

**Dexamethasone Down-regulates Endothelin Receptors and Reduces
Endothelin-induced Production of Matrix Metalloproteinases in Cultured Rat
Astrocytes**

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Abbreviations: Dex,dexamethasone; ECE, endothelin converting enzyme; ERK,extracellular signal regulated kinase; ET,endothelin; GC,glucocorticoid; GFAP, glial fibrillary acidic protein; MMP,matrix metalloproteinase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate

ABSTRACT

In brain disorders, astrocytes change phenotype to reactive astrocytes, and are involved in the induction of neuroinflammation and brain edema. The administration of glucocorticoids (GCs), such as dexamethasone (Dex), reduces astrocytic activation, but the mechanisms underlying this inhibitory action are not well understood. Endothelins (ETs) promote astrocytic activation. Therefore, the effects of Dex on ET receptor expressions were examined in cultured rat astrocytes. Treatment with 300 nM Dex for 6–48 hours reduced the mRNA expression of astrocytic ET_A and ET_B receptors to 30-40 % of non-treated cells. Levels of ET_A and ET_B receptor proteins became about 50 % of non-treated cells after Dex treatment. Astrocytic ET_A and ET_B receptor mRNAs were decreased by 300 nM hydrocortisone. The effects of Dex and hydrocortisone on astrocytic ET receptors were abolished in the presence of mifepristone, a GC receptor antagonist. Although Dex did not decrease the basal levels of matrix metalloproteinase 3 (MMP3) and MMP9 mRNAs, pre-treatment with Dex reduced ET-induced increases in MMP mRNAs. The effects of ET-1 on release of MMP3 and MMP9 proteins were attenuated by pre-treatment with Dex. ET-1 stimulated the phosphorylation of extracellular signal regulated kinase 1/2 (ERK1/2) in cultured astrocytes. Pre-treatment with Dex reduced the ET-induced increases in ERK1/2 phosphorylation. In contrast, pre-treatment with Dex did not affect MMP production or ERK1/2 phosphorylation induced by phorbol myristate acetate, a protein kinase C activator. These results indicate that Dex down-regulates astrocytic ET receptors and reduces ET-induced MMP production.

INTRODUCTION

Glucocorticoids (GCs), such as dexamethasone (Dex), have various pharmacological actions including anti-inflammation, immunosuppression, anabolism, and fluid homeostasis, and are widely used in the clinic. Studies using in animal models reported that GCs prevented the impairment of nerve functions by intracerebral hemorrhage (Yang et al.,2011; Lee et al.,2015), nerve trauma (Holmin and Mathiesen,1996; Genovese et al.,2007) or brain tumor (Gu et al.,2009; Fan et al.,2014), by preventing disruption of the blood-brain barrier (BBB), neuroinflammation and brain edema. Based on these findings, clinical trials to examine the beneficial actions of GCs were undertaken for patients with spinal cord injury (Bracken et al.,1990;1997) or brain tumor (Piette et al.,2006). However, the beneficial actions of GCs are still controversial (Nichols et al.,2005). Thus, further investigation of the actions of GCs in brain disorders is required.

Astrocytes play an important role in the induction of various pathophysiological responses in injured nerve tissues. During brain disorders, astrocytes change phenotype to reactive astrocytes, which are characterized by hypertrophy of the cell body and the increased expression of glial fibrillary acidic protein (GFAP) (Koyama,2014). Accompanying the phenotypic conversion, astrocytes produce various soluble factors that affect the functions of other brain cells and modulate pathophysiological responses (Buffo et al.,2010; Burda et al.,2016). Because these astrocyte-derived factors include pro-inflammatory substances and vascular permeability factors, the induction of reactive astrocytes aggravates nerve injury mediated by neuroinflammation and brain edema (Lopes Pinheiro et al.,2016; Stokum et al.,2016). Astrocytic GC receptors are upregulated by brain injury (Yan et al.,1999; Hwang et al.,2006) and GCs were suggested to affect the pathophysiological functions of astrocytes. In animal models of brain injury, the administration of Dex prevented

the induction of GFAP-positive reactive astrocytes (Imai et al.,2001; Unemura et al.,2012; Spataro et al.,2005). Dex also reduced brain edema formation and the infiltration of inflammatory cells in damaged brain areas (Yang et al.,2011; Lee et a.,2015; Holmin and Mathiesen,1996; Genovese et al.,2007). In addition to its actions on inflammatory cells and brain microvessels, GCs inhibit brain edema and neuroinflammation by attenuating astrocytic activation. However, the mechanisms underlying the inhibitory actions of GCs on astrocytic activation are poorly understood.

In brain disorders, the production of endothelin-1 (ET-1) is increased in damaged nerve tissues and increases in brain ET-1 modulate various pathophysiological responses of the brain (Koyama and Michinaga,2012). Receptors for ETs, especially ET_B, are highly expressed in astrocytes (Peters et al.,2003; Rogers et al.,2003; Wilhelmsson et al.,2004). The administration of a selective ET_B agonist increased the number of GFAP-positive reactive astrocytes in rat brain (Ishikawa et al.,1997; Koyama et al.,2003). Furthermore, ET_B antagonists reduced the induction of reactive astrocytes in animal models of brain injury (Gadea et al.,2008; Koyama et al.,1999; Michinaga et al.,2014). These observations indicate that the activation of astrocytic ET receptors promotes the phenotypic conversion to reactive astrocytes. In addition, activation of ET receptors stimulated the production of astrocytic factors that affect vascular permeability and neuroinflammation, including matrix metalloproteinases (MMPs) (Koyama and Tanaka,2008; 2010; Koyama et al.,2011; 2012; 2013). Because the excessive production of MMPs disrupts the BBB, the activation of astrocytic ET receptors is thought to promote brain edema formation in injured nerve tissues. In support of this, ET antagonists ameliorated the disruption of BBB and brain edema formation in several types of brain injury (Moldes et al.,2012; Kim et al.,2013; Michinaga et al.,2014). Thus, GCs and ET-1 have opposing roles in the regulation of astrocytic functions,

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but interactions between GC and ET signals in astrocytes have not been reported. To clarify the mechanisms underlying the inhibitory action of GCs on astrocytic functions, the present study examined the effects of Dex on the expressions of ET-related molecules in cultured rat astrocytes. We report that Dex decreased astrocytic ET_A and ET_B receptor expression and that the down-regulation of ET receptors was accompanied with a reduction of MMP3 and MMP9 production by ET-1.

