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Endothelin-1 enhances cell migration via matrix metalloproteinase-9 up-regulation in brain astrocytes

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Abstract

The bioactivity of endothelin-1 (ET-1) has been suggested in the development of CNS diseases, including disturbance of water homeostasis and blood-brain barrier integrity. Recent studies suggest that hypoxic/ischemic injury of the brain induces release of ET-1, behaving through a G-protein coupled ET receptor family. The deleterious effects of ET-1 on astrocytes may aggravate brain inflammation. Increased plasma levels of matrix metalloproteinases (MMPs), in particular MMP-9, have been observed in patients with neuroinflammatory disorders. However, the detailed mechanisms underlying ET-1-induced MMP-9 expression remain unknown. In this study, the data obtained with zymographic, western blotting, real-time PCR, and immunofluorescent staining analyses showed that ET-1-induced MMP-9 expression was mediated through an ET_B-dependent transcriptional activation. Engagement of $G_{i/o}$- and G_q-coupled ET_B receptor by ET-1 led to activation of p42/p44 MAPK and then activated transcription factors including Ets-like kinase, nuclear factor-kappa B, and activator protein-1 (c-*Jun*/c-*Fos*). These activated transcription factors translocated into nucleus and bound to their corresponding binding sites in MMP-9 promoter, thereby turning on MMP-9 gene transcription. Eventually, up-regulation of MMP-9 by ET-1 enhanced the migration of astrocytes. Taken together, these results suggested that in astrocytes, activation of Ets-like kinase, nuclear factor-kappa B, and activator protein-1 by ET_B-dependent p42/p44 MAPK signaling is necessary for ET-1-induced MMP-9 gene up-regulation. Understanding the mechanisms of MMP-9 expression and functional changes regulated by ET-1/ET_B system on astrocytes may provide rational therapeutic interventions for brain injury associated with increased MMP-9 expression.

Keywords: AP-1, astrocytes, Elk-1, endothelin-1, matrix metalloproteinases, NF-κB, p42/p44 mitogen-activated protein kinase.

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Endothelial cells are known to produce vasoactive peptides that contribute to vasotone regulation. One of such vasoactive mediators is the endothelin (ET) family, composed of ET-1, -2, and -3 that cause vasoconstriction. Among the ET members, the bioactivity of ET-1 is considered as a potent vasoconstrictor and pro-inflammatory peptide and has been implicated in the development of cardiovascular diseases (Levin 1995; Böhm and Pernow 2007). ET-1 exerts its biological effects via two types of ET receptor, ET type A (ET_A) and type B (ET_B), which are members of G protein-coupled receptor (GPCR) superfamily (Rubanyi and Polokoff 1994). Besides the significance in cardiovascular pathology, ET-1 plays a potential role in either the normal development or in CNS diseases. Several lines of evidence suggest the substantial role of ET-1 in brain inflammation. Hypoxic/ischemic injury of the brain may induce release of ET-1 from endothelial cells (Chen *et al.* 2001) and astrocytes (Hasselblatt *et al.* 2001). On astrocytes, the ET_B receptor is predominantly expressed and may mediate astrocytic hypertrophy in the injured CNS (Nakagomi *et al.* 2000; Rogers *et al.* 2003). Current data have further demonstrated that over-expression of ET-1 on astrocytes

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Abbreviations used: AP-1, activator protein-1; BBB, blood-brain barrier; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; Elk-1, Ets-like kinase; ET, endothelin; F-12, Ham's nutrient mixture F-12; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; GPCR, G protein-coupled receptor; IKK, I Kappa B kinase; IL-1β, interleukin-1β; MEK1/2, mitogen-activated protein kinase kinase 1/2; MMP, matrix metalloproteinase; NF-κB, nuclear factor-kappa B; RBA-1, rat brain astrocyte-1; S6C, sarafotoxin 6C; SDS–PAGE, sodium doecyl sulfate–polyacrylamide gel electrophoresis; shRNA, short hairpin RNA; siRNA, small interfering RNA; TIMP, tissue inhibitors of MMPs; TSIIA, tanshinone IIA.

has deleterious effects on water homeostasis, cerebral edema and blood-brain barrier (BBB) integrity, which contribute to more severe ischemic brain injury like stroke (Lo *et al.* 2005). Moreover, a study model based on rabbit optic nerve suggests that ET-1 induces a hypertrophy of ET_B/glial fibrillary acid protein (GFAP)-immunoreactive astrocytes, a typical characteristic of astrogliosis, in the normal optic nerve, leading to glial scar formation following CNS injury (Rogers *et al.* 2003). Accumulating evidence suggests that cytokine production may be involved in the effects of ET-1 in brain inflammation. It has been demonstrated that endothelial ET-1 induces interleukin-1β (IL-1β) secretion by astrocytes, which directly contributes to BBB breakdown during CNS inflammation (Didier *et al.* 2003). However, the detailed mechanisms responsible for ET-1 action remain to be elucidated.

The matrix metalloproteinase (MMP) family is zinc-dependent proteinases which are recognized as crucial determinants in normal development and wound healing, as well as in pathophysiological implications, such as atherosclerosis, metastasis, and inflammation. Contributions of MMPs, MMP-9 especially, to the CNS diseases have been identified in the progression of neural dysfunction and neuroinflammation in the CNS (Yong *et al.* 1998, 2001; Aoki *et al.* 2002; Rosenberg 2002). The expression of MMP-9 is induced by extracellular stimuli such as cytokines, endotoxins, and growth factors in malignant glioma and neurological diseases (Yong *et al.* 1998; Harkness *et al.* 2000; Rosenberg 2002). Moreover, IL-1β, bradykinin and endotoxins have been reported to up-regulate MMP-9 expression and activity in cultured rat astrocytes (Lee *et al.* 2003; Hsieh *et al.* 2004; Wu *et al.* 2004), indicating that the expression and activation of MMP-9 may be modulated by various factors during neuroinflammation.

There is evidence to support the role of MMP-9 in the pathogenesis of CNS disorders (Yong *et al.* 2001; Rosenberg 2002; Gu *et al.* 2005). The activity of MMP-9 is strictly regulated at diverse levels, including transcription, translation, activation, and inhibition. Moreover, the MMP-9 promoter has been identified to contain potential binding elements for recognition of transcription factors such as nuclear factor-kappa B (NF-κB), Ets, and activator protein-1 (AP-1) families (Yong *et al.* 2001; Rosenberg 2002). However, the roles of these transcription factors in astrocytic MMP-9 gene induced by ET-1 remain unclear.

In this study, we investigated the molecular mechanisms underlying ET-1-induced MMP-9 expression in rat brain astrocytes. These findings suggested that ET-1 induced MMP-9 expression at the transcriptional and translational levels, which was mediated through the ET_B receptor-dependent activation of p42/p44 MAPK/Ets-like kinase (Elk-1), NF-κB, and AP-1 pathways, leading to cell migration in rat brain astrocytes.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM)/Ham's nutrient mixture F-12 (F-12) medium, fetal bovine serum, and TRIzol were from Invitrogen (Carlsbad, CA, USA). Hybond C membrane and enhanced chemiluminescence (ECL) western blotting detection system were from GE Healthcare Biosciences (Buckinghamshire, UK). MMP-9 antibody was from NeoMarker (Fremont, CA, USA). Phospho-(Thr202/Tyr204)-p42/p44 MAPK, and phospho-(Ser176/180)-IKKα/β antibody kits were from Cell Signaling (Danver, MA, USA). Phospho-(Ser383)-Elk-1, p42, I Kappa B kinase (IKK) α/β, p65, phospho-c-*Jun*, c-*Jun*, and c-*Fos* antibody kits were from Santa Cruz (Santa Cruz, CA, USA). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was from Biogenesis (Boumemouth, UK). MMP-2/9i, BQ-123, BQ-788, GP antagonist-2, GP antagonist-2A, U0126, Bay11-7082, and tanshinone IIA were from Biomol (Plymouth Meeting, PA, USA). Bicinchoninic acid protein assay reagent was from Pierce (Rockford, IL, USA). Enzymes, ET-1, XTT assay kit, and other chemicals were from Sigma (St. Louis, MO, USA).

Rat brain astrocyte culture

Rat brain astrocyte-1 (RBA-1) cells were used throughout this study. This cell line was originated from a primary astrocyte culture of neonatal rat cerebrum and naturally developed through successive cell passages (Jou *et al.* 1985). Staining of RBA-1 with the astrocyte-specific marker, GFAP, showed over 95% positive staining. In this study, the RBA-1 cells were used within 40 passages that show normal cellular morphological characteristics and had steady growth and proliferation in the monolayer system. Cells were cultured and treated as previously described (Hsieh *et al.* 2004). Primary astrocyte cultures were prepared from the cortex of 6-day-old Wistar rat pups, handled according to the guidelines of Animal Care Committee of Chang Gung University and NIH Guides for the Care and Use of Laboratory. As previously described (Hsieh *et al.* 2008), cortex was dissected and dissociated by mechanical chopping and trypsinization (0.125% trypsin at 37°C for 30 min) and then filtered through a 75 μm pore size sterilized nylon mesh to obtain a single-cell suspension. Cells were plated into poly-L-lysine solution-coated plastic culture dishes and routinely cultured in DMEM/F-12 medium containing 10% fetal bovine serum at 37°C in a humidified 5% CO_2 atmosphere. Forty minutes later, the unattached cells (i.e., astroglia cells) were transferred to another plastic dish with the same medium. The cells were plated on 12-well plates and 10-cm culture dishes for MMP gelatin zymography and PCR, respectively. The culture medium was changed every 3 days. The purity of primary astrocyte cultures was assessed with the astrocyte-specific marker, GFAP, showing over 90% GFAP-positive astrocytes.

MMP gelatin zymography

After ET-1 treatment, the culture medium was collected, mixed with equal amounts of non-reduced sample buffer and electrophoresed on 10% sodium doecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) containing 1 mg/mL gelatin as a protease substrate. Following electrophoresis, gelatinolytic activity was determined as previously described (Hsieh *et al.* 2004). Mixed human MMP-2 and MMP-9 standards (Chemicon, Temecula, CA, USA) are used as

positive controls. Because cleaved MMPs were not reliably detectable, only proform zymogens were quantified. When inhibitors were used, they were added 1 h prior to the application of ET-1. Treatment of RBA-1 cells with pharmacological inhibitors alone had no significant effect on cell viability determined by an XTT assay (data not shown).

