Nox activity in intact cells was assayed by lucigenin chemiluminescence [33]. After incubation, the cells were gently scraped and centrifuged at 400×*g* for 10 min at 4 °C. The cell pellet was resuspended in a known volume (35 μl/well) of ice-cold RPMI 1640 medium, and the cell suspension was kept on ice. To a final 200 μl of pre-warmed (37 °C) RPMI-1640 medium containing either NADPH (1 μM) or lucigenin (20 μM), 5 μl of cell suspension (2×10^4 cells) was added to initiate the reaction followed by immediate measurement of chemiluminescence using an Appliskan luminometer (Thermo®) in an out-of-coincidence mode. Appropriate blanks and controls were established, and chemiluminescence was recorded. Neither NADPH nor NADH enhanced the background chemiluminescence of lucigenin alone (30–40 cpm). Chemiluminescence was continuously measured for 12 min, and the activity of Nox was expressed as counts per million cells.

Chromatin Immunoprecipitation Assay

To detect the in vivo association of nuclear proteins with rat MMP-9 promoter, chromatin immunoprecipitation (ChIP) analysis was conducted as previously described [30]. Briefly, RBA cells were cross-linked with 1% formaldehyde for 10 min at 37 °C and washed thrice with ice-cold PBS containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1% aprotinin. Soluble chromatin was prepared using a ChIP assay kit (Upstate) according to the manufacturer's recommendations and immunoprecipitated without (control) or with anti-c-Fos and normal goat immunoglobulin G (IgG). Following washes and elution, precipitates were heated overnight at 65 °C to reverse cross-linking of DNA and protein. DNA fragments were purified by phenol-chloroform extraction and ethanol precipitation. The purified DNA was subjected to PCR amplification using the primers specific for the region (− 597 to − 318) containing the distal AP-1 binding site (− 503 to − 497) present in the MMP-9 promoter region, sense primer: 5′-AGAGCCTGCTCCCAGAGGGC-3′; antisense primer: 5′-GCCAAGTCAGGCAGGACCCC-3′. PCR fragments were analyzed on 2% agarose in 1× TAE gel containing ethidium bromide and the size (279 bp) was compared to a molecular weight marker.

Plasmid Construction, Transfection, and Luciferase Reporter Gene Assays

The upstream region (− 1280 to + 19) of the rat MMP-9 promoter was cloned to the pGL3-basic vector containing the luciferase reporter system [30]. All plasmids were prepared by using QIAGEN plasmid DNA preparation kits. These constructs were transfected into RBA cells by using a Lipofectamine reagent according to the instructions of manufacture. The transfection efficiency (~ 60%) was determined by transfection with enhanced GFP. After incubation with BK, cells were collected and disrupted by sonication in lysis buffer (25 mM Tris, pH 7.8, 2 mM EDTA, 1% Triton X-100, and 10% glycerol). After centrifugation, aliquots of the supernatants were tested for promoter activity using a luciferase assay system (Promega, Madison, WI). Firefly luciferase activities were standardized for β-galactosidase activity.

Preparation of BK-Challenged Astrocytic-Conditioned Culture Medium

For collection of conditioned media, RBA cells were plated and incubated with BK for 24 h (BK-CM). Cell-free supernatant fractions were applied to human neuroblastoma SK-N-SH cells to evaluate the changes in cell viability and related parameters in the study.

Cytotoxicity Assay

Cells were grown in 24-well culture plates at a concentration of 5×10^4 cells/well followed by treatment. Five-microliter Cell Counting Kit-8 (CCK-8) solution (Sigma) in 500-μl growth medium was added to each well. After incubation with CCK-8 at 37 °C for 2 h, cultured medium was collected. The water-soluble tetrazolium salt, a product of CCK-8 by the action of mitochondrial dehydrogenases, was solubilized in cultured medium and quantified spectrophotometrically at 450 nm, reference at 650 nm.

Apoptosis Assay

Hoechst 33342 staining was performed as previously described [31], with some modifications. In brief, attached cells were washed with PBS and stained with 1 μg/ml Hoechst 33342 in the dark for 10 min. Following rinsing with PBS and mounting on glass slides, at least 300 cells per condition were observed using a fluorescence microscope. The two cell populations (normal and apoptotic cells) were counted for Hoechst 33342 staining with chromatin. The Hoechst 33342, a kind of blue fluorescent dye, which stains the condensed chromatin in apoptotic cells, was brighter than that of normal cells. Apoptotic cells were also identified on the basis of morphology and condensation and fragmentation of nuclei.

Statistical Analysis of Data

All data were estimated using GraphPad Prism 5 Program (GraphPad, San Diego, CA). Quantitative data were analyzed by one-way ANOVA followed by Tukey's honestly significant difference tests between individual groups. Data were expressed as mean ± SEM. A value of $P < 0.05$ was considered significant.