MATERIALS AND METHODS

Preparation of primary cultured astrocytes from rat brain: All experimental protocols conformed to the Guide for the Care and Use of Laboratory Animals by the U.S. National Institute of Health, and were approved by the Animal Experiment Committee of Osaka Ohtani University. Astrocytes were prepared from the cerebra of 1–2-day-old Wistar mixed rats as described previously (Koyama et al.,2012). Isolated cells were seeded at 1×10^4 cells/cm² in 75 cm² culture flasks and grown in minimal essential medium (MEM) supplemented with 10% fetal calf serum. To remove small process-bearing cells (mainly oligodendrocyte progenitors and microglia from the protoplasmic cell layer), the culture flasks were shaken at 250 rpm overnight, 10–14 days after seeding. The monolayer cells were trypsinized and seeded on 6-well culture plates or 6-cm culture dishes. At this stage, approximately 95% of the cells showed immunoreactivity for GFAP.

Treatment of cultured astrocytes with GCs and ET-1: Before treatment with GCs, astrocytes in 6-well culture plates were incubated in serum-free MEM for 24 h. Dex (Nacalai Tesque, Osaka, Japan) and hydrocortisone (Nacalai Tesque) were dissolved in dimethyl sulfoxide to make stock solutions. After the culture medium was replaced with fresh serum-free MEM, an aliquot of Dex or hydrocortisone solution was added to the medium. For controls, an equal amount of dimethyl sulfoxide was added. Then, astrocytes were treated for the time indicated at 37°C. In some experiments, astrocytes treated with Dex were further treated with ET-1 (Peninsula Lab. Inc. Belmont, CA, USA) or phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, St Louis, MO, USA), where Dex-containing MEM was replaced with fresh serum-free MEM. After treatment with the agents, cultured astrocytes were rinsed with ice-cold phosphate-buffered saline and used to prepare total RNA and cell lysates.

Measurement of mRNA levels by quantitative RT-PCR: Total RNA in cultured astrocytes was extracted using a total RNA extraction kit (Favorgen Biotech Corp., Ping-Tung, Taiwan). First-strand cDNA was synthesized from total RNA (1 µg) using MMLV reverse transcriptase (200 U; Invitrogen, Carlsbad, CA, USA), random hexanucleotides (0.2 µg; Invitrogen) and an RNase inhibitor (20 U; Takara, Tokyo, Japan) in 10 µl of a buffer supplied by the enzyme manufacturer. mRNA levels of ET related signal molecules in each sample were determined by quantitative PCR using SYBR Green fluorescent probes. Each reverse transcription product was added to Sybr Green Master Mix (Toyobo, Tokyo, Japan) along with the primer pairs, and the mixture was then placed in a thermal cycler (Opticom 2; BioRad, Hercules, CA, USA). The following primer pairs were used:

preproET-1, 5'-TGTGTCTACTTCTGCCACCT-3' and 5'-CACCAGCTGCTGATAGATAC-3';
preproET-3, 5'-GGGACCAGGAGGAAAAGAGGGTG-3' and 5'-
ACTGGGAACCTTTCTGGAACTGG-3'; endothelin converting enzyme (ECE) 1,
5'-GAGAAGCGCCGGGATGA-3' and 5'-GGCATTTCAGAAAGGGTAACCAG-3'; ECE2, 5'-
AATGAAATCGTCTTCC-3' and 5'-GTGAGTGAATTCATTC-3'; ET_A receptor,
5'-TGCCCTCAGCGAACACC-3' and 5'-CATAGACGGTTTTCTTCAA-3'; ET_B receptor,
5'-GATACGACAACTTCCGCTCCA-3' and 5'-GTCCACGATGAGGACAATGAG-3'; MMP2,
5'-CTATTCTGTCAGCACTTTGG-3' and 5'-CAGACTTTGGTTCTCCA-3'; MMP3,
5'-GAGGACAAATTCTGGAGATTTGATG-3' and 5'-GTGAAGATCCGCTGAAGAAGTAAAG
-3'; MMP9, 5'-AAATGTGGGTGTACACAGGC-3' and 5'-TTCACCCGGTTGTGGAACT-3';
G3PDH, 5'-CTCATGACCACAGTCCATGC-3' and 5'-TACATTGGGGGTAGGAACAC-3'.

As a standard for the copy numbers of PCR products, serial dilutions of each amplicon were amplified in the same manner. The amount of mRNA was calculated as the copy number of each reverse-transcription product equivalent to 1 µg of total RNA, and normalized to the

value for G3PDH.

Measurement of protein levels by immunoblotting: Cultured astrocytes in 6-well culture plates were dissolved in 100 μ l of ice-cold homogenization buffer (20 mM Tris/HCl, pH 7.4, 1% sodium dodecyl sulfate (SDS), 2 mM ethylene-diaminetetra acetic acid, 2 mM phenylmethylsulfonyl fluoride, 20 μ g/ml aprotinin) at 4°C. The lysates were centrifuged at 15,000 $\times g$ for 10 min, and the protein contents of the supernatants were measured. The cell lysates were applied to SDS polyacrylamide gel electrophoresis and electroblotted onto polyvinylidene fluoride membranes. For the detection of ET_A and ET_B receptor proteins, the membranes were first probed with rabbit anti-ET_A receptor (1:1000 dilution, H-60, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit anti-ET_B receptor (1:4,000 dilution, M-74, Santa Cruz Biotechnology), respectively. Then, membranes were incubated with peroxidase-conjugated secondary antibodies. The exposed X-ray films were scanned, and the densities of the protein bands were measured using ImageJ 1.45 software (US. NIH, Bethesda, Maryland, USA). After the detection of ET receptor proteins, the membranes were re-probed with mouse anti- β -actin primary antibody (1:4000 dilution, Chemicon, Temecula, CA, USA) and the protein bands were quantified. Expression levels of ET receptor proteins were determined as a ratio to β -actin proteins. To detect the phosphorylation levels of extracellular signal regulated kinase 1/2 (ERK1/2), the membranes were first probed with rabbit anti-phospho-ERK1/2 (1:4000 dilution, Cell Signal Tech Inc., Danver, MA, USA) and re-probed with rabbit anti-ERK1/2 (1:4000 dilution, Cell Signal Tech Inc.). Levels of protein phosphorylation were indicated as a ratio of phosphorylated ERK1/2 protein to total ERK1/2 protein.