Total RNA extraction and gene expression studies

For RT-PCR analysis, total RNA was extracted from RBA-1 cells stimulated by ET-1 as previously described (Hsieh *et al.* 2004). The cDNA obtained from 0.5 μg total RNA was used as a template for PCR amplification. Oligonucleotide primers were designed based on Genbank entries for rat MMP-9, tissue inhibitors of MMP (TIMP)-1, TIMP-2, c-*fos*, and β-actin. The following primers were used for amplification reaction: for MMP-9: 5′-AGTTTGGTGTCGCGG AGCAC-3′ (sense), 5′-TACATGAGCGCTTCCGGCAC-3′ (anti-sense); for TIMP-1: 5′-CTGGCATCCTCTTGTTGCTA-3′ (sense), 5′-GTAGCCCTTCTCAGAGCCCA-3′ (anti-sense); for TIMP-2: 5′-CGGACCCAGGCCCCTAGCGC-3′ (sense), 5′-AGCATGGGAT-CATAGGGCAG-3′ (anti-sense); for c-*fos*: 5′-AGACGAAGGA-AGACGTGTAAGCACTGCAGCT-3′ (sense), 5′-AAGGAGAATC-CGAAGGGAAAGGAATAAGATG-3′ (anti-sense); for β-actin: 5′-GAACCCTAAGGCCAACCGTG-3′ (sense), 5′-TGGCATAGAG-GTCTTTACGG-3′ (anti-sense). PCR mixes contained 10 μL of 5× PCR buffer, 1.25 mM of each dNTP, 100 pmol of each forward and reverse primer, and 2.5 units of Taq polymerase (Takara, Shiga, Japan). The final reaction volume was 50 μL. Amplification was performed in 30 cycles at 55°C, 30 s; 72°C, 1 min; 94°C, 30 s. After the last cycle, all samples were incubated for an additional 10 min at 72°C. PCR fragments were analyzed on 2% agarose 1× Tris-acetate-EDTA (TAE) gel containing ethidium bromide and their size was compared to a molecular weight marker. 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. These primer sets specifically recognized only the genes of interest as indicated by amplification of a single band of the expected size (754 bp for MMP-9, 600 bp for c-*fos*, and 514 bp for β-actin) and direct sequence analysis of the PCR products. Real-time PCR was performed with the TaqMan gene expression assay system, using primer and probe mixes for MMP-9, ET_A, ET_B, and endogenous GAPDH control genes. The primers were: for MMP-9: 5′-TGATGCCATTGCTGATATCCA-3′ (sense), 5′-CGGATCCTCAAAGGCTGAGT-3′ (anti-sense); for ET_A: 5′-AATACAAGGGCGAGCAGCAC-3′ (sense), 5′-GCAAGCTCC-CATTCCTTCTGT-3′ (anti-sense); for ET_B: 5′-AGACGAGAAG-TGGCCAAGACA-3′ (sense), 5′-GGAATTCAAAGAAGCCATGT-TG-3′ (anti-sense); for GAPDH: 5′-AACTTTGGCATCGTGGA-AGG-3′ (sense), 5′-GTGGATGCAGGGATGATGTTC-3′ (anti-sense). PCRs were performed using the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Relative gene expression was determined by the ΔΔCt method, where Ct meant threshold cycle. All experiments were performed in triplicate.

ELISA analysis

The culture medium samples were collected from ET-1-incubated RBA-1 cells and analyzed for MMP-9 levels using the rat MMP-9 ELISA kit (Cusabio Biotech, Wuhan, Hubei, China) according to the manufacturer's instructions.

Migration assay

Rat brain astrocyte-1 cell monolayers were grown on coverslips in 6-well culture plates. After starvation with serum-free DMEM/F-12 medium for 24 h, the coverslips were inverted with the cell monolayers facing down onto a new culture plate. Serum-free DMEM/F-12 medium with or without ET-1 was added to each dish as indicated after pre-treatment with the inhibitors for 1 h. Hydroxyurea, an inhibitor of DNA synthesis (Yarbro 1992), was added to prevent cell proliferation during the period of observation. Images of migratory cells from the coverslip boundary were observed and acquired at 0 and 48 h with a digital camera and a light microscope (Olympus, Tokyo, Japan). Number of migratory cells was counted from the resulting four phase images for each point and then averaged for each experimental condition. The data presented are generated from three separate assays.

Western blotting analysis of MMP-9

Conditioned media derived from untreated or ET-1-treated RBA-1 cells were mixed with equal amounts of reduced sample buffer at 95°C for 5 min before electrophoresis with 10% SDS–PAGE. The resolved band was electrotransferred onto nitrocellulose membrane which was incubated overnight at 4°C with monoclonal antibody against MMP-9 as described by Hsieh *et al.* (2004).

Preparation of cell extracts and western blot analysis

Growth-arrested RBA-1 cells were incubated with ET-1 at 37°C for various times. The cells were washed with ice-cold phosphate-buffered saline, scraped, and collected by centrifugation at 45 000 *g* for 1 h at 4°C to yield the whole cell extract, as described previously (Hsieh *et al.* 2004). Samples were denatured, subjected to SDS–PAGE using a 10% (w/v) running gel, and transferred to nitrocellulose membrane. Membranes were incubated overnight using anti-phospho-p42/p44 MAPK, phospho-Elk-1, phospho-IKKα/β, or phospho-c-*Jun* antibody. Membranes were washed with Tris-Tween buffered saline (TTBS) four times for 5 min each, incubated with a 1 : 2000 dilution of anti-rabbit horseradish peroxidase antibody for 1 h. The immunoreactive bands detected by ECL reagents were developed by Hyperfilm-ECL.

Immunofluorescence staining

Rat brain astrocyte-1 cells were plated on 6-well culture plates with coverslips. Cells were treated with 100 nM ET-1 and washed twice with ice-cold phosphate-buffered saline. Immunofluorescence staining using a primary anti-p65 monoclonal antibody was performed as described previously (Hsieh *et al.* 2004).

Plasmid construction, transient transfection, and promoter assays

The rat MMP-9 promoter was constructed as previously described (Eberhardt *et al.* 2002) with some modifications. The upstream region (−1280 to +108) of the rat MMP-9 promoter was cloned into the pGL3-basic vector containing the luciferase reporter system. pGL-MMP-9-ΔEts and pGL-MMP-9-ΔAP1 were constructed as previously described (Wang *et al.* 2010; Wu *et al.* 2009). Introduction of a double-point mutation into the NF-κB-binding site (κB domain; GGAATTCC to GGAATTGG) to generate pGL-MMP-9-ΔκB (mt-κB-MMP-9) was performed using the following (forward) primer: 5′-GGGTTGCCCCGTGGAATTGGCCCAAATCCTGC-3′ (corresponding to a region from −572 to −541). The underlined

nucleotides indicate the positions of substituted bases. All plasmids were prepared by using QIAGEN plasmid DNA preparation kits (Valencia, CA, USA). The dominant negative mutant of IKKα/β, short hairpin RNA (shRNA) for p42, c-*Jun*, and c-*Fos*, small interfering RNA (siRNA) for p65 (Rela-RSS358497, Rela-RSS317831, Rela-RSS317830; Invitrogen), and MMP-9 promoter reporter constructs were transfected into RBA-1 cells using the Lipofetamine™ RNAiMAX reagent according to the instructions of manufacture (Invitrogen). The transfection efficiency (~60%) was determined by transfection with enhanced green fluorescent protein (GFP). To assess promoter activity, cells were collected and disrupted by sonication in lysis buffer (25 mM Tris-phosphate, pH 7.8, 2 mM EDTA, 1% Triton X-100, and 10% glycerol). After centrifugation, aliquots of the supernatants were tested for luciferase activity using a luciferase assay system. Firefly luciferase activities were standardized to β-galactosidase activity.

Analysis of data

Concentration-effect curves were made using GraphPad Prism Program (GraphPad, San Diego, CA, USA). 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

ET-1-induced proMMP-9 up-regulation enhances RBA-1 cell migration

To investigate the effects of ET-1 on proMMP-9 expression, RBA-1 cells were treated with various concentration of ET-1 for the indicated time intervals. The condition media were collected and analyzed by gelatin zymography. As shown in Fig. 1(a), ET-1 induced proMMP-9 expression in a concentration- and time-dependent manner. There was a significant increase within 16 h and sustained over 24 h. In contrast, the expression of proMMP-2 was not significantly changed during incubation with ET-1 (Fig. 1a). We also determined whether the regulatory proteins of MMP activity such as TIMPs could be regulated by ET-1 in RBA-1 cells. The