Results

Effects of DHEAS on BK-Induced MMP-9 Expression in Rat Brain Astrocytes

Our previous study has indicated that BK can up-regulate MMP-9 expression in brain astrocytes [29]. First, to evaluate whether DHEAS reduces BK-induced MMP-9 expression in RBA cells, cells were pretreated with DHEAS (1 mM) and then incubated with BK (10 nM) for the indicated time intervals. As shown in Fig. 1a, pretreatment with DHEAS significantly reduced (~ 65.8% at 24 h) BK-induced MMP-9 expression by the gelatin zymography. Pretreatment with DHEAS also inhibited (~ 60.8% at 24 h) BK-induced MMP-9 protein expression by Western blot analysis (Fig. 1b). Moreover, pretreated with various concentrations of DHEAS

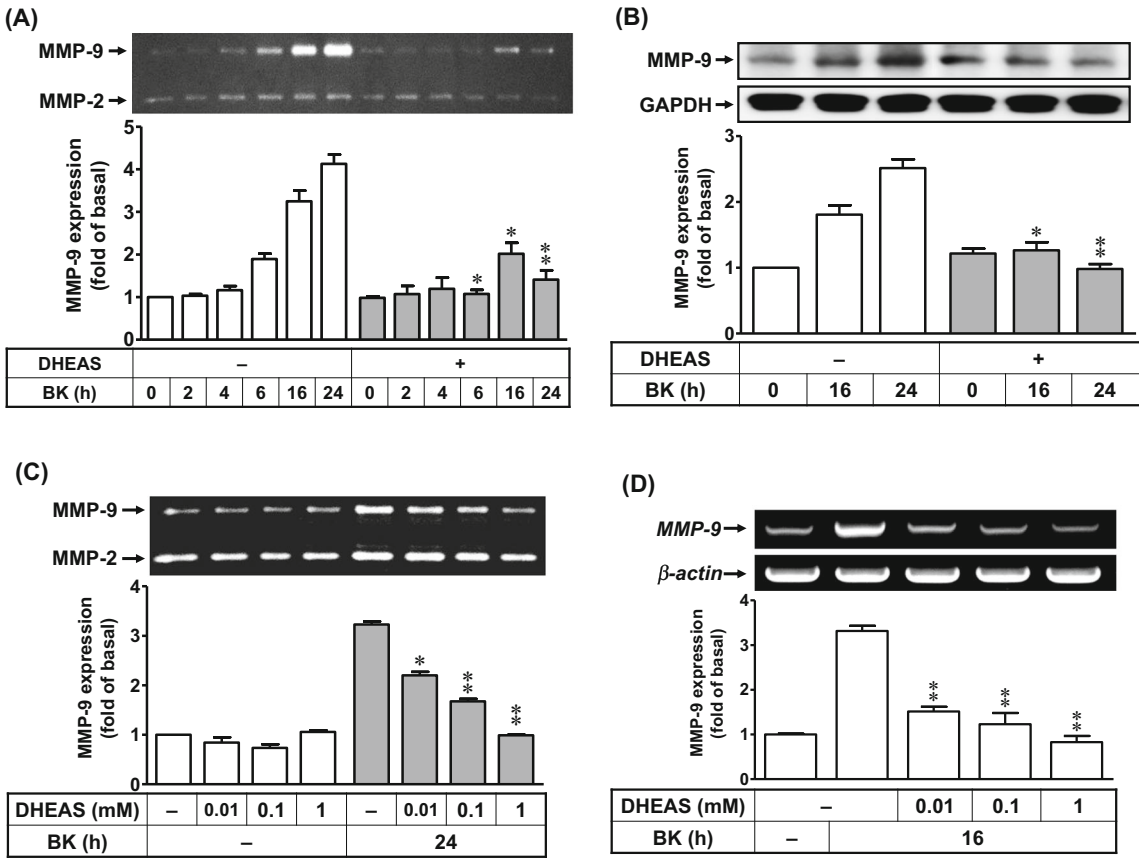

Fig. 1 Effect of DHEAS on BK-induced MMP-9 expression in rat brain astrocytes (RBA). **a**, **b** Time dependence of DHEAS inhibited BK-induced MMP-9 activity and expression, cells were pretreated with or without DHEAS (1 mM) and then incubated with BK (10 nM) for the indicated time intervals. **c** Cells were pretreated with various concentrations of DHEAS (0.01, 0.1, and 1 mM) and then incubated with BK (10 nM) for 24 h. The conditioned media were collected and assayed for MMP-9 expression and activity by gelatin zymography (**a**, **c**) and Western blot (**b**). **d** DHEAS concentration-dependently inhibited BK-induced MMP-9 mRNA expression. The MMP-9 mRNA was analyzed by RT-PCR as described in Methods. Data are expressed as the mean ± SEM ($N = 3$). $^{*}P < 0.05$; $^{**}P < 0.01$, as compared with the respective values of cells stimulated with BK only. The figure represents one of three individual experiments

(0.01, 0.1, or 1 mM) attenuated (~ 32.9, ~ 49.1, and ~ 70.1%) BK-induced MMP-9 expression in a concentration-dependent manner by the gelatin zymography (Fig. 1c). Next, to check whether DHEAS affect the BK-induced MMP-9 mRNA expression in RBA cells, cells were pretreated with various concentrations of DHEAS (0.01, 0.1, or 1 mM) for 1 h and then treated with BK for 16 h. The data showed that DHEAS concentration-dependently blocked BK-induced MMP-9 mRNA expression by RT-PCR analysis (Fig. 1d, ~ 54.5, ~ 63.6, and ~ 75.8%). These results demonstrated that DHEAS can repress BK-induced MMP-9 protein and mRNA expression in RBA cells.