Determinations of MMP3 and MMP9 protein release by enzyme-linked immunosorbent assay: Cultured astrocytes in 6 cm culture dishes were treated with Dex and ET-1 in

serum-free MEM. The concentration of MMP3 and MMP9 proteins in the culture medium were measured by enzyme-linked immunosorbent assay (ELISA) kits for rat MMP3 (Cloud-Clone Corp., Houston, TX, USA) and rat MMP9 (R&D Systems, Minneapolis, MN, USA) according to the suppliers' protocols. After the culture medium was collected, astrocytes in 6 cm dishes were dissolved with 0.1 N NaOH. The cell lysate was used to determine the total protein content. The amount of released MMP3 and MMP9 protein in culture medium was normalized to the total protein content of each dish.

Statistical Analysis: Results are presented as the mean \pm standard deviation (SD). Results were analyzed by one-way ANOVA followed by post-hoc analysis. *P*-values were calculated using Ekuseru-Toukei2015 ver 1.02 (Social Survey Research Information Co., Ltd, Tokyo, Japan). *P*-values less than 0.05 were considered to be statistically significant.

RESULTS

Expression levels of ET ligands, ECEs, and ET receptors in cultured rat astrocytes:

Astrocytes, the major target cells of brain ETs, produce and release ET ligands. First, we investigated the expression levels of ET signal related molecules, i.e., ET ligands, ECEs, and ET receptors in cultured astrocytes. In non-treated astrocytes, mRNAs for preproET-1 and preproET-3 were detected, and the copy number of prepro-ET-1 was about 100 times higher than that of prepro-ET-3 (Table 1). ECEs are a peptidase family that process ET precursor peptides (i.e. big ETs) to bioactive mature ETs. Copy numbers of ECE1 and ECE2 in cultured astrocytes were similar. Although both ET_A and ET_B receptor mRNAs were detected in cultured astrocytes, the copy number of ET_B receptors was about 10 times higher than that of ET_A receptors.

Effects of Dex on the expression of ET system related molecules: Treatment with 300 nM Dex for 3–48 hours had no obvious effect on the expression of preproET-1, preproET-3, ECE1, or ECE2 mRNAs in cultured rat astrocytes (Fig. 1A). ET_A receptor mRNA expression was decreased by treatment with Dex for 3 hours, and became about 30% of non-treated cells in 12–28 hours. ET_B receptor mRNA expression was also decreased to 30–40% of the non-treated cells by treatment with Dex for 12–48 hours. Immunoblot analysis showed that Dex decreased protein levels of ET_A and ET_B receptors in cultured astrocytes (Fig. 1B). Decreases in ET receptor mRNAs by Dex were dose-dependent, and statistically significant decreases in ET_A and ET_B receptors were obtained at concentrations greater than 30 nM (Fig. 2A). Dex also dose-dependently decreased protein levels of astrocytic ET_A and ET_B receptors (Fig. 2B). Hydrocortisone (300 nM), an endogenous GC, decreased mRNA levels of astrocytic ET_A and ET_B receptors (Fig. 3A). Decreased ET receptor mRNA expressions by

Dex and hydrocortisone were abolished by the addition of 50 nM mifepristone, a GC receptor antagonist (Fig. 3B and 3C).

Reduction of ET-induced MMP production by pre-treatment with Dex: MMPs are zinc endopeptidases that degenerate several extracellular matrix and membrane proteins on the cell surface. The excessive production of brain MMPs cause brain edema and infiltration of inflammatory cells through disruption of the BBB. Astrocytes produce MMP2, MMP3 and MMP9, whose functions are stimulated by ETs (Koyama and Tanaka,2008;2010). To clarify whether the Dex-induced down-regulation of astrocytic ET receptors would affect astrocytic functions, the effect of Dex on MMP production by ET-1 was examined. Treatment of cultured astrocytes with 300 nM Dex for 3–48 hours did not decrease MMP3 mRNA expression, but transient increases were observed after 3–6 hours treatment (Fig. 4A). MMP9 mRNA expression was not altered by Dex treatment for 3–48 hours, whereas MMP2 mRNA was decreased.

ET-1 (100 nM) increased astrocytic MMP3 and MMP9 mRNA expression (Fig. 4B). Two-way ANOVA showed that statistically significant interactions between ET-1 and Dex treatments on MMP3 and MMP9 mRNA expression (MMP3: $F(1,3) = 5.419, p < 0.05$, MMP9: $F(1,3) = 5.086, p < 0.05$), where effects of ET-1 were attenuated (Fig. 5A). ETs stimulated astrocytic MMP3 and MMP9 production through the activation of protein kinase C (PKC) (Koyama and Tanaka,2008;2010). Phorbol 12-myristate 13-acetate (PMA, 100 nM), a PKC activator, increased MMP3 and MMP9 mRNA expression in cultured astrocytes (Fig. 5B). Pre-treatment with Dex showed no interaction with effect of PMA on MMP9 expression ($F(1,3) = 0.148, p = 0.930$, by two-way ANOVA). On the other hand, a statistically significant interaction between Dex and PMA treatments were shown in MMP3 expression ($F(1,3) = 9.284, p < 0.001$, by two-way ANOVA, Fig. 5B), where effect of PMA was enhanced,

but not attenuated (Fig. 5B). ET-1 increased the release of MMP3 and MMP9 proteins from cultured astrocytes (Fig. 6). The ET-induced release of MMP3 and MMP9 proteins were reduced by pre-treatment with Dex.