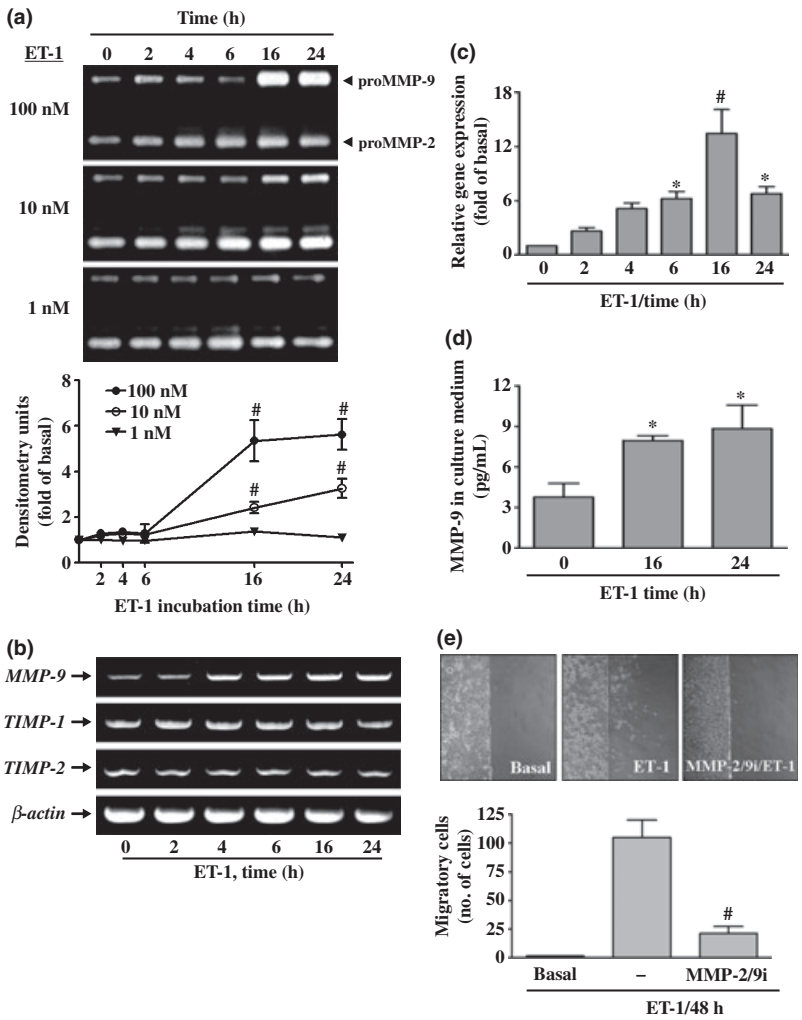

Fig. 1 ET-1 induced proMMP-9 expression and astrocytic migration. (a) Time and concentration dependence of ET-1-induced proMMP-9 expression, cells were treated with various concentration ET-1 for the indicated time intervals. The condition media were collected to assay proMMP-9 expression by gelatin zymography. (b) Time dependence of ET-1-induced gene expression, cells were treated with 100 nM ET-1 for the indicated time intervals. MMP-9, TIMP-1, and TIMP-2 mRNA were analyzed by RT-PCR. (c) The MMP-9 mRNA was also analyzed by real-time PCR. (d) The cultured media were collected and analyzed the level of MMP-9 protein by MMP-9 ELISA kit. (e) ET-1-induced cell migration was evaluated by migration assay. Cells were pre-treated with MMP2/9 inhibitor (2/9i, 3 μM) for 1 h and then incubated with ET-1 (100 nM) for 48 h. The random phase contrast images of RBA-1 cells were taken (upper part, $n = 3$). The number of ET-1-induced cell migration was counted as described in 'Materials and Methods' (lower part). Data are expressed as mean ± SEM of three independent experiments. $^{*}p < 0.05$; $^{\#}p < 0.01$, as compared with vehicle (a, b, c, d) or ET-1 alone (e). The image represents one of at least three individual experiments.

mRNA levels of MMP-9, TIMP-1, and TIMP-2 in ET-1-treated astrocytes were analyzed by RT-PCR. The results showed that ET-1 induced an increase in MMP-9 expression, but not TIMP-1 and TIMP-2 in RBA-1 cells (Fig. 1b). To further examine whether the increase of proMMP-9 expression by ET-1 resulted from the induction of MMP-9 mRNA expression, the real-time PCR analysis was performed. The data showed that ET-1 time-dependently induced MMP-9 mRNA expression in RBA-1 cells (Fig. 1c). There was a significant increase in MMP-9 mRNA within 6 h, reached a maximal response within 16 h, and sustained over 24 h during the period of observation. We further evaluated the protein level of MMP-9 in the culture medium using an ELISA analysis. Data in Fig. 1(d) showed that the levels of MMP-9 in the medium collected from the cells treated without or with ET-1 for 16 and 24 h were 3.76 ± 1.02, 7.96 ± 0.35, and 8.83 ± 1.74 pg/mL. These results suggested that ET-1-induced proMMP-9 expression occurred at transcriptional and translational levels. Moreover, to demonstrate the consequence of proMMP-9 expression induced by ET-1, we evaluated its functional effect on RBA-1 cells by a migration assay. The images showed that ET-1 induced RBA-1 cell migration after 48 h of incubation (Fig. 1e, upper part). The number of migratory RBA-1 cells was summarized as shown in Fig. 1(e). We found that ET-1-induced cell migration was significantly blocked by pre-treatment with the inhibitor of MMP-2/9 activity (MMP-2/9i, 3 μM) (Fig. 1e, lower part), suggesting that up-regulation of proMMP-9 and its activity are required for enhancing RBA-1 cell migration induced by ET-1.

ET-1 induces proMMP-9 expression via $G_{i/o}$ and G_q protein-coupled receptors

Endothelin-1 exerts its biological effects via the ET receptors, ET_A and ET_B, which are members of GPCR superfamily (Rubanyi and Polokoff 1994). Among the ET receptors, the ET_B receptor is predominantly expressed in astrocytes and may mediate astrocytic hypertrophy in the injured CNS (Nakagomi *et al.* 2000; Rogers *et al.* 2003). We determined the expression of ET_A and ET_B receptors on RBA-1 cells by a real-time PCR analysis. As shown in Fig. 2(a), both ET_A and ET_B receptors were expressed in RBA-1 cells, and the latter was expressed to a greater extent. Next, to identify the subtypes of ET receptors responsible for ET-1-induced proMMP-9 expression, cells were pre-treated with either BQ-123 (an ET_A antagonist) or BQ-788 (an ET_B antagonist) (IC_{50} = 0.78 ± 0.30 μM) (Saadoun and García 1999) for 1 h and then incubated with ET-1 for 16 h. These data showed that selective blockade of ET_B receptor, but not ET_A receptor, inhibited the ET-1-induced proMMP-9 expression in RBA-1 cells (Fig. 2b). Because ET receptors are characterized as GPCRs, we used G protein antagonists to determine the subtypes of G proteins involved in ET-1-induced proMMP-9 expression in RBA-1 cells. Pre-treatment with GP antagonist-2 (GPAnt2, a $G_{i/o}$ protein antagonist) or GP antagonist-2A (GPAnt2A, a G_q protein antagonist) significantly inhibited ET-1-induced proMMP-9 expression (Fig. 2c). These data indicated that ET_B receptors coupled to $G_{i/o}$ and G_q proteins may predominantly mediate ET-1-induced proMMP-9 expression in RBA-1 cells. To substantiate the role of ET_B receptors in ET-1-induced functional response, we evaluated cell migration of RBA-1 cell using BQ-788. As shown in Fig. 2(d), the images showed that ET-1-induced cell migration was significantly attenuated by pre-treatment with 1 μM BQ-788, suggesting that ET_B receptors mediated the cell migration induced by ET-1 in RBA-1 cells.

Furthermore, we investigated the ET-1-induced proMMP-9 expression in primary culture of rat astrocytes. The characteristics of primary cultured astrocytes were identified by immunofluorescence staining using an anti-GFAP antibody (Fig. 2e). The zymographic data showed that the increases in proMMP-9 expression induced by ET-1 were significantly reduced by pre-treatment with BQ-788, GPAnt2, or GPAnt2A (Fig. 2f), suggesting that ET-1 induces proMMP-9 expression via ET_B receptors coupled with $G_{i/o}$ and G_q proteins in primary culture of astrocytes.

To confirm the contribution of ET_B to MMP-9 induction, we tested the effects of sarafotoxin 6C (S6C), an ET_B agonist, on ET-1-induced proMMP-9 expression in primary astrocytes. Data in Fig. 2(g) showed that S6C induced proMMP-9 expression in a time-dependent manner. There was a significant increase within 6 h and sustained over 24 h. In addition, blockades of ET_B receptor and G_q protein inhibited the S6C-induced proMMP-9 expression in primary astrocytes. However, blockade of $G_{i/o}$ protein delayed the S6C-induced proMMP-9 expression as compared with that of control (Fig. 2g).

ET-1-induced proMMP-9 expression and cell migration is mediated through p42/p44 MAPK/Elk-1 cascade

Accumulating evidence has suggested that activation of p42/p44 MAPK by ET-1 modulates cellular functions of astrocytes (Schinelli *et al.* 2001; He *et al.* 2007). To investigate the role of p42/p44 MAPK in ET-1-induced proMMP-9 expression in RBA-1, cells were pre-treated with the inhibitor of mitogen-activated protein kinase kinase 1/2 (MEK1/2) (U0126) for 1 h and then incubated with ET-1 for 16 h. These data showed that pre-treatment with U0126 attenuated ET-1-induced proMMP-9 expression in RBA-1 cells (Fig. 3a, left part). In addition, pre-treatment with U0126 concentration-dependently inhibited ET-1-induced proMMP-9 expression in primary culture of astrocytes (Fig. 3a, right part), suggesting that ET-1-stimulated p42/p44 MAPK is involved in proMMP-9 induction in astrocytes.

Moreover, we determined whether phosphorylation of p42/p44 MAPK was involved in the ET-1-induced responses, the kinetics of p42/p44 MAPK phosphorylation was assessed by western blotting using an anti-phospho-p42/p44 MAPK