DHEAS Reduces BK-Stimulated Phosphorylation of Protein Kinases, Including c-Src and PYK2 in RBA Cells

Several protein kinases, including c-Src and PYK2, have been shown to participate in MMP-9 expression in various cell types [34]. Our recent study has indicated that BK-stimulated several protein kinases activation in brain astrocytes, including c-Src [35]. Here, we investigated whether DHEAS attenuates BK-induced MMP-9 expression via blocking activation of these related protein kinases. The cells were pretreated with DHEAS (1 mM) for 1 h and then incubated with BK for the indicated times. As shown in Fig. 2, BK stimulated time-dependently phosphorylation of c-Src and PYK2 at 1–5 min in RBA cells, which were significantly inhibited (p-c-Src: ~ 23.3% at 3 min; p-PYK2: ~ 35.0% at 3 min) by pretreatment with DHEAS. The results suggested that in RBA, DHEAS-reduced BK-induced MMP-9 expression may be mediated through attenuating activation of several protein kinases (i.e., c-Src and PYK2)-dependent pathways.

Anti-Oxidative Effects of DHEAS on BK-Induced Nox-Dependent ROS Production

Recent report has indicated that ROS may contribute to MMP expression in various cell types [36]. The NADPH oxidase (Nox) is considered to be a major source of ROS in many

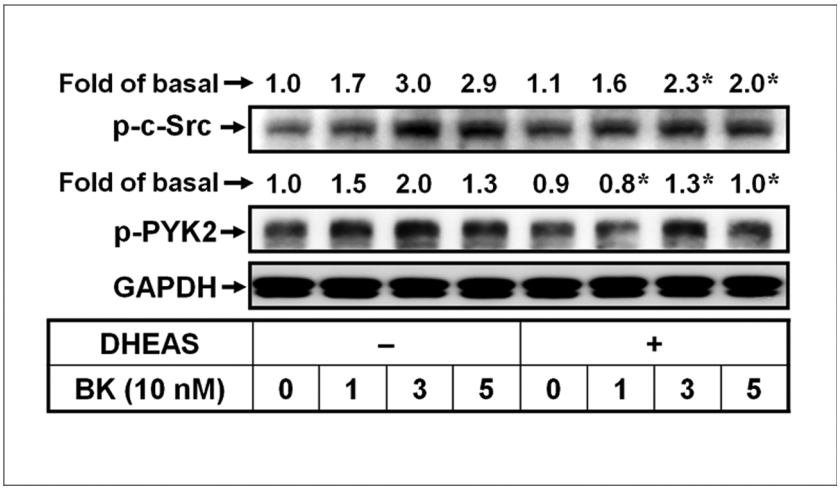

Fig. 2 DHEAS inhibits BK-stimulated phosphorylation of protein kinases, including c-Src and PYK2 in RBA cells. Cells were pretreated with or without DHEAS (1 mM) and then incubated with BK (10 nM) for the indicated time intervals. The cell lysates were collected and analyzed by Western blot as described in Methods. Data are expressed as the mean ± SEM ($N = 3$). $^{*}P < 0.05$; $^{**}P < 0.01$, as compared with the respective values of cells stimulated with BK only. The figure represents one of three individual experiments

physiological and pathological processes [22, 37]. Previous studies have demonstrated that Nox-derived ROS signaling cascade is involved in BK-induced MMP-9 expression in astrocytes [28]. Thus, to determine whether DHEAS-reduced MMP-9 induction by BK is due to decreasing Nox-dependent ROS production, the Nox activity and ROS production were detected. As shown in Fig. 3 (open bar), pretreatment with DHEAS (1 mM) markedly reduced (~ 51.5%) BK-stimulated Nox activity. Next, to observe whether DHEAS also influence on BK-increased ROS production, a ROS probe DCF-DA was used. The data showed that BK-stimulated ROS production was attenuated (~ 56.4%) by pretreatment with DHEAS (Fig. 3, gray bar). These data demonstrated that DHEAS can inhibit BK-stimulated Nox/ROS signal in RBA cells, suggesting that DHEAS may be possessed of anti-oxidative activity in the event.

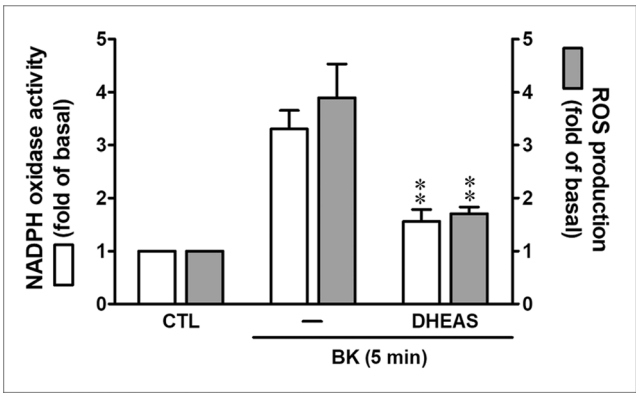

Fig. 3 Roles of DHEAS in BK-stimulated NADPH oxidase activity and ROS generation in RBA cells. Cells were pretreated with DHEAS (1 mM) and then incubated with BK (10 nM) for 5 min. The Nox activity (open bar) was analyzed as described in Methods. Moreover, cells were incubated with the DCF-DA (5 μM) for 45 min, followed by pretreatment with DHEAS (1 mM) and stimulation with BK for 5 min. The fluorescence intensity (gray bar: ROS generation) of cells was determined as described in Methods. Data are expressed as the mean ± SEM ($N = 3$). $^{**}P < 0.01$, as compared with the respective values of cells stimulated with BK only