Reduction of ET-induced ERK1/2 activation by pre-treatment with Dex: Stimulation of astrocytic ET receptors induces the activation of ERK1/2, which involves astrocytic MMP3 and MMP9 production (Koyama and Tanaka,2008;2010). The effects of pre-treatment with Dex on ET-induced ERK1/2 activation were examined. ET-1 (100 nM) increased the phosphorylated (activated) forms of ERK1/2 proteins in cultured astrocytes (Fig. 7A). Pre-treatment with 300 nM Dex had no effect on the basal levels of astrocytic ERK1/2 phosphorylation. However, two-way ANOVA showed a statistically significant interaction between ET-1 and Dex treatments on ERK1/2 phosphorylation ($F(1,3) = 4.174, p < 0.05$, Fig. 7A). In contrast, pre-treatment with Dex showed no interaction with effect of PMA on ERK1/2 phosphorylation in cultured astrocytes ($F(1,3) = 0.682, p = 0.563$, Fig. 7B).

DISCUSSION

Dex down-regulates ET_A and ET_B receptors in cultured rat astrocytes: In brain disorders, reactive astrocytes produce and release soluble factors to increase the permeability of brain microvessels, which causes brain edema and allows the infiltration of inflammatory cells (Lopes Pinheiro et al.,2016; Stokum et al.,2016). Therefore, the management of astrocytic activation might be a promising target for novel neuroprotective drugs (Buffo et al.,2010; Karimi-Abdolrezaee and Billakanti,2012). Activation of ET signaling promotes the phenotypic conversion of normal astrocytes to reactive astrocytes and the production of vascular permeability factors (Koyama and Tanaka,2008; 2010; Koyama et al.,2011; 2012; 2013). In contrast, the administration of GCs, such as Dex, reduces the induction of reactive astrocytes accompanied by an amelioration of brain edema and neuroinflammation (Yang et al.,2011; Lee et a.,2015; Holmin and Mathiesen,1996; Genovese et al.,2007). However, interactions between GC and ET signals in astrocytes have not been reported. In this study, treatment with Dex and hydrocortisone decreased the expressions of ET_A and ET_B receptors in cultured rat astrocytes (Figs. 1–3). The effects of Dex and hydrocortisone were antagonized by mifepristone (Fig. 3B and 3C), suggesting the involvement of nuclear GC receptors. Both ET_A and ET_B receptors, which are G-protein coupled receptors and linked to Gq sub-types, are present in the brain. However, these ET receptors are differently expressed among brain cells. Previous studies showed that ET_B types were highly expressed in astrocytes (Peter et al.,2003; Rogers et al.,2003; Wilhelmsson et al.,2004). The higher expression of ET_B receptors compared with ET_A receptors by astrocytes was confirmed by comparing their mRNA copy numbers (Table 1). The administration of an ET_B selective agonist promoted the induction of GFAP-positive reactive astrocytes in rat brain (Ishikawa et al.,1997; Koyama et al.,2003), whereas ET_B

antagonists, but not ET_A antagonists, decreased reactive astrocytes in injured brain (Koyama et al.,1999; Michinaga et al.,2014;2015). These findings suggest that ET_B receptors are predominantly involved in the ET-induced phenotypic conversion to reactive astrocytes. In contrast to the actions of ETs, Dex inhibited the induction of GFAP-positive reactive astrocytes and their functions (Imai et al.,2001; Unemura et al.,2012; Spataro et al.,2005). As for GFAP expression, we found that treatments with Dex had no effect on GFAP mRNA levels in cultured astrocytes by itself (Supplemental figure 1). Therefore, the down-regulation of astrocytic ET receptors, especially ET_B, by Dex might be related to the inhibitory actions of Dex on astrocytic phenotypic conversion.

Shibata et al. (1995) reported that the administration of Dex decreased ET_B receptor expression in the rat hypothalamus, although the cell types showing ET receptor down-regulation were not examined. In this report, the authors suggested that the increased production of brain ET-1 by Dex indirectly caused the decrease in ET_B receptors. Supporting this mechanism, the production of ET-1 in cultured astrocytes was increased by a high concentration (10 μM) of Dex (Hasselblatt et al.,2001). However, the Dex-induced production of ET-1 is unlikely to mediate the effects of Dex, because the concentrations of Dex used in the present study (300 nM) did not increase preproET-1 mRNA levels in cultured astrocytes (Fig. 1A). In addition, we confirmed that treatment with 300 nM Dex for 6-24 hours did not stimulate the extracellular release of ET-1 peptide from cultured astrocytes (Supplemental figure 2). Therefore, mechanisms other than ET receptor activation are thought to underlie the Dex-induced down-regulation of astrocytic ET receptors.

Dex reduced ET-induced production of MMP3 and MMP9: MMPs are endopeptidases that regulate tissue re-modeling by degrading extracellular matrix proteins. In brain