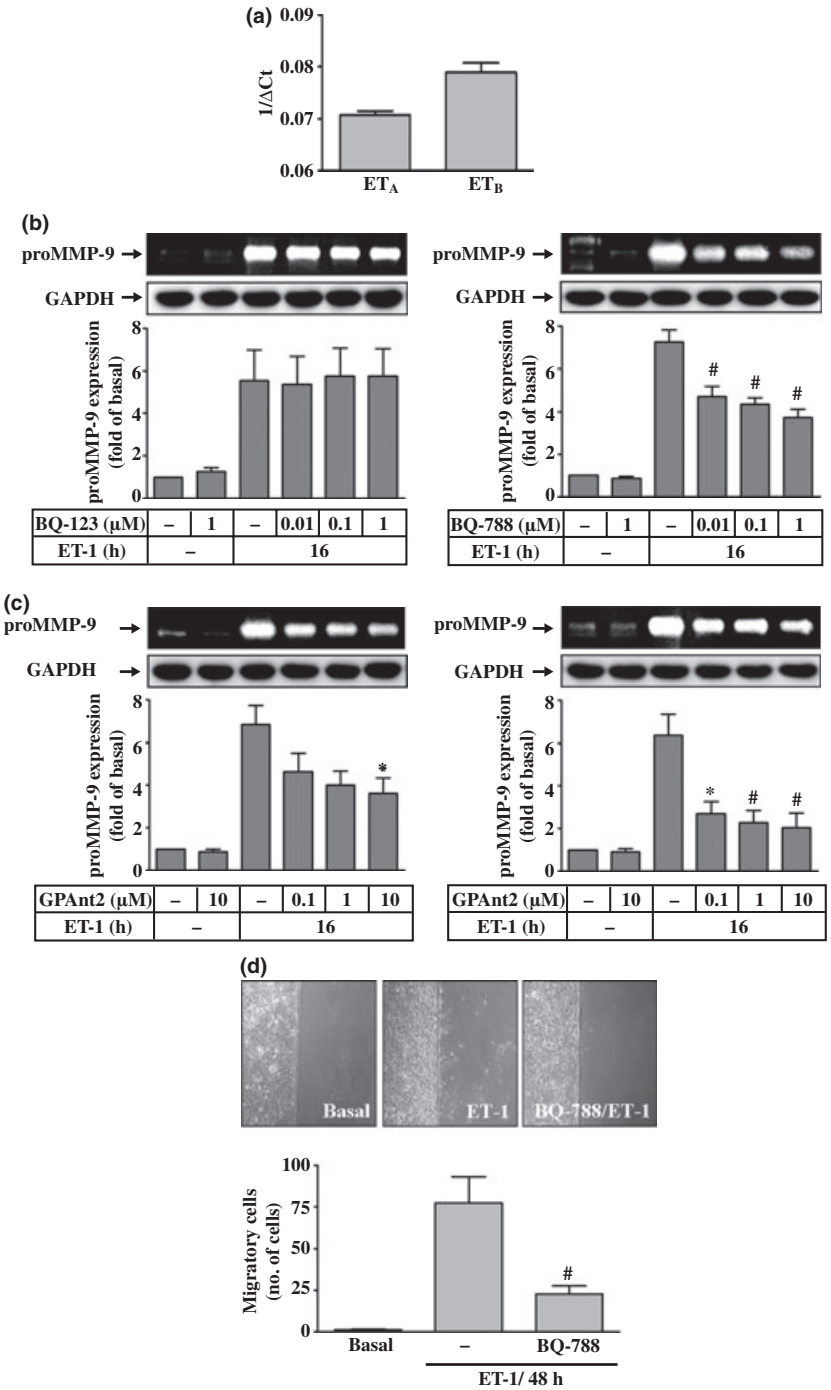

Fig. 2 Involvement of G_i and G_q proteins coupling to ET_B receptors in ET-1-induced proMMP-9 expression in RBA-1 cells. (a) Real-time PCR analysis of ET receptor expression in RBA-1 cells. (b) The cells were pre-treated with BQ-123 or BQ-788 for 1 h and then incubated with ET-1 for 16 h. (c) RBA-1 cells were pre-treated with G_i antagonist (GPAnt2) or G_q antagonist (GPAnt2A) for 1 h and then exposed to ET-1 for the indication time intervals. (d) For cell migration, cells were pre-treated with BQ-788 for 1 h and then incubated with ET-1 (100 nM) for 48 h. The random phase contrast images of RBA-1 cells were taken (upper part, $n = 3$). The number of ET-1-induced cell migration was counted as described in 'Materials and Methods'. (e) The primary rat brain culture of astrocytes (RBA) were prepared, cultured and analyzed by immunofluorescence staining using an anti-GFAP antibody. Moreover, cells were pre-treated with BQ-788, GPAnt2, or GPAnt2A for 1 h and then exposed to ET-1 for the indicated time intervals. The proMMP-9 protein was analyzed by gelatin zymography (f). (g) ET_B agonist (S6C) induced proMMP-9 expression in primary astrocytes. Data are expressed as mean ± SEM of three individual experiments. $^*p < 0.05$; $^\#p < 0.01$, as compared with the respective values of cells stimulated by ET-1 alone. The figure represents one of three individual experiments.

antibody. The results showed that ET-1 stimulated p42/p44 MAPK phosphorylation in a time-dependent manner and with a maximal response within 3–5 min which was inhibited by pre-treatment with 10 μM U0126 or 1 μM BQ-788 (Fig. 3b, upper part). Recent studies have suggested that phosphorylation of Ets family transcription factors may be the consequence of activation of MAPK. Activation of Ets family members, particularly Elk-1, has been identified in cerebral insults, and participates in production of inflammatory mediators (Hu *et al.* 2004; Krupinski *et al.* 2005; Hsieh *et al.* 2008). Therefore, we investigated the role of transcription factor Elk-1 in ET-1-induced proMMP-9 expression in RBA-1 cells. The results showed that ET-1 stimulated a time-dependent phosphorylation of Elk-1 with a maximal response within 10 min and sustained for 15 min during the period of observation in RBA-1 cells (Fig. 3b, lower part). To identify related upstream components in these responses, cells were pre-treated with inhibitors of MEK1/2 (U0126, 10 μM) or

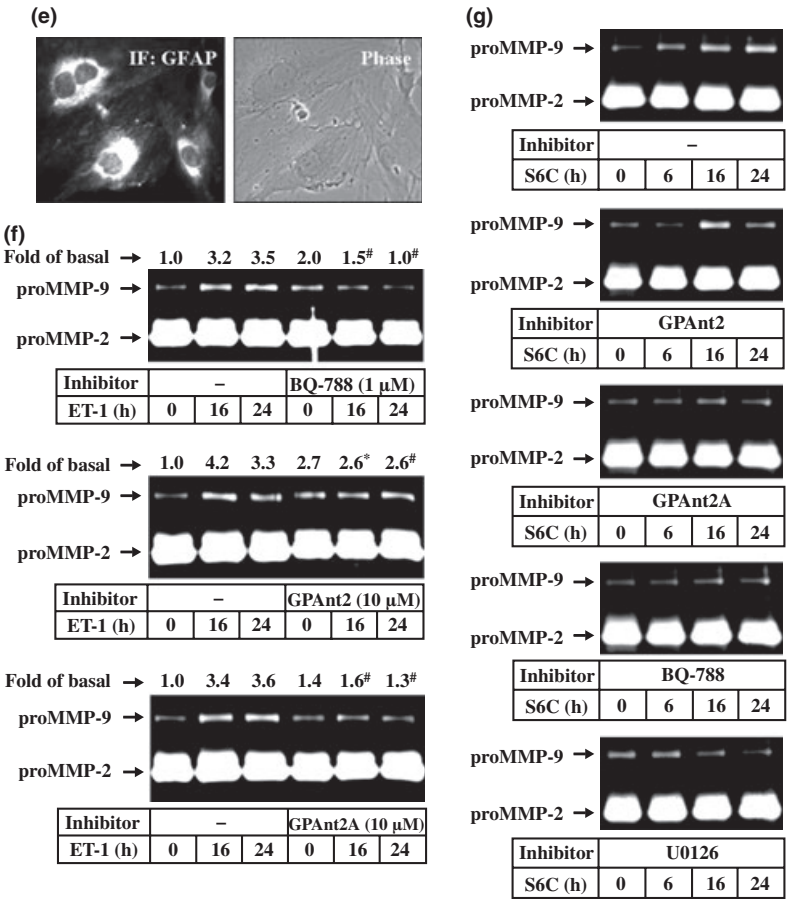

Fig. 2 (*Continued*).

ET_B receptor (BQ-788, 1 μM) for 1 h and then stimulated by ET-1 for the indicated time intervals. The results showed that pre-treatment with U0126 or BQ-788 blocked ET-1-stimulated Elk-1 phosphorylation (Fig. 3b, lower part), suggesting that ET_B-mediated p42/p44 MAPK activation is crucial for Elk-1 activation in RBA-1 cells. To further ensure the role of p42/p44 MAPK in ET-1-induced proMMP-9 expression, cells were transfected with shRNA for p42 MAPK. As shown in Fig. 3(c), transfection with p42 shRNA knocked-downed the expression of total p42 protein and attenuated ET-1-induced proMMP-9 expression, demonstrating that p42/p44 MAPK was essential for ET-1-induced proMMP-9 expression in RBA-1 cells.

To examine the functional response of ET-1-stimulated p42/p44 MAPK activation, we evaluated cell migration of RBA-1 cells. As shown in Fig. 3(d), the images showed that ET-1-induced cell migration was significantly attenuated by pre-treatment with 10 μM U0126, suggesting that p42/p44 MAPK participated in the cell migration induced by ET-1 in RBA-1 cells. These results demonstrated that p42/p44 MAPK/Elk-1 cascade was involved in ET-1-stimulated proMMP-9 expression and migration of RBA-1 cells.

ET-1 induces cell migration via NF-κB-dependent proMMP-9 induction

As shown in Fig. 1(c), ET-1 induced proMMP-9 expression at transcriptional level. The promoter region of MMP-9 possesses serial binding elements for recognition of transcription factors including NF-κB and AP-1 (Yong *et al.* 2001; Rosenberg 2002). The NF-κB family is considered as an essential regulator of cellular activities associated with inflammation (Ghosh and Hayden 2008). ET-1 has been shown to stimulate NF-κB activation, which modulates cellular functions in various cell types (Morigi *et al.* 2006; Banerjee *et al.* 2007). Moreover, MMP-9 promoter contains the κB binding site that is crucial for induction of MMP-9 (Rosenberg 2002; Hsieh *et al.* 2008). Thus, we examined whether activation of NF-κB was required for proMMP-9 expression induced by ET-1 in RBA-1 cells. First, cells were pre-treated with a selective NF-κB inhibitor, Bay11-7082, which blocks activation of NF-κB signaling (Huang *et al.* 2002), and then incubated with ET-1 for 16 h. The zymographic data showed that pre-treatment with Bay11-7082 concentration-dependently reduced ET-1-induced proMMP-9 expression in RBA-1 cells (Fig. 4a, left part) and primary culture of astrocytes (Fig. 4a, right part), suggesting the