DHEAS Attenuates BK-Stimulated ERK1/2 and Akt Activation in RBA Cells

Recently, the ROS-dependent activation of MAPKs has been indicated to participate in MMP-9 expression induced by various stimuli in brain astrocytes [38, 39]. Our previous data have also demonstrated that MAPKs such as ERK1/2 contribute to BK-induced MMP-9 expression in astrocytes [29]. Therefore, we further explained whether DHEAS affects the BK-stimulated activation of MAPKs, in particular ERK1/2, the anti-phospho-MAPKs, including ERK1/2, JNK1/2, and p38 MAPK antibodies were used. As shown in Fig. 4a, pretreatment with DHEAS (1 mM) attenuated BK-stimulated phosphorylation of ERK1/2 (~ 54.2% at 3 min), but not p38 MAPK and JNK1/2 (data not shown). Moreover, previous data also indicated that BK induces MMP-9 expression via Akt pathway in astrocytes [29]. Here, we also investigated the effect of DHEAS on BK-stimulated Akt phosphorylation by Western blotting. The result showed that pretreatment with DHEAS (1 mM) reduced (~ 66.7% at 3 min) BK-stimulated phosphorylation of Akt (Fig. 4b). These results suggested that DHEAS inhibits BK-induced MMP-9 expression that is mediated through suppressing activation of ERK1/2 and Akt in RBA cells.

Roles of DHEAS in BK-Stimulated Activation of Transcription Factors Such as AP-1

The AP-1-dependent pathways have been demonstrated to involve in MMP-9 expression in various cell types [40]. To determine whether DHEAS-reduced BK-induced MMP-9 gene expression is mediated through blocking up-regulation of transcription factor AP-1 (i.e., c-Fos), these signals were detected by RT-PCR analysis. The results showed that pretreatment of RBA with DHEAS significantly reduced BK-stimulated c-Fos/AP-1 gene expression (Fig. 5a). Moreover, previous studies reported that MMP-9 promoter region

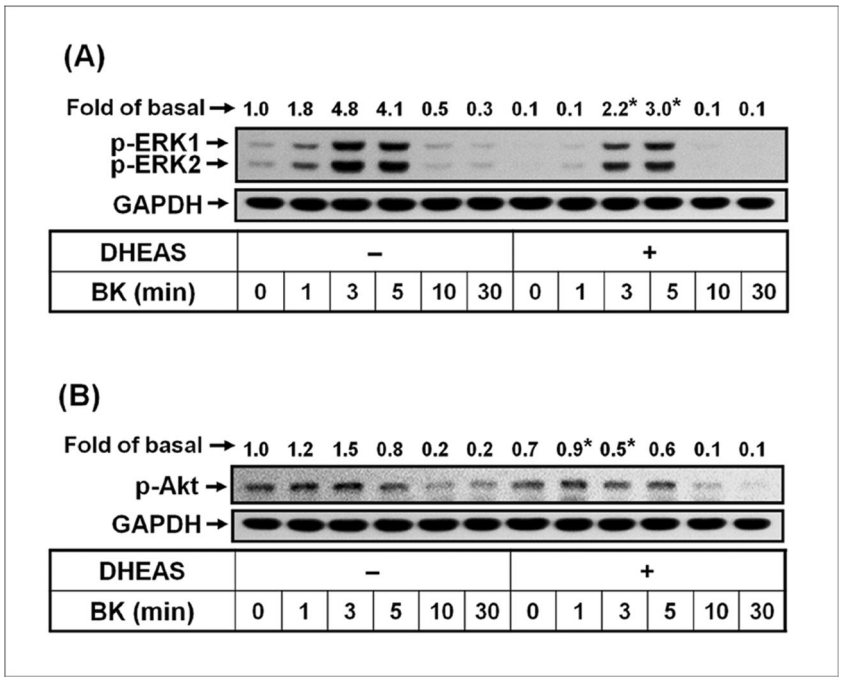

Fig. 4 DHEAS attenuates BK-stimulated activation of MAPKs (e.g., ERK1/2) and PI3K/Akt cascade in RBA cells. **a**, **b** Cells were pretreated with or without DHEAS (1 mM) and then incubated with BK (10 nM) for the indicated time intervals. The cell lysates were collected and analyzed phosphorylation of ERK1/2 (**a**) and Akt (**b**) by Western blot as described in Methods. Data are expressed as the mean ± SEM ($N = 3$). $^{*}P < 0.05$; $^{**}P < 0.01$, as compared with the respective values of cells stimulated with BK only. The figure represents one of three individual experiments