disorders, the production of MMP2, MMP3 and MMP9 are increased, causing brain edema and the infiltration of inflammatory cells by disrupting the BBB (Rosell and Ho,2008; Planas et al.,2001). Astrocytes produce MMP2, MMP3 and MMP9, which are stimulated with their conversion to reactive astrocytes (Muir et al.,2002; Rivera et al.,2002; Koyama and Tanaka,2010). The administration of Dex decreased MMP3 and MMP9 production induced by brain injury and cytokines in nerve tissue and glial cells (Yang et al.,2011; Liu et al.,2008; Tenenbaum et al.,2008; Green et al.,2010). We found that treatment with Dex did not decrease basal levels of MMP3 and MMP9 expression in cultured astrocytes (Fig. 4A). However, pre-treatment with Dex reduced the effects of ET-1 on MMP3 and MMP9 production (Fig. 5A). These results suggest that Dex attenuated ET receptor-mediated signals leading to MMP3 and MMP9 production. The activation of PKC and ERK signaling are involved in astrocytic MMP3 and MMP9 production (Kunapuli et al.,2004; Lee et al.,2003; Arai et al.,2003). In agreement with these observations, PMA activated astrocytic ERK1/2 and stimulated MMP expression (Figs. 5B and 7B) and ET-induced astrocytic MMP3 and MMP9 production was mediated by PKC/ERK signals (Koyama and Tanaka,2008;2010). Although both ET-1 and PMA activated ERK1/2, different effects of Dex on ERK1/2 activation were obtained (Fig. 7). Dex reduced the activation of ERK1/2 by ET-1, but not by PMA. In addition, the inhibitory actions of Dex on MMP production were shown during ET-1, but not PMA, treatment (Fig. 5). These findings suggest that Dex attenuates ET signals leading to astrocytic MMP production upstream of PKC/ERK activation. Treatment with Dex decreased the expression of astrocytic ET_A and ET_B receptors (Figs. 1 and 2). Considering the effects of Dex on ET receptors, the selective reduction of ET signals might be caused by the down-regulation of ET receptors. Because the stimulation of either ET_A or ET_B receptors activated PKC/ERK signals through Gq protein (Koyama,2013), both ET

receptor types are involved in ET-induced MMP production. However, the levels of ET_B receptors in cultured astrocytes were much higher than that of ET_A receptors (Table 1). Moreover, ET-induced astrocytic MMP3 and MMP9 production was selectively inhibited by an ET_B antagonist, but not by an ET_A antagonist (Koyama and Tanaka,2008;2010). Thus, although an involvement of ET_A receptors cannot be excluded, the major effect of Dex on ET-induced MMP3 and MMP9 productions is mediated by the down-regulation of ET_B receptors. Differing from MMP3 and MMP9, astrocytic MMP2 was decreased by Dex treatment (Fig. 4A). MMP2 cleaves an inactive form of MMP9 to activate it. So, in addition to the actions on ET receptors, Dex may decrease MMP9 activity also by reduction of astrocytic MMP2 production.

While prolonged treatments with Dex attenuated effects of ET-1 on MMP3 expression, a transient increase in MMP3 mRNA expression was also observed (Fig. 4B). In addition, pre-treatment with Dex enhanced PMA-induced MMP3 expression (Fig. 5B). These observations suggest a stimulatory action of Dex on astrocytic MMP3 production. The transient increase in MMP3 mRNA levels was not reduced by mifepristone (Supplemental figure 3). Pre-treatment with Dex did not affect PMA-induced ERK activation (Fig. 7B). These observations indicate that activation of neither PKC/ERK nor nuclear CG receptor mediates the stimulatory action on MMP3 production by Dex. Because stimulatory actions of Dex and other GCs on MMP3 production have not been reported in any cell types, at present, we cannot refer possible mechanisms clearly. However, in hippocampal neurons, treatments with Dex caused an increase in cytosolic Ca²⁺ and enhanced neurotoxicity through mifepristone-insensitive and non-genomic mechanisms (Takahashi et al.,2002; Xiao et al.,2010). As for the non-genomic actions of Dex, an involvement of membrane-bound GC receptors, which have not been identified yet, was

suggested (Groeneweg et al.,2012). So, it may be possible that the stimulatory action on MMP3 expression is mediated by membrane-bound GC receptors and related to neurotoxicity of Dex.

Significance of Dex-induced down-regulation of astrocytic ET receptors; a mechanism of anti-brain edema action: Brain edema is a mortal pathological state that occurs during the acute phase of stroke and head trauma, and management of brain edema is critical to improve the prognosis of patients. GCs, such as Dex, were shown to prevent disruption of the BBB and brain edema formation in animal models of intracerebral hemorrhage (Yang et al.,2011;Lee et al.,2015), nerve trauma (Holmin and Mathiesen,1996; Genovese et al.,2007) and brain tumor (Gu et al.,2009; Fan et al.,2014). However, the mechanisms underlying the neuroprotective actions of GCs have not been fully determined. Investigations on the anti-brain edema action of GCs have proposed several mechanisms including inhibitory actions on pro-inflammatory cells and the stabilization of tight junctions in brain endothelial cells (Salvador et al.,2014). In addition, astrocytes are thought to be involved in the actions of GCs, because their conversion to reactive astrocytes was inhibited by GCs (Imai et al.,2001; Unemura et al.,2012; Spataro et al.,2005). In perivascular areas, astrocytes surround brain microvessels with a specialized structure called “end-feet”. The astrocyte-endothelial cell unit has a major role in BBB functions (Alvarez et al.,2013; Wolburg et al.,2009), where barrier functions of the BBB are dynamically controlled by astrocyte-derived permeability factors. The production of astrocyte-derived permeability factors are stimulated by the conversion to reactive astrocytes (Buffo et al.,2010; Burda et al.,2016) and this disrupts the BBB leading to brain edema and the infiltration of inflammatory cells (Lopes Pinheiro et al.,2016; Stokum et al.,2016). In injured nerve tissues, reactive astrocytes produce MMP3 and MMP9 (Muir et al.,2002; Rivera et al.,2002; Koyama

and Tanaka,2010), which increase vascular permeability by degrading the basal lamina and endothelial tight junctions (Rosell and Ho,2008; Planas et al.,2001). The administration of an ET_B antagonist prevented the induction of reactive astrocytes after brain injury (Koyama et al.,1999; Kim et al.,2013). Moldes et al. (2012) showed that ET antagonists alleviated brain infarct and edema in a brain ischemia model. Our studies on brain edema formation and BBB disruption after brain cold-injury showed that the protective actions of ET antagonists were mediated by a reduction of astrocytic permeability factors, including MMPs (Michinaga et al.,2015). These studies indicate that a reduction of astrocytic ET signals is beneficial to prevent brain edema formation and BBB disruption. The present study showed that Dex down-regulated ET receptors and attenuated ET signals in astrocytes. The administration of GCs is known to have beneficial effects against disruption of the BBB, brain edema and neuroinflammation in several brain disorders (Kim et al.,2008; Salvador et al.,2014). Although further examinations by using *in vivo* brain injury models are required, the Dex induced down-regulation of astrocytic ET receptors may be involved in the neuroprotective actions of GCs.