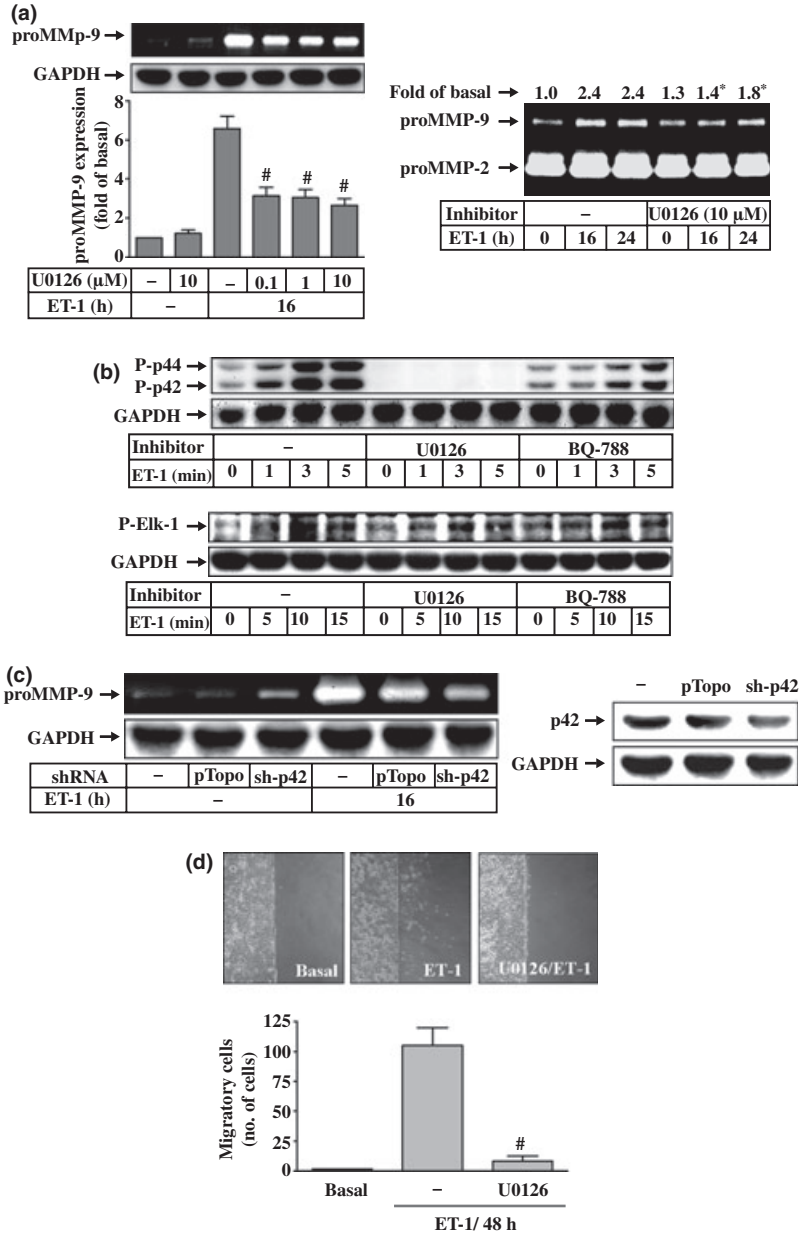

Fig. 3 Involvement of p42/p44 MAPK/Elk-1 cascade in ET-1-induced proMMP-9 expression in RBA-1 cells. (a) Cells, RBA-1 cell line (left part) or primary culture of RBA (right part), were treated with 100 nM ET-1 for 16 h in the absence or presence of U0126. ProMMP-9 expression was determined by zymography as described in Fig. 1. (b) Time dependence of ET-1-stimulated p42/p44 MAPK and Elk-1 phosphorylation, cells were incubated with 100 nM ET-1 for the indicated times in the absence or presence of U0126 (10 μM) or BQ-788 (1 μM). (c) Cells were transfected with p42 shRNA for 24 h and then exposed to ET-1 for 16 h. The condition media and cell lysates were collected and analyzed by gelatin zymography and western blotting using an anti-phospho-p42/p44 MAPK, anti-phospho-Elk-1, anti-p42, anti-Elk-1, or anti-GAPDH (as an internal control) antibody. (d) For cell migration, cells were pre-treated with 10 μM U0126 for 1 h and then incubated without (Basal) or with ET-1 (100 nM) for 48 h. Data are expressed as mean ± SEM of at least three individual experiments. $^{*}p < 0.05$; $^{\#}p < 0.01$, as compared with ET-1 alone. The figure represents one of at least three individual experiments.

involvement of NF-κB in ET-1-induced proMMP-9 expression. To further verify that NF-κB is important in ET-1-induced proMMP-9 expression, cells were transfected with p65 siRNA and then stimulated by ET-1 for 16 h. As shown in Fig. 4(b), transfection with p65 siRNA knock-downed the expression of p65 NF-κB protein and attenuated the ET-1-induced proMMP-9 expression, whereas a housekeeping protein GAPDH was not changed. These data suggested that p65 NF-κB was involved in proMMP-9 induction by ET-1. Moreover, cell migration assay was performed to determine whether NF-κB was involved in ET-1-induced RBA-1 migration. The images showed that ET-1-induced cell migration was significantly inhibited by pre-treatment with 1 μM Bay11-7082 (Fig. 4c). These data demonstrated that NF-κB is necessary for ET-1-induced proMMP-9 expression and cell migration in RBA-1 cells.

The NF-κB translocation from cytosolic fraction into nucleus is an effective index in reflecting the state of NF-κB activation. Thus, we determined whether ET-1 stimulated the translocation of NF-κB (p65 subunit) by immunofluorescent staining. As shown in Fig. 5(a), the imaging data showed that ET-1 stimulated translocation of p65 NF-κB into nucleus with a maximal response within 60 min and sustained over 120 min, which was

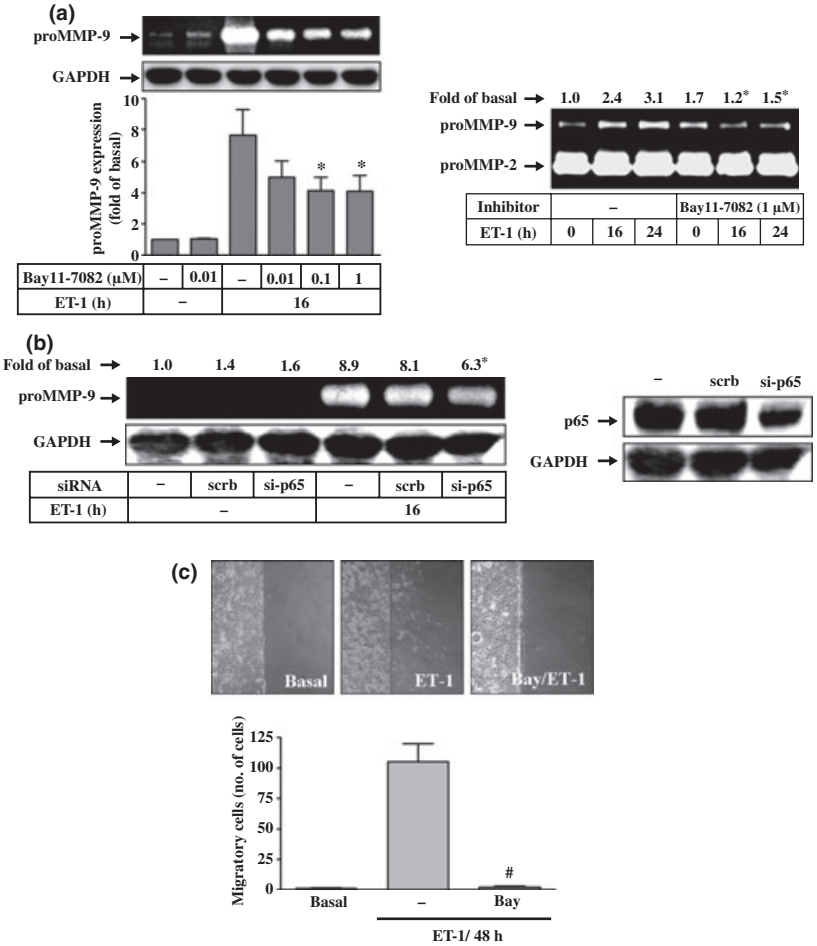

Fig. 4 NF-κB (p65) is essential for ET1-1-induced proMMP-9 expression in RBA-1 cells. (a) Cells, RBA-1 cell line (left part) or primary RBA (right part), were treated with 100 nM ET-1 for 16 h in the absence or presence of Bay11-7082 (0.01, 0.1 or 1 μM). ProMMP-9 expression was determined by zymography as described in Fig. 1. (b) Cells were transfected with scramble (scrb) or p65 siRNA for 24 h and then exposed to ET-1 for 16 h. The condition media and cell lysates were collected and analyzed by zymography for proMMP-9 and western blotting for p65 and GAPDH (as an internal control), as described in Methods. (c) For cell migration, cells were pre-treated with 1 μM Bay11-7082 for 1 h and incubated without (Basal) or with ET-1 (100 nM) for 48 h. Data are expressed as mean ± SEM of at least three independent experiments. $^{*}p < 0.05$; $^{\#}p < 0.01$, as compared with ET-1 alone. The figure represents one of at least three individual experiments.

significantly attenuated by pre-treatment with the inhibitors of NF-κB (Bay11-7082, 1 μM), ET_B receptors (BQ-788, 1 μM) or MEK1/2 (U0126, 10 μM) (Fig. 5b). To further determine whether the activation of NF-κB signaling was mediated through phosphorylation of IKKα/β, the phosphorylation of IKKα/β was determined by western blot using an anti-phospho-IKKα/β antibody. As shown in Fig. 5(c), ET-1 stimulated phosphorylation of IKKα/β in a time-dependent manner, which was inhibited by pre-treatment with Bay11-7082, BQ-788, or U0126. Collectively, these results revealed that ET-1-activated IKKα/β/NF-κB cascade participated in proMMP-9 induction and cell migration in RBA-1 cells.

To further ensure the involvement of IKKα/β in ET-1-induced proMMP-9 expression, cells were transfected with a dominant negative IKKα/β mutant (ΔIKKα/β) and then incubated with ET-1 for 16 h. As shown in Fig. 5(d), the protein level of IKKα/β was increased by transfection with ΔIKKα/β, which attenuated the ET-1-induced proMMP-9 expression. These data suggested that IKKα/β was involved in proMMP-9 induction by ET-1. These results indicated that ET-1 activated the IKKα/β/NF-κB cascade through ET_B and p42/p44 MAPK signaling in RBA-1 cells.

ET-1 up-regulates proMMP-9 expression via AP-1 activation

Recent studies suggest that the binding elements of the MMP-9 promoter region include two AP-1 sites, which are required for MMP-9 induction (Wang *et al.* 2009; Wu *et al.* 2009). To examine whether AP-1 contributed to ET-1-induced proMMP-9 expression, cells were pre-treated with tanshinone IIA (TSIIA, an inhibitor of AP-1) for 1 h and then incubated with ET-1 for 16 h. These data revealed that ET-1-induced proMMP-9 expression was blocked by pre-treatment with TSIIA in RBA-1 cells (Fig. 6a, left part) and primary culture of astrocytes (Fig. 6a, right part). These results indicated that the transcription factor AP-1 may contribute to induction of proMMP-9 by ET-1.