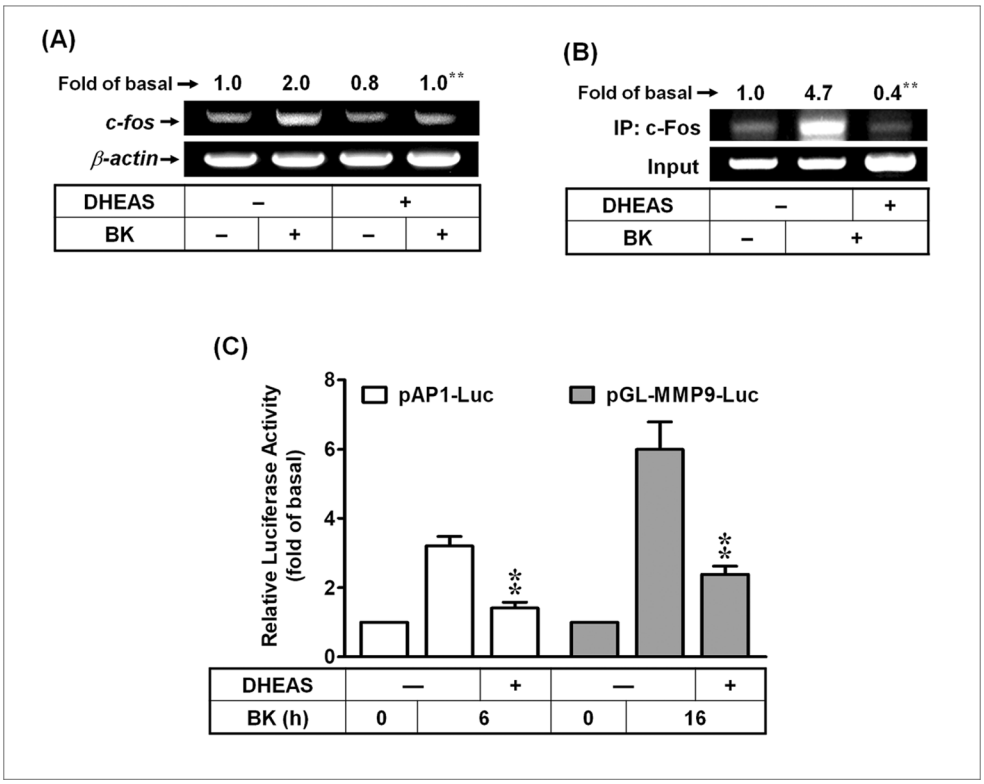

Fig. 5 DHEAS blocks BK-upregulated the transcription factor *c-fos* expression and the recruitment of c-Fos to the MMP-9 promoter in RBA cells. **a** Cells were pretreated with DHEAS (1 mM) and then incubated with BK (10 nM) for 15 min. The total RNA was collected and analyzed c-fos mRNA expression by RT-PCR analysis as described in Methods. **b** BK stimulated the recruitment of c-Fos to the MMP-9 promoter, cells were pretreated with DHEAS (1 mM) and then incubated with BK (10 nM) for 30 min. The ChIP-PCR assays were performed using an anti-c-Fos antibody. **c** Cells were transiently cotransfected with pAP1-Luc or pGL-MMP9-Luc and pGal for 24 h, pretreated with DHEAS for 1 h, and then incubated with BK for 6 or 16 h. After stimulation, luciferase activity of AP-1- or MMP-9-promoter construct were measured as relative promoter activity to that of β-galactosidase. Data are expressed as the mean ± SEM ($N = 3$). $^{*}P < 0.05$; $^{**}P < 0.01$, as compared with the respective values of cells stimulated with BK only. The figure represents one of three individual experiments

contains AP-1 binding sites [41]. Hence, we used ChIP-PCR assay to determine the effects of DHEAS on BK-stimulated recruitment of c-Fos/AP-1 to MMP-9 promoter. We designed a pair of primers for MMP-9 promoter (− 597 to − 318) region, containing an AP-1 binding site. Chromatin was immunoprecipitated using an anti-c-Fos antibody, and then, the MMP-9 promoter region (− 597 to − 318) was amplified by PCR. As shown in Fig. 5b, BK stimulated binding of c-Fos to the MMP-9 promoter at 30 min, which was blocked by pretreatment with DHEAS (1 mM), indicating that DHEAS repressed BK-enhanced recruitment of c-Fos/AP-1 to MMP-9 promoter in RBA cells. We next examined whether DHEAS also reduces BK-induced AP-1 and MMP-9 promoter activity, a promoter containing AP-1 binding sites (pAP1-Luc) and a rat MMP-9 promoter reporter constructs (pGL-MMP-9-Luc) were used [28, 41]. The data showed that pretreatment with DHEAS significantly attenuated BK-increased AP-1 (~ 54.8%) and MMP-9 (~ 60.0%) promoter activity (Fig. 5c), suggesting that DHEAS plays a suppressor in BK-induced MMP-9 expression via inhibiting c-Fos/AP-1-mediated MMP-9 transcription activity in RBA cells.

DHEAS May Play a Neuroprotective Role via Inhibiting BK-Induced MMP-9-Related Events

MMP-9 has been reported to be elevated in various brain injuries and participates in the pathogenesis of several CNS disorders. BK-induced MMP-9 increase has been shown to involve in astrocyte migration and neuronal cell apoptosis [30, 31]. Therefore, we further investigated the effects of DHEAS on BK-induced MMP-9-mediated cellular function changes, including the RBA cell migration and neuronal cell death. First, the images of RBA cell migration induced by BK (10 nM) were observed and taken at 48 h. Pretreatment with DHEAS (1 mM) significantly blocked (~ 57.9%) BK-induced RBA cell migration (Fig. 6a, upper panel). The number of migratory RBA cells was counted, and the statistical data are presented in Fig. 6a (lower panel). The results demonstrated that DHEAS can block BK-induced cell migration via reducing expression of MMP-9 in brain astrocytes.