Authorship Contribution

Participated in research design: Koyama and Tokuyama.

Conducted experiments: Koyama, Ukita, Abe, Iwamae and Kotake.

Performed data analysis: Tanaka and Kotake

Wrote or contributed to the writing of the manuscript: Koyama.

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FOOTNOTES

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The authors have no conflicts of interest with this study.

FIGURE LEGENDS

Fig. 1. (A) Effects of Dex on ECE, ET ligand and ET receptor mRNA expression in cultured rat astrocytes. Cultured rat astrocytes were treated with 300 nM Dex for the times indicated. The expression levels of ECE, preproET, and ET receptor (ET-R) mRNAs were normalized to that of G3PDH. The results are expressed as the mean \pm SD of 8–12 experiments. Dex caused statistically significant decreases in ET_A and ET_B receptor mRNAs. Expression levels of ET_A receptor mRNA in Dex treatment for 12 and 24 hours were 29.5% (95% confidence interval (CI) 16.6–42.4%) and 26.1% (95% CI 15.7–36.5%) of 0-time, respectively. ET_B receptor mRNA in Dex treatment for 24 and 48 hours were 30.8% (95% CI 19.3–42.3%) and 31.4% (95% CI 26.8–36.0%) of 0-time, respectively. * $p < 0.05$, ** $p < 0.01$ vs. 0-time by one-way ANOVA followed by Dunnett's test. **(B) Effects of Dex on levels of ET receptor proteins in cultured astrocytes.** Cultured astrocytes were treated with 300 nM Dex for 12, 24, and 48 hours. The levels of ET receptor (ET-R) proteins were measured by immunoblotting. After the detection of ET receptors, blots were re-probed by an anti- β -actin antibody to confirm that equal amounts of protein were loaded in each lane. Typical patterns of immunoblots for ET_A and ET_B receptors are indicated above the graphs. The graphs show the densitometric analysis of ET_A and ET_B receptor proteins. The results are shown as the mean \pm SD of 6–7 different preparations, and presented as ratios of ET receptor/ β -actin proteins. Dex caused statistically significant decreases in ET_A and ET_B receptor proteins. Expression levels of ET_A receptor protein in Dex treatment for 24 and 48 hours were 41.5% (95% CI 23.0–60.0%) and 35.5% (95% CI 22.0–49.1%) of none, respectively. ET_B receptor protein in Dex treatment for 24 and 48 hours were 75.5% (95% CI 64.8–86.2%) and 57.4% (95% CI 47.5–67.2%) of none, respectively. * $p < 0.05$, ** $p < 0.01$ vs none by one-way ANOVA followed by Dunnett's test.

Fig. 2. (A) Dose-responses of Dex-induced decreases in astrocytic ET receptor mRNAs: Cultured astrocytes were treated with the indicated concentrations of Dex for 24 hours. The results are the mean \pm SD of 7–8 experiments. $*p < 0.05$, $**p < 0.01$ vs none by one-way ANOVA followed by Dunnett's test. **(B) Dose-responses of Dex-induced decreases in astrocytic ET receptor proteins:** Cultured astrocytes were treated with the indicated concentrations of Dex for 24 hours. The results are the mean \pm SD of 6–7 experiments. $*p < 0.05$, $**p < 0.01$ vs none by one-way ANOVA followed by Dunnett's test.

Fig. 3. (A) Effects of hydrocortisone on ET receptor mRNA expression. Cultured astrocytes were treated with 300 nM hydrocortisone for the times indicated. The expression of ET receptor (ET-R) mRNAs was normalized to G3PDH. The results are expressed as the mean \pm SD of 7 experiments. $*p < 0.05$, $**p < 0.01$ vs 0-time by one-way ANOVA followed by Dunnett's test. **(B) Effects of mifepristone on Dex-induced decreases in ET receptor mRNAs.** Cultured astrocytes were treated with 30 nM Dex for 48 hours in the presence or absence of 50 nM mifepristone. The results are expressed as the mean \pm SD of 13–14 experiments. $*p < 0.05$ vs. non-treatment (no Dex in the absence of mifepristone), $^{\#}p < 0.05$ vs no mifepristone by two-way ANOVA followed by Tukey's test. **(C) Effects of mifepristone on hydrocortisone-induced decreases in ET receptor mRNAs.** Cultured astrocytes were treated with 50 nM hydrocortisone for 48 hours in the presence or absence of 50 nM mifepristone. The results are expressed as the mean \pm SD of 11–12 experiments. $**p < 0.01$ vs non-treatment, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs no mifepristone by two-way ANOVA followed by Tukey's test.

Fig. 4. (A) Effects of Dex on MMP3 and MMP9 mRNA expression in cultured rat astrocytes. Cultured astrocytes were treated with 300 nM Dex for the times indicated. The expression of MMP3 (black circle) and MMP9 (white circle) mRNAs were normalized to that of G3PDH. The results are expressed as the mean \pm SD of 11–12 experiments. * p < 0.05 vs. 0-time by one-way ANOVA followed by Dunnett's test. **(A) Effects of ET-1 on MMP3 and MMP9 mRNA expression in cultured rat astrocytes.** Cultured astrocytes were treated with 100 nM ET-1 for the times indicated. The expression of MMP3 (black circle) and MMP9 (white circle) mRNAs were normalized to that of G3PDH. The results are expressed as the mean \pm SD of 7–12 experiments. * p < 0.05, ** p < 0.01 vs. 0-time by one-way ANOVA followed by Dunnett's test.