We further examined whether ET-1 affected c-*Jun* phosphorylation and c-*Fos* expression in RBA-1 cells. First, ET-1-stimulated c-*Jun* phosphorylation was analyzed by western blotting using an anti-phospho-c-*Jun* antibody. As expected,

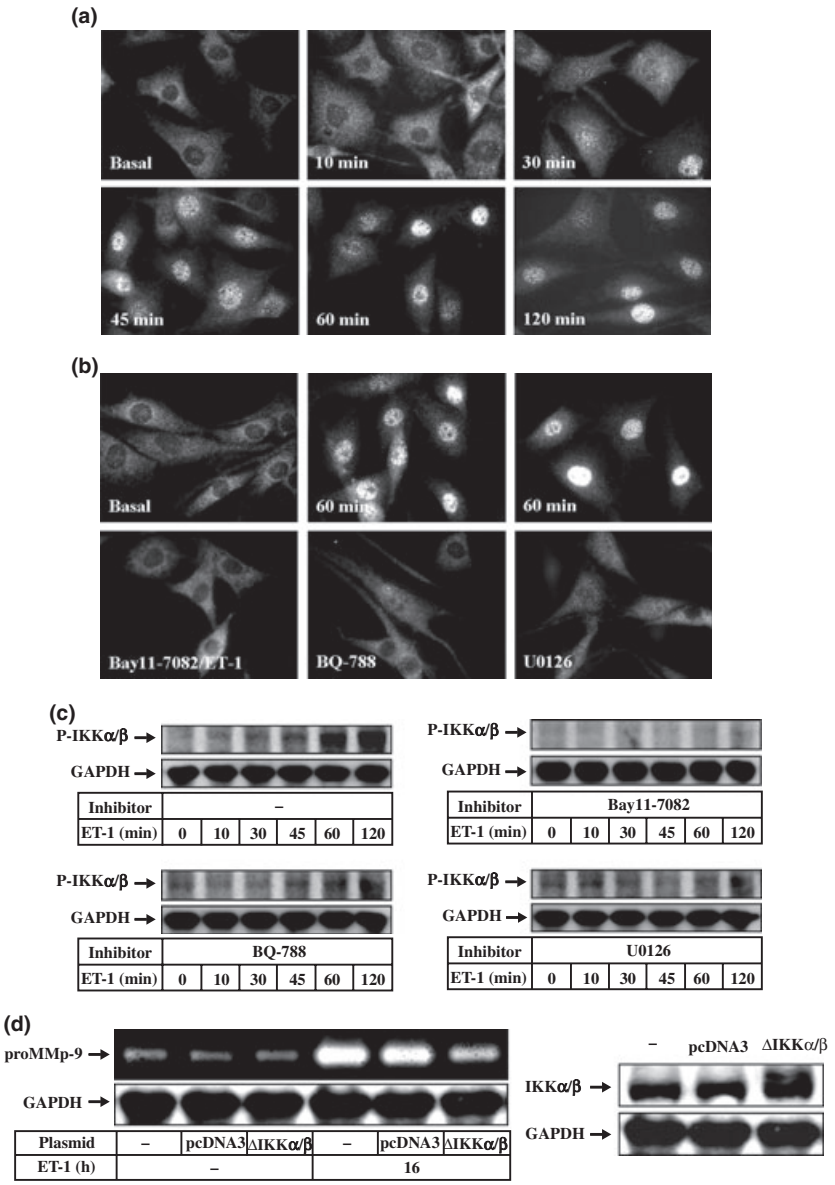

Fig. 5 ET-1 induces proMMP-9 expression via an IKK/NF-κB pathway in RBA-1 cells. (a) Time dependence of ET-1-induced NF-κB p65 subunit translocation. (b) Cells were treated with 100 nM ET-1 for 60 min in the absence or presence of Bay11-7082 (1 μM), BQ-788 (1 μM) or U0126 (10 μM). The p65 NF-κB translocation was determined by an immunofluorescence staining using anti-p65 antibody. (c) Time dependence of ET-1-stimulated IKKα/β phosphorylation, cells were incubated with 100 nM ET-1 for the indicated time intervals in the absence or presence of Bay11-7082, BQ-788 or U0126. The IKKα/β phosphorylation were determined by western blot as described in Methods. (d) Cells were transfected with an empty vector (pcDNA3) or dominant negative IKK mutants (ΔIKKα/β) for 24 h and then exposed to ET-1 for 16 h. The condition media and cell lysates were collected and analyzed by gelatin zymography for proMMP-9 and western blotting for IKKα/β phosphorylation and GAPDH (as an internal control). The figure represents one of at least three individual experiments.

ET-1 stimulated c-*Jun* phosphorylation in a time-dependent manner, which was significantly inhibited by pre-treatment with TSIIA and BQ-788 during the period of observation (Fig. 6b). To examine whether ET-1 also induced immediate early gene c-*Fos*/AP-1 expression, RT-PCR analysis was performed. As shown in Fig. 6(c), ET-1-induced *c-fos* mRNA expression was significantly inhibited by pre-treatment with TSIIA or BQ-788. These results demonstrated that ET-1 induced c-*Jun* phosphorylation and *c-fos* expression through ET_B receptors and AP-1. To further verify the roles of c-*Jun* and c-*Fos* in ET-1-induced proMMP-9 expression, cells were transfected with shRNA for c-*Jun* or c-*Fos* and then incubated with ET-1 for 16 h. The data showed that transfection with siRNA for c-*Jun* and c-*Fos* significantly knock-downed the expression of c-*Jun* (Fig. 6e, right part) and c-*Fos* (Fig. 6f, right part), and reduced the ET-1-induced proMMP-9 expression (Fig. 6e and f). These data suggested that c-*Jun* and c-*Fos* are involved in proMMP-9 induction by ET-1. Next, cell migration assay was performed to determine the role of AP-1 in ET-1-induced proMMP-9 expression associated with cell migration. The images showed that ET-1-induced cell migration was significantly inhibited by pre-treatment with 10 μM TSIIA (Fig. 6d), suggesting that AP-1 is involved in the ET-1-induced cell migration. These results indicated that AP-1 (*i.e.* c-*Jun* and c-*Fos*) activity was required for ET-1-induced proMMP-9 expression and cell migration in RBA-1 cells.

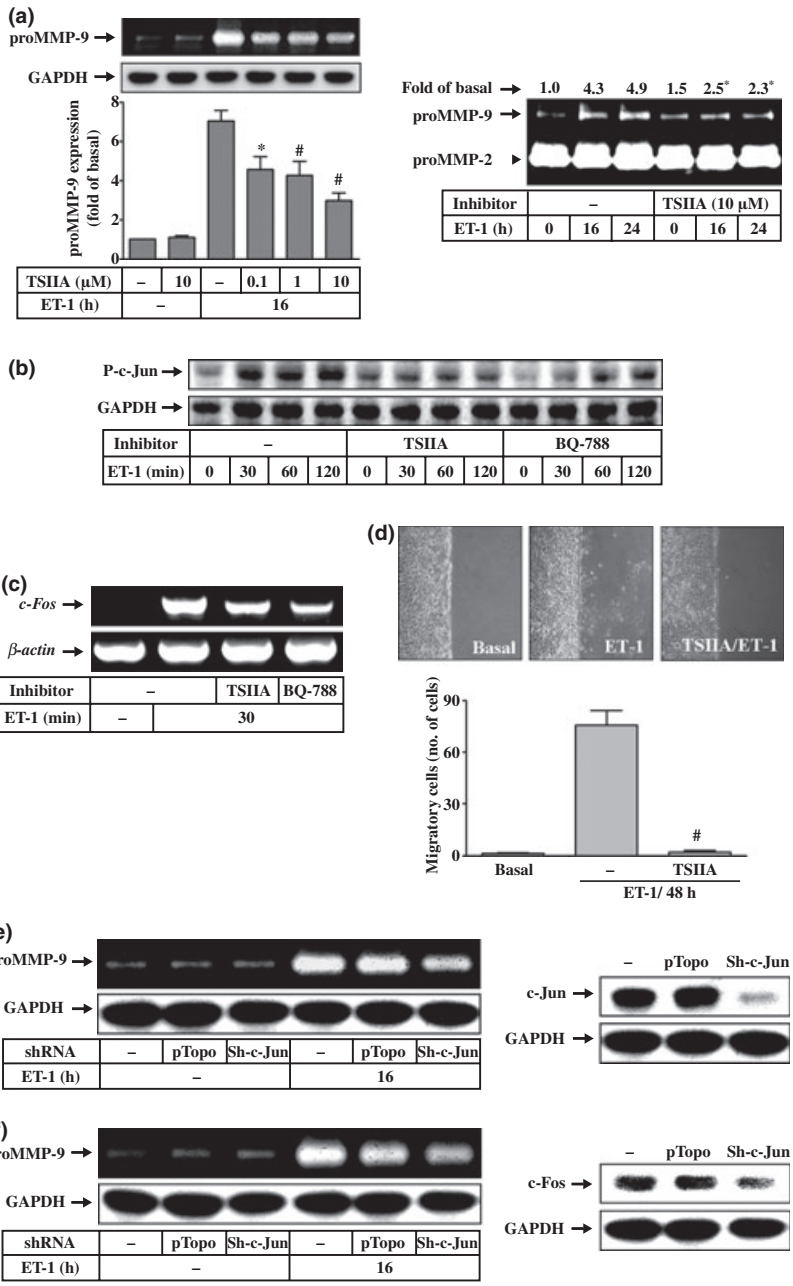

Fig. 6 AP-1 is crucial for ET-1-induced proMMP-9 expression in RBA-1 cells. (a) Cells, RBA-1 cell line (left part) or primary culture of RBA (right part), were treated with 100 nM ET-1 for 16 h in the absence or presence of tanshinone IIA (TSIIA). ProMMP-9 expression was determined by gelatin zymography. (b) Cells were pre-treated with 10 μM TSIIA or 1 μM BQ-788 for 1 h and then exposed to ET-1 for the indicated time intervals. The cell lysates were analyzed by western blotting using an anti-phospho-c-*Jun* antibody or anti-GAPDH (as an internal control). (c) Cells were pre-treated with 10 μM TSIIA or 1 μM BQ-788 for 1 h and then exposed to ET-1 for the indicated times. RNA was extracted and analyzed by RT-PCR to determine c-*Fos* gene expression. To ensure the involvement of c-*Jun* and c-*Fos* in ET-1-induced proMMP-9 expression, cells were transfected with (e) c-*Jun* or (f) c-*Fos* shRNA for 24 h and then exposed to ET-1 for 16 h. ProMMP-9 expression was determined by gelatin zymography. (d) For cell migration, cells were pre-treated with 10 μM TSIIA for 1 h and then incubated without (Basal) or with ET-1 (100 nM) for 48 h. Data are expressed as mean ± SEM of at least three independent experiments. $^{*}p < 0.05$; $^{\#}p < 0.01$, as compared with ET-1 alone. The figure represents one of at least three individual experiments.