Next, to investigate the effects of DHEAS on BK-challenged astroglial MMP-9-mediated neuronal cell viability, various astrocytic conditioned culture media were preparation and a human neuroblastoma SK-N-SH cell line was used. The procedure for the experiments is shown in Fig. 6b. First, the RBA cells were pretreated with or without DHEAS (1 mM) for 1 h and treated by BK (10 nM) for 24 h, and then, the conditioned media (CM) were collected as CTL-CM, BK-CM, and DHEAS/BK-CM for the following experiments. Subsequently, the SK-N-SH cells were incubated with CTL-CM, BK-CM, or DHEAS/BK-CM for 24 h, respectively, and the cell viability was detected by XTT assay. As shown in Fig. 6c, the cell viability of SK-N-SH was reduced about 40% (from 97 to 58%) by incubation with BK-CM. Compared to the result of BK-CM, treatment of cells with DHEAS/BK-CM was ineffective in the SK-N-SH cell viability (about 97%). Here, to demonstrate the effects of DHEAS on BK-challenged astroglial MMP-9-mediated neuronal cell apoptosis, the apoptotic events were observed and detected by a cell permeable blue-fluorescence DNA dye, Hoechst 33342 after treatment of SK-N-SH cells with CTL-CM, BK-CM, or DHEAS/BK-CM for 24 h. The statistical data of apoptotic nuclei counting are shown in Fig. 6d, indicating that BK-CM markedly induced SK-N-SH cell apoptosis about 35% (from 1 to 36%). Similarly, DHEAS/BK-CM has not significantly induced the SK-N-SH cell apoptosis (about 7%). These results suggested that the endogenous natural steroid DHEAS may play a critical role in anti-inflammation and neuroprotection through inhibiting up-regulation of MMP-9 from BK-challenged RBA cells.

Discussion

MMPs contribute to a wide range of biological activities in different tissues, including several CNS diseases, such as stroke, Alzheimer's disease, and malignant glioma [16]. Among MMPs, MMP-9 expression and activation play a critical role in tissue remodeling in the pathogenesis of brain diseases [16]. Reduction of MMP activity by pharmacological inhibitors or gene knock-out strategies protects the brain from advanced neuroinflammation [42]. These studies suggest that up-regulation of MMP-9 by pro-inflammatory factors may be a great effect upon brain inflammation and neurodegeneration. Moreover, BK and related peptides are simultaneously produced and released following brain injury [43]. Our previous data have demonstrated that BK induces MMP-9 expression in astrocytes which may change astrocytic functions such as cell motility and neuroinflammation [28, 31]. These findings imply that BK may play an important role in brain injury, astroglioma, or CNS diseases. Pharmacological and knockout-mouse approaches suggest that targeting MMP-9 and their upstream signaling pathways should yield useful therapeutic targets for brain injury and inflammation. Herein, we evaluate whether DHEAS possess anti-inflammatory and neuroprotective effects on BK-induced MMP-9 expression in brain astrocytes and its mechanism. In this study, we found that DHEAS is a water-soluble product, its maximum solubility of about 0.2 M, thus the highest concentration of this study used only to 1 mM. Moreover, because the collected conditioned medium is then used to treat neuronal cells, the conditioned medium is collected to maintain sterility throughout the process.

First, we found that DHEAS can inhibit BK-induced MMP-9 gene expression in RBA cells (Fig. 1). This result is the first finding that DHEAS can suppress MMP-9 up-