Fig. 5. Effects of pre-treatment with Dex on ET- or PMA-induced increases in MMP mRNAs. (A) ET-1: Cultured astrocytes were treated with 300 nM Dex for 48 hours in serum-free MEM. After the pre-treatment, fresh serum-free MEM replaced the Dex-containing MEM and astrocytes were further incubated for 3 hours. Then, astrocytes were treated with ET-1 for 2 hours at the concentrations indicated. MMP3 and MMP9 mRNA expressions were determined as described above. The results are the mean \pm SD of 6 and 10 experiments for MMP3 and MMP9, respectively. * p < 0.05, ** p < 0.01 vs 0 nM ET-1, # p < 0.05, ### p < 0.01 vs no Dex pre-treatment by two-way ANOVA followed by Fisher's test. **(B) PMA:** Cultured astrocytes were treated with Dex as described above. Then, cultured astrocytes were treated with PMA for 6 hours at the concentrations indicated, and the expression of MMP3 and MMP9 mRNAs were determined. The results are the mean \pm SD of 7 and 9 experiments for MMP3 and MMP9, respectively. * p < 0.05, ** p < 0.01 vs 0 nM PMA, # p < 0.05, ### p < 0.01 vs no Dex pre-treatment by two-way ANOVA followed by Fisher's

test.

Fig. 6. Effects of pre-treatment with Dex on ET-induced release of MMP3 and MMP9 proteins from cultured astrocytes. Cultured astrocytes were treated with 300 nM Dex for 48 hours in serum-free MEM. After Dex pre-treatment, fresh serum-free MEM replaced the Dex-containing MEM and the astrocytes were further incubated for 3 hours. Then, astrocytes were further cultured in the presence or absence of 100 nM ET-1 for 12 hours. Concentrations of MMP3 **(A)** and MMP9 **(B)** proteins in cultured medium were determined by ELISA. The results are the mean \pm SD of 8 experiments and are expressed as a percentage of the control with no Dex treatment. The results are the mean \pm SD of 6 experiments. $**p < 0.01$ vs control, $\#p < 0.05$ vs no Dex treatment by two-way ANOVA followed by Tukey's test.

Fig. 7. Effects of pre-treatment with Dex on the phosphorylation of ERK1/2 by ET-1 and PMA. (A) ET-1: Cultured astrocytes were treated with 300 nM Dex for 48 hours in serum-free MEM. After Dex pre-treatment, fresh serum-free MEM replaced the Dex-containing MEM and the astrocytes were further incubated for 3 hours. Then, astrocytes were treated with ET-1 for 20 min at the concentrations indicated. Phosphorylated and total ERK1/2 proteins were detected by immunoblotting and quantified. Phosphorylation of ERK1/2 is presented as the ratio of phosphorylated/total proteins. The results are the mean \pm SD of 7 experiments. $**p < 0.01$ vs respective 0 nM ET-1, $\#p < 0.05$, $##p < 0.01$ vs no Dex treatment by two-way ANOVA followed by Tukey's test. **(B) PMA:** Cultured astrocytes were treated with 300 nM Dex for 48 hours in serum-free MEM. After Dex pre-treatment, fresh serum-free MEM replaced the Dex-containing MEM and the astrocytes were further

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incubated for 3 hours. Then, astrocytes were treated by PMA for 20 min at the concentrations indicated. The results are the mean \pm SD of 6 experiments. * p < 0.05, ** p < 0.01 vs respective 0 nM PMA by two-way ANOVA followed by Tukey's test.

Table 1. Comparison of the mRNA copy numbers of ET ligands, ECEs and ET receptors in rat cultured astrocytes.

	mRNA copy number (x 10 ³ /μg total RNA)		
Prepro-ET-1	126.0	± 20.7	(9)
Prepro-ET-3	1.21	± 1.32	(9)
ECE1	1,781.6	± 1077.6	(9)
ECE2	499.2	± 141.0	(9)
ET _A receptor	604.2	± 261.3	(9)
ET _B receptor	7,072.1	± 4041.2	(12)
G3PDH	39,834.3	± 30545.0	(12)

Cultured astrocytes were prepared from the cerebra of Wistar rats, and total RNA was extracted. The mRNA copy numbers of ET ligands, ECEs and ET receptors were determined by quantitative RT-PCR. The copy numbers of G3PDH mRNA in the same samples were also determined. The data are the means ± SD and are presented as ×10³ copy numbers/μg total RNA. Numbers of total RNA preparations are given in the parenthesis.