Involvement of binding elements in the activation of the rat MMP-9 gene promoter by ET-1

We have found that ET-1 stimulates activation of various transcription factors including Elk-1, NF-κB, and AP-1 in RBA-1 cells. Next, we examined whether the binding of these transcription factors to their promoter binding elements was essential for ET-1-induced MMP-9 gene regulation. The rat MMP-9 promoter luciferase reporter was constructed and its activity was evaluated by promoter-luciferase activity assay. The rat MMP-9 promoter was constructed into a pGL3-basic vector containing a luciferase reporter system (as illustrated in Fig. 7a, upper part; pGL-MMP-9-Luc), which contains several putative recognition elements for a variety of transcription factors that include NF-κB, Ets, and AP-1 families. Thus, to determine the effect of ET-1 on the MMP-9 promoter activity, cells were transfected with a pGL-MMP-9-Luc construct and then incubated with ET-1 (100 nM) for the indicated time intervals. As shown in Fig. 7(a), ET-1 increased the MMP-9 promoter activity in a time-dependent manner. A maximal response was obtained within 16 h, which was significantly inhibited by pre-treatment with an ET_B antagonist (BQ-788, 1 μM) and the inhibitors of MEK1/2 (U0126, 10 μM), NF-κB (Bay11-7082, 1 μM), or AP-1 (TSIIA, 10 μM) (Fig. 7b). To further ensure that Elk-1,

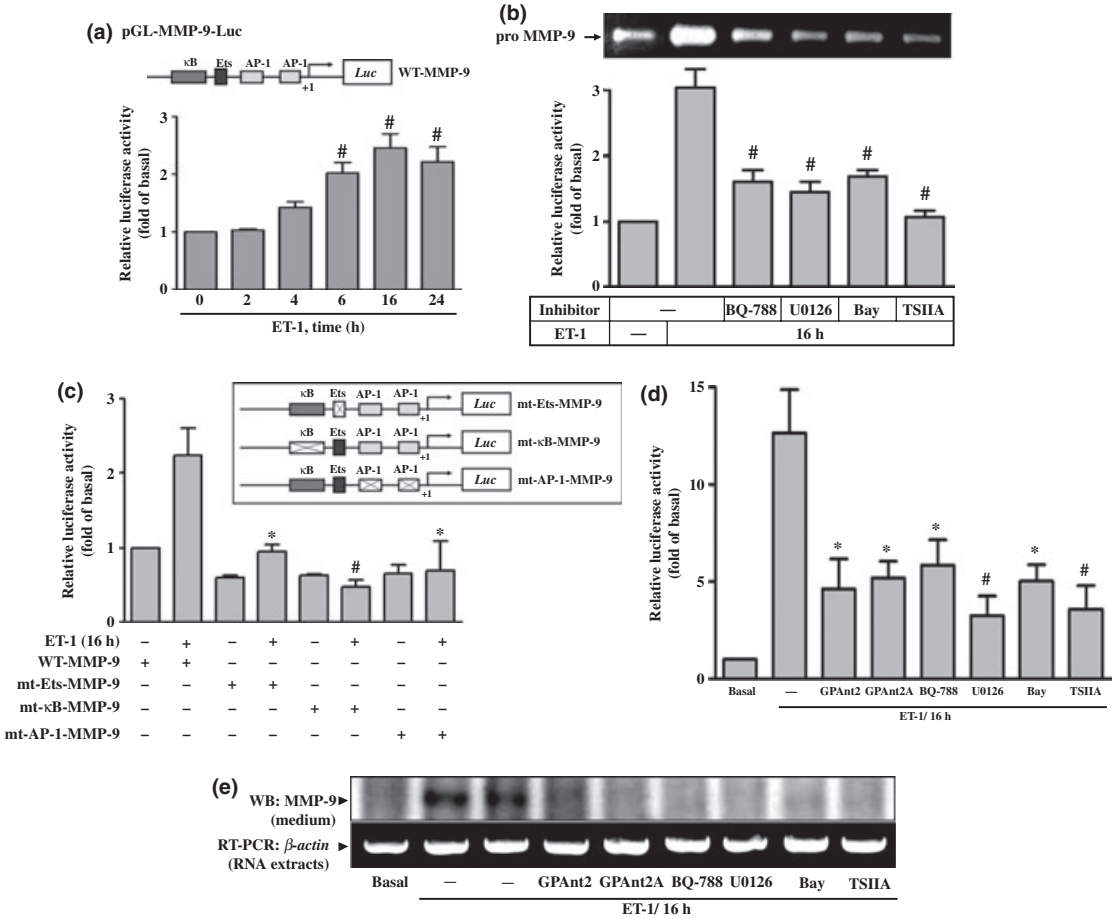

Fig. 7 ET-1-stimulated MMP-9 promoter activity, mRNA expression and protein secretion is mediated through Elk-1/NF-κB/AP-1 pathways in RBA-1 cells. (a) Schematic representation of a 5′-promoter regions of the rat MMP-9 gene fused to the pGL-luciferase reporter gene (pGL-MMP-9-Luc), the translational start site (+1) of the luciferase reporter gene was indicated by an arrow. RBA-1 cells were transiently co-transfected with pGL-MMP9-Luc and pGal encoding for β-galactosidase for 24 h. The cells were treated with or without ET-1 (100 nM) for the indicated time intervals. (b) After co-transfection, cells were pre-treated with BQ-788 (1 μM), U0126 (10 μM), Bay11-7082 (Bay, 1 μM) or TSIIA (10 μM) for 1 h, and then incubated with ET-1 for 16 h. (c) Activation of wild-type (WT), Ets-(mt-Ets), κB-(mt-κB), or AP-1-point-mutated (mt-AP-1) MMP-9 promoter constructs by ET-1. Schematic representation of the different MMP-9-luciferase constructs, either wild-type (WT) or modified by single-point mutation of the Ets, κB, or AP-1 binding site (inset panel). After co-transfection for 24 h, promoter activities of different MMP-9-promoter constructs stimulated with or without ET-1 (100 nM) for 16 h, were measured as relative MMP-9 promoter activity to that of β-galactosidase. The relative increase in MMP-9 promoter activity induced by ET-1 normalized to un-stimulated cells is indicated as fold increase. Cells were pre-treated with GPAnt2 (10 μM), GPAnt2A (10 μM), BQ-788 (1 μM), U0126 (10 μM), Bay11-7082 (Bay, 1 μM), or TSIIA (10 μM) for 1 h, and then incubated with 100 nM ET-1 for 16 h. The expression of MMP-9 mRNA induced by ET-1 was analyzed by real-time PCR (d). Condition media were analyzed by western blotting using an anti-MMP-9 antibody (e). Data are expressed as mean ± SEM of at least three independent experiments. $^{*}p < 0.05$; $^{\#}p < 0.01$, as compared with ET-1 alone. The figure represents one of at least three individual experiments.

NF-κB, and AP-1 indeed mediated ET-1-induced MMP-9 promoter activity through binding to their regulatory elements within the MMP-9 promoter region, the wild-type (WT) MMP-9 promoter mutated by a single-point mutation of the Ets binding site (mt-Ets-MMP-9), the κB binding site (mt-κB-MMP-9), and the AP-1 binding site (mt-AP-1-MMP-9) were constructed (as indicated in Fig. 7c, insert panel), ET-1 stimulated MMP-9 promoter activity was significantly attenuated in RBA-1 cells transfected with mt-Ets-MMP-9, mt-κB-MMP-9, or mt-AP-1-MMP-9, indicating that Ets, κB, and AP-1 elements were essential for ET-1-induced MMP-9 promoter activity. These results further confirmed that ET-1 induced MMP-9 promoter activity via enhancing Elk-1, NF-κB, and AP-1 binding to the Ets, κB, and AP-1 elements of the MMP-9 promoter, respectively, in RBA-1 cells.

Furthermore, we determined the roles of ET-1 receptors and related transcription factors in ET-1-induced MMP-9 gene expression. RBA-1 cells were pre-treated with GPAnt2

(10 μM), GPAnt2A (10 μM), BQ-788 (1 μM), U0126 (10 μM), Bay11-7082 (1 μM), or TSIIA (10 μM) for 1 h and then incubated with ET-1 (100 nM) for 16 h. Pretreatment with these inhibitors significantly attenuated ET-1-induced MMP-9 mRNA (Fig. 7d) and protein (Fig. 7e) expression, suggesting that ET-1 induces MMP-9 gene expression via ET_B receptors linking to activation of p42/p44 MAPK/Elk-1, NF-κB, AP-1 cascades in RBA-1 cells.

Discussion

The MMP-9 activity has been implicated in neuroinflammation, which involves BBB disruption and cell death in the CNS (Yong *et al.* 2001; Rosenberg 2002). It is reported that elevated MMP-9 activity contributes to the progression of CNS pathology (Aoki *et al.* 2002; Wang *et al.* 2002; Harris *et al.* 2007). Using pharmacological blockades or gene knock-out strategies, prevention of MMP-9 activity exhibits protective effects on the brain after cerebral ischemia (Svedin *et al.* 2007; McColl *et al.* 2008). In the CNS, ischemic injury elicits ET-1 production from astrocytes (Hasselblatt *et al.* 2001), related to the destructive outcomes of ischemic brain injury (Lo *et al.* 2005). The physiopathological effects of ET-1 on astrocytes are substantially mediated through ET_B receptors (Nakagomi *et al.* 2000; Rogers *et al.* 2003). However, the role of ET-1 activity on astrocytic functions in the CNS diseases remains elusive. Here, we use cultured models of rat astroglial cell line (RBA-1) and primary rat brain culture of astrocytes to investigate the mechanisms underlying ET-1-induced MMP-9 expression and functional changes. These results suggest that in rat astrocytes, activation of ET_B receptor-dependent p42/p44 MAPK/Elk-1, NF-κB, and AP-1 signaling cascades is essential for ET-1-induced MMP-9 gene expression and cell migration.

In addition to maintain the functional homeostasis in CNS microenvironment, astrocytes have been demonstrated to participate in the regulation of cerebral blood flow. It represents control mechanisms that match oxygen and glucose delivery through blood flow with the local metabolic demands that are imposed by neural activity (Iadecola and Nedergaard 2007). As a fundamental component of the neurovascular unit, recent work has revealed the role of astrocyte dysfunction in neurodegenerative diseases (Iadecola 2004; Guo and Lo 2009). Thus, we study how ET-1 modulates astrocytic functions.

First, we demonstrated that ET-1-triggered activation of signaling molecules is mediated via ET_B receptor in rat astrocytes. By real-time PCR, zymographic, and migration analyses, we found that activation of ET_B receptor coupled to $G_{i/o}$ and G_q proteins was essential for ET-1-induced proMMP-9 expression in rat astrocytes (Fig. 2). These data were consistent with the findings that activation of ET_B receptor is responsible for characteristics of reactive gliosis (Rogers *et al.* 2003; Gadea *et al.* 2008) and of resistance artery remodeling in diabetes (Sachidanandam *et al.* 2007). However, in respiratory and cardiovascular systems, both ET receptor subtypes, ET_A in particular, are involved in progression of airway diseases (De Lagausie *et al.* 2005; Lund *et al.* 2009). The data from real-time PCR showed a predominant expression of ET_B receptor in RBA-1 cells (Fig. 2a). As there was no statistical significance of ET_A-selective antagonist BQ-123 on ET-1-induced proMMP-9 expression (Fig. 2b), we suggested that ET_B receptor played a more important role than ET_A receptor in ET-1-induced MMP-9 induction, consistent with the significance of ET_B receptor in the modulation of astrocytic hypertrophy in the injured CNS (Nakagomi *et al.* 2000; Rogers *et al.* 2003).