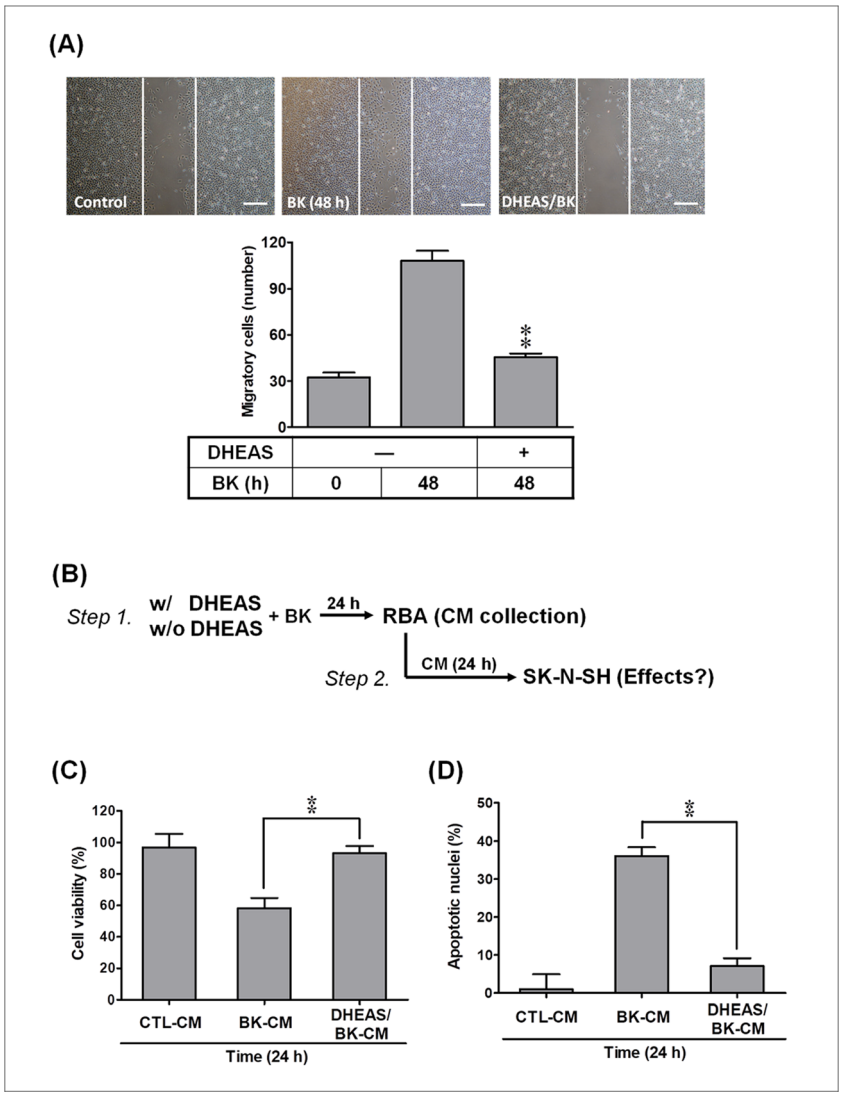

Fig. 6 Effects of DHEAS on BK-regulated MMP-9-related events, including cell migration or neuronal apoptosis. **a** Cells were plated on 6-well culture plates, grew to confluence, and starved with serum-free medium for 24 h. Cells were pretreated with DHEAS for 1 h and the monolayer cells were manually scratched with a blue tip as described in Methods, and then incubated with BK (10 nM) for 48 h. Phase contrast images of cells were taken at 48 h and the number of cell migration was counted as described in Methods (scale bar = 50 μm). **b**, **c** Cells were pretreated with or without MMP2/9 inhibitor (2/9i, 1 μM) or DHEAS (1 mM) for 1 h before exposure to BK-CM for 24 h. The cell viability was analyzed (**b**) and the number of apoptotic nuclei was counted (**c**), and the percentage was calculated as described in the Methods. Data are expressed as mean ± SEM of three independent experiments ($N = 3$). $^{**}P < 0.01$, as compared with the values of cells stimulated with BK (**a**) or BK-CM (**b**, **c**) alone

regulation by BK in brain astrocytes. Next, many reports and our previous data have indicated that several protein kinases (e.g., c-Src or PKCs) may contribute to various stimuli-induced MMP-9 expression in brain astrocytes [28, 30, 44]. Moreover, several reports also demonstrate that PYK2 is crucial for MMP-9 expression [35]. Thus, we investigated whether the inhibition of DHEAS is mediated through blocking the activation of protein kinase signals by BK in brain astrocytes. The results showed that pretreatment with DHEAS attenuated BK-stimulated phosphorylation of c-Src and PYK2 in RBA cells (Fig. 2), demonstrating that DHEAS may inhibit BK-induced MMP-9 expression via reducing protein kinases (i.e., c-Src and PYK2)-relative pathways in RBA cells.

Redox imbalance has been shown to play a causative role in numerous pathologies of degenerative diseases [25]. ROS concentration dependently exerts a key role in the normal physiological functions and the inflammatory responses [23]. In the brain, ROS also extend to the control of vascular tone which is tightly modulated by metabolic activity within neurons [24]. Moreover, increasing ROS generation by diverse stimuli can regulate the expression of inflammatory genes in pathogenesis of brain disorders [45]. Recently, the cellular damage in neurodegenerative disorders such as Alzheimer's disease (AD) is attributed to oxidative stress in brain inflammatory disorders [21, 25]. In astrocytes, our recent data have demonstrated that in both in vitro and in vivo studies, BK induced MMP-9 expression via Nox-dependent ROS generation in brain astrocytes [28]. In the study, we further demonstrated that DHEAS may have an anti-oxidative activity function (Fig. 3). Herein, we are the first group to establish that DHEAS reduce Nox/ROS signal induced by BK in brain astrocytes. The finding is consistent with previous studies that indicated that a DHEAS analog, DHEA, has an anti-oxidative effect on endothelial cells and Leydig cells [46, 47].

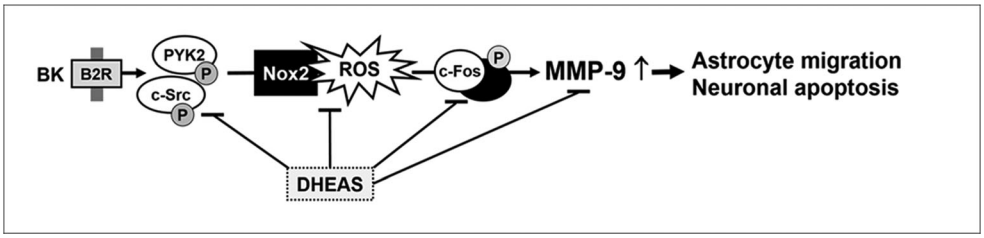

Fig. 7 Schematic presentation of the effects of DHEAS on the BK-induced MMP-9 expression and its related events. In brain astrocytes (RBA cells), BK induces ERK1/2 activation through c-Src/PYK2, and Nox-derived ROS signals resulting in c-Fos/AP-1-dependent MMP-9 expression. This result leads to RBA cell migration and neuronal cell death. The RBA-derived MMP-9-related events, including cell migration and neuronal apoptosis, were suppressed by DHEAS through reducing activation of c-Src/PYK2, Nox/ROS, ERK1/2, and c-Fos/AP-1 signaling pathways

Abnormal MAPK regulation might be implicated in several models of CNS inflammation and injury [18]. Moreover, BK has been reported to act as an important inflammatory mediator through activation of MAPK cascades in different cell types [48, 49]. Previously, we have demonstrated that MAPKs such as ERK were essentially required for BK-induced MMP-9 expression [28, 30]. Here, our data showed that DHEAS may reduce MMP-9 expression via inhibiting BK-stimulated ERK1/2 MAPKs activation in RBA cells (Fig. 4a). In addition to MAPKs, BK-stimulated activation of PKB/Akt has also been demonstrated in many cell types [50, 51]. Moreover, PKB/Akt has been linked to induction of MMPs such as MMP-9 [52]. The previous study has demonstrated that BK induces MMP-9 expression that is mediated through PI3K/Akt cascades in brain astrocytes [29]. Here, we found that DHEAS may inhibit MMP-9 expression through blocking BK-stimulated Akt activation in RBA cells (Fig. 4b). These findings are consistent with previous reports that showed that DHEAS can inhibit thrombin-dependent activation of Akt and ERK1/2 in platelet function [53]. Moreover, another study indicated that the antiviral activity of DHEA occurs via a mechanism independent of its ability to modulate ERK phosphorylation [54]. In contrast, the previous study showed that midazolam anesthesia protects neuronal cells from oxidative stress-induced death via activation of the MAPKs (JNK and ERK) pathway. [55]. These differences suggest that the nature of its effects may vary in a stimuli-dependent or cell-type-specific manner.

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. The transcription factors such as AP-1 play a key role in the regulation of several gene expressions including MMP-9 associated with physiological and pathological events [56]. In addition, several reports also indicate that AP-1 is involved in the pathogenesis of brain inflammation [40]. In the CNS, various stimuli (e.g., BK) can induce expression of several inflammatory mediators such as MMP-9 through ROS-mediated activation of AP-1 manner in astrocytes [40]. Recently, we have demonstrated that AP-1 participates in the expression of several genes including MMP-9 by BK through ROS-dependent manner [28]. These results implicate that AP-1 play a central role in regulating MMP-9 expression and lead to inflammatory gene expression in pathological events including the CNS inflammation. Therefore, we focus on the effects of DHEAS on BK-stimulated activation of these transcription factors (e.g., AP-1) in RBA cells. The results showed that BK-stimulated activation of AP-1 (c-Fos induction), recruitment of c-Fos/AP-1 to the MMP-9 promoter, and AP-1 and MMP-9 promoter activity were inhibited by DHEAS (Fig. 5a–c). These results suggested that DHEAS may alleviate up-regulation of MMP-9 by BK through inhibiting activation of the transcription factor AP-1 in brain astrocytes.

In conclusions, our previous study showed that BK directly induces MMP-9 expression via c-Src/PYK2-mediated Nox/ROS and ERK1/2 signals, linking to activation of c-Fos/AP-1, which results in the brain astrocytes (RBA cells) migration and neuronal cell (SK-N-SH) apoptosis. Based on the observations from literatures and our findings, Fig. 7 depicts a model for the inhibitory action of DHEAS on BK-induced MMP-9-dependent events, including RBA cell migration and neuronal cell apoptosis. Herein, the results showed that in brain astrocytes, DHEAS reduced BK-induced MMP-9-dependent astrocytic migration is mediated through inhibition of the protein kinases (e.g., c-Src and PYK2)-activated Nox/ROS and ERK1/2 signals leading to induction of c-Fos/AP-1 pathways. Moreover, DHEAS can also inhibit BK-induced MMP-9-mediated neuronal cell apoptosis via the same mechanism. These findings concerning the endogenous steroid DHEAS-reduced BK-induced MMP-9 expression in brain astrocytes imply that DHEAS may play a critical role in the anti-oxidative and anti-inflammatory properties which may contribute to its protective effects in several brain inflammatory disorders. Therefore, the inhibition of MMP-9-mediated inflammatory pathways by DHEAS may provide therapeutic strategies to brain inflammation and neurodegenerative diseases. In the future, the results may be applied to clinic by increase DHEAS levels in vivo by diet or giving DHEAS

directly to brain disordered patients with low DHEAS, which might reduce or treat the brain-related inflammatory disorders.

Acknowledgements This work was supported by the Ministry of Science and Technology, Taiwan; Grant number: NSC102-2320-B-255-005-MY3 and MOST106-2320-B-255-005; Chang Gung Medical Research Foundation, Grant number: CMRPF1C0193, CMRPF3D0033, CMRPF1F0131, and CMRPF1F0132. We thank Ms. Yin-Chen Chen and Shu-Ching Hsu for their technical assistance. We also thank Professor Ying-Tung Lau for his support.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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