Within the MMP-9 promoter, there are numerous transcription factor binding sites, which control MMP-9 gene expression. Additionally, increasing studies have demonstrated that diverse extracellular stimuli, such as IL-1β, bradykinin (BK), and oxidized low-density lipoprotein (oxLDL), prompt MMP-9 induction through Elk-1 (a member of Ets family), NF-κB, and AP-1, respectively (Wu *et al.* 2004; Hsieh *et al.* 2008; Wang *et al.* 2009, 2010). These extensive researches indicate that Ets, NF-κB, and AP-1 may regulate MMP-9 expression through direct, indirect (*e.g.*, induction of other transcription factors such as c-*Fos* or c-*Jun*), or synergistic action. Moreover, a current study has identified that ET-1 induces MMP-9 expression via NF-κB in human osteosarcoma (Felx *et al.* 2006). However, very few data indicate that ET-1 regulates MMP-9 through AP-1 or Ets family. In this study, we demonstrated that ET-1-induced MMP-9 expression is mediated through NF-κB, AP-1, and Elk-1 transcription factors in RBA-1 cells. All these three transcription factors may be essential for MMP-9 up-regulation by ET-1.

Several lines of evidence have suggested that activation of p42/p44 MAPK by ET-1 regulates cellular functions of astrocytes (Schinelli *et al.* 2001; He *et al.* 2007). Activation of MAPK/Elk-1 pathway has also been shown to be involved in MMP-9 induction in different cell types (Tanimura *et al.* 2002; Hsieh *et al.* 2008). We further demonstrated that ET-1 stimulated an ET_B receptor-dependent cascade of sequential p42/p44 MAPK/Elk-1 phosphorylation (Fig. 3b), which contributes to induction of MMP-9 promoter activity (Fig. 7b and c), mRNA, and protein levels (Fig. 7d and e). These results were consistent with those of obtained with BK- and oxLDL-induced MMP-9 expression in RBA-1 cells (Hsieh *et al.* 2008; Wang *et al.* 2010).

The transcription factor NF-κB is a ubiquitous regulator of gene expression associated with inflammatory responses. Our results revealed that IKKα/β and NF-κB activation were required for ET-1-induced proMMP-9 expression by blockade of NF-κB signaling using the pharmacological and transfection strategies (Figs 4 and 5). It has been confirmed that NF-κB signaling is required for MMP-9 induction both *in vitro* and *in vivo* (Hsieh *et al.* 2004; Tai *et al.* 2008), which

enhances cell motility (Hsieh *et al.* 2008) and tumor invasion (Tai *et al.* 2008). The mechanism of NF-κB activation stimulated by ET-1 remains elusive. Our data suggested that ET_B-mediated p42/p44 MAPK activation promoted IKKα/β phosphorylation (Fig. 5c) and NF-κB translocation (Fig. 5b) in RBA-1 cells. In human U87 astrocytoma cells, p42/p44 MAPK has been suggested to be necessary for IKK phosphorylation (Kam *et al.* 2007). Besides, another MAPK member p38 MAPK, has been demonstrated to mediate tumor necrosis factor (TNF)-α-triggered IKK activation in skeletal muscle (De Alvaro *et al.* 2004). These findings imply that in different cell types, distinct MAPK members are potentially associated with IKK phosphorylation. We further provided evidence to ensure that ET-1-triggered NF-κB activity was obligatory for MMP-9 induction. We found that pre-treatment with Bay11-7082 attenuated ET-1-stimulated MMP-9 promoter activity (Fig. 7b), mRNA, and protein levels (Fig. 7d and e), as well as mutation of the NF-κB element retarded ET-1-induced MMP-9 promoter activity (Fig. 7c). These data were consistent with the mechanisms of MMP-9 expression in the human breast cancer cell line MCF-7 (Tai *et al.* 2008; Park *et al.* 2009).

The results from Fig. 5(a) showed that ET-1 stimulated translocation of p65 NF-κB at 30 min and sustained over 120 min. Moreover, the rapid activation of NF-κB is essential for MMP-9 induction in astrocytes (Hsieh *et al.* 2004). According to these results, we considered that ET-1-mediated responses, including NF-κB activation, proMMP-9 mRNA and protein expression occur in a lineage relationship. ET-1 has been shown to induce IL-1β release in astrocytes (Didier *et al.* 2003). Moreover, IL-1β also promotes MMP-9 expression in rat brain astrocytes (Wu *et al.* 2004). These reports further support that other possible mechanisms, such as secretion of inflammatory cytokines, may be involved in ET-1-induced proMMP-9 expression.

Accumulating evidence indicates that MMP-9 is produced via AP-1-dependent mechanisms in various cell types (Byun *et al.* 2006; Wang *et al.* 2009). AP-1 family proteins are important transcription factors that regulate the MMP-9 gene expression by different stimuli (Xu *et al.* 2001; Woo *et al.* 2004). c-*Jun*/c-*Fos* has been shown to be one of the well-characterized examples of AP-1 family. Upon stimulation, c-*Jun* could dimerize with c-*Fos* to form stable heterodimers that bind to a specific AP-1 site in the promoter region of target genes and enhances the gene transcription (Shaulian and Karin 2001). Therefore, we investigated the role of c-*Jun* and c-*Fos* in ET-1-induced proMMP-9 expression. Our data showed that c-*Jun* phosphorylation and c-*Fos* up-regulation were essential for ET-1-induced proMMP-9 expression (Fig. 6), as well as MMP-9 promoter activity (Fig. 7b), mRNA and protein levels (Fig. 7d and e). Such inhibitory effects were also achieved by mutation of the AP-1 element on MMP-9 promoter activity (Fig. 7c). The data were consistent with the mechanisms of MMP-9 expression in human breast cancer cells (Byun *et al.* 2006) and astrocytes (Wang *et al.* 2009).

Cell motility is a vital process involved in embryonic development, wound healing, inflammatory responses, and tumor metastasis (Lauffenburger and Horwitz 1996). Recent studies further suggest the correlation between MMP-9, cell migration, and glial scar formation (Takenaga and Kozlova 2006; Hsu *et al.* 2008), implying that MMP-9 may be involved in brain remodeling after injury. It has been reported that p42/p44 MAPK/Elk-1, NF-κB, and AP-1 are involved in MMP-9 up-regulation, which is crucial for regulating cell motility in different cell types (Byun *et al.* 2006; Hsieh *et al.* 2008; Mantuano *et al.* 2008; Weng *et al.* 2008; Wang *et al.* 2009). In this study, we demonstrated that ET-1 induced cell migration required MMP-9 activity (Fig. 1c) via coordination of transcription factors including Elk-1, NF-κB, and AP-1 (Figs 3d, 4c, and 6d). To rule out the possibility of cell proliferation in ET-1-induced cell migration, hydroxyurea, an inhibitor of DNA synthesis (Yarbro 1992), was used to prevent proliferation of astrocytes during the period of observation in the migration assay. Therefore, we further suggested that up-regulation of proMMP-9 by ET-1 is essential for enhancing RBA-1 migration. Moreover, we found that the pharmacological studies showed ET-1-induced MMP-9 expression is only partially blocked by NF-κB inhibitor Bay11-7082 and AP-1 inhibitor TSIIA. On the other hand, ET-1-induced RBA-1 cell migration is completely blocked by Bay11-7082 and TSIIA. We considered that the discrepancy is because of the different experimental conditions, or ET-1-mediated cell migration may require other proteins besides MMP-9.

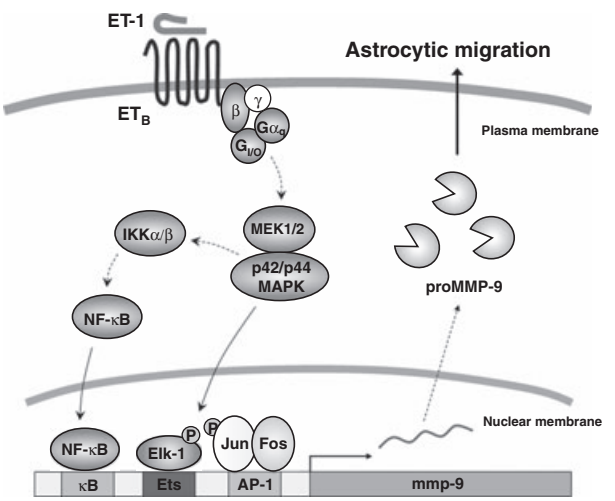

Fig. 8 Schematic representation of signaling pathways for ET-1-induced proMMP-9 expression and cell migration in astrocytes. ET-1 functionally regulates proMMP-9 expression and cell migration via ET_B receptor-dependent p42/p44 MAPK/Elk-1, NF-κB, and AP-1 signaling cascades in astrocytes.

Based on the typical characteristics of astrogliosis, the ability of ET-1 to promote DNA synthesis and hypertrophy has been identified in astrocyte models (Cazaubon *et al.* 1997; Rogers *et al.* 2003). In endothelial cells, ET-1 has been shown to prompt ET_B-mediated migration, which is ultimately correlated with angiogenesis (Daher *et al.* 2008). However, ET-1 also exhibits an anti-migratory effect on neural progenitor cell (Mizuno *et al.* 2005) and endothelial cells (Castañares *et al.* 2007). These findings suggest that the biological effects of ET-1 may be specific differences under distinct conditions.

In conclusion, we demonstrated that ET-1 induces proMMP-9 expression via activation of ET_B receptors coupled to $G_{i/o}$ and G_q and transcription factors like Elk-1, NF-κB, and AP-1 (c-*Jun*/c-*Fos*), resulting in the promotion of cell migration in astrocytes. Based on the observations from literatures and our findings, Fig. 8 depicts a model for the molecular mechanisms implicated in ET-1-induced proMMP-9 expression in RBA-1 cells. These findings imply that ET-1 functionally regulates the progression of brain diseases. The molecular mechanisms underlying ET-1-induced proMMP-9 expression and consequent cell migration may provide potential targets for the treatment of brain injury.

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