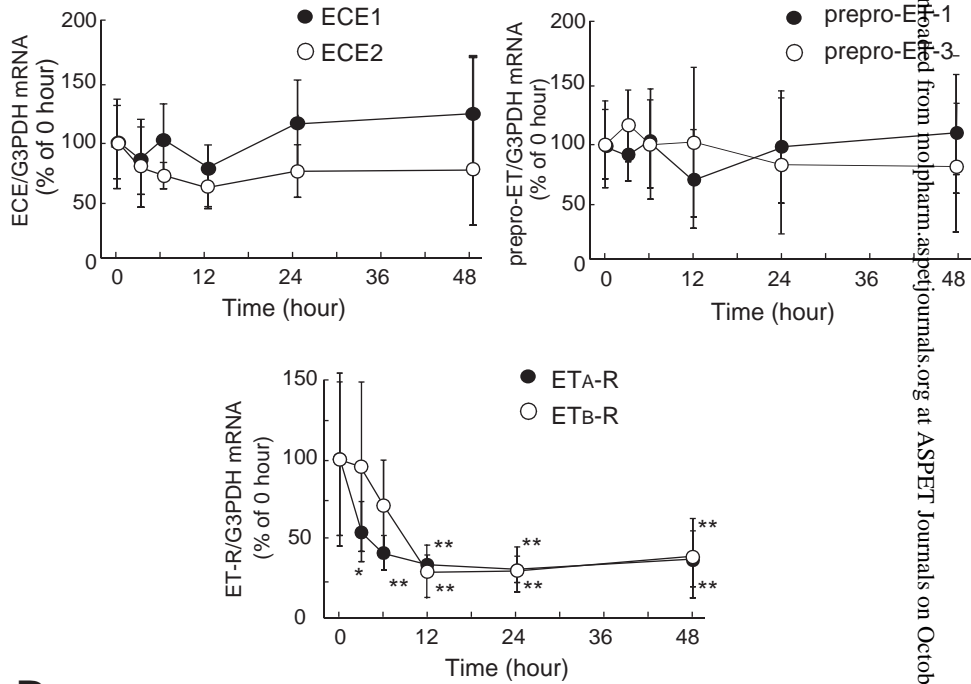
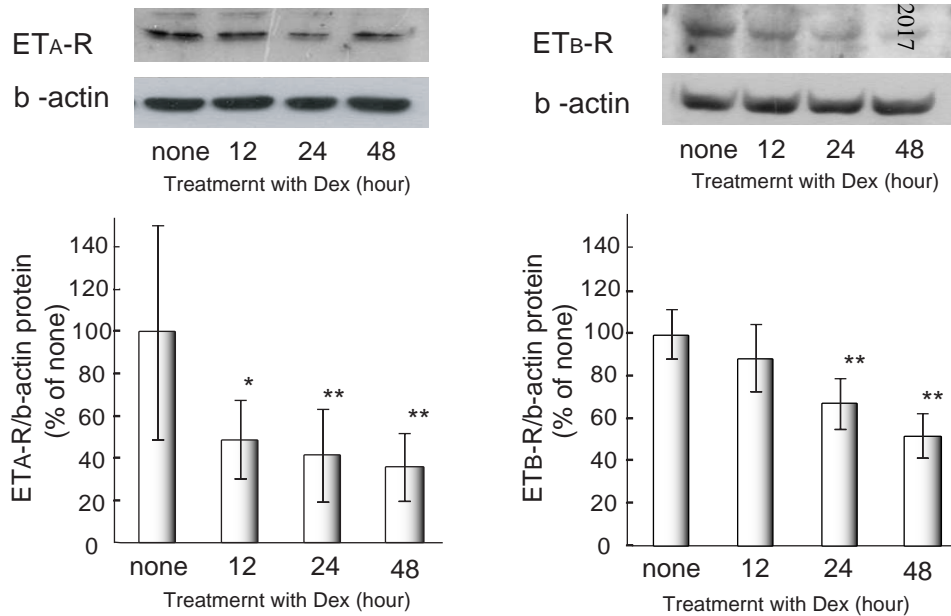
A**B**

Figure 1

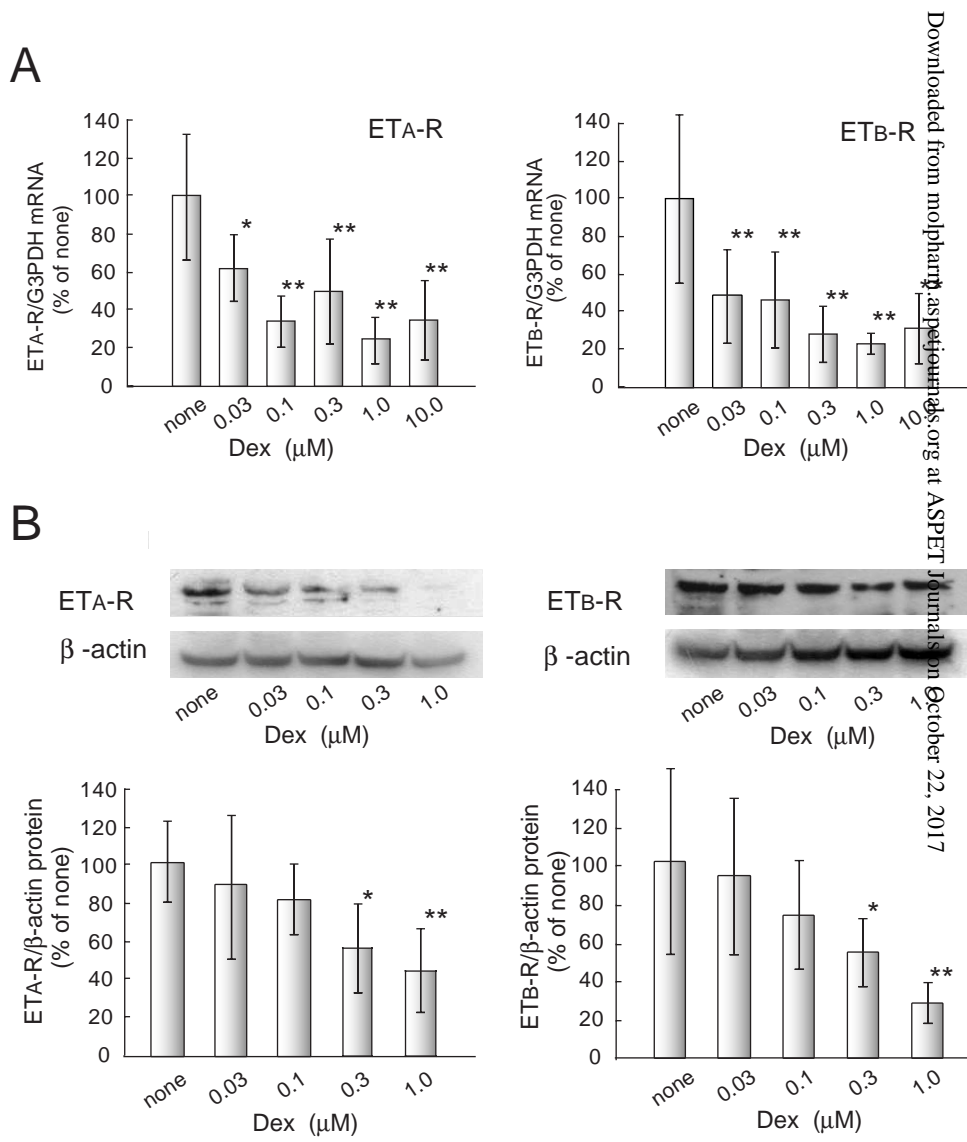


Figure 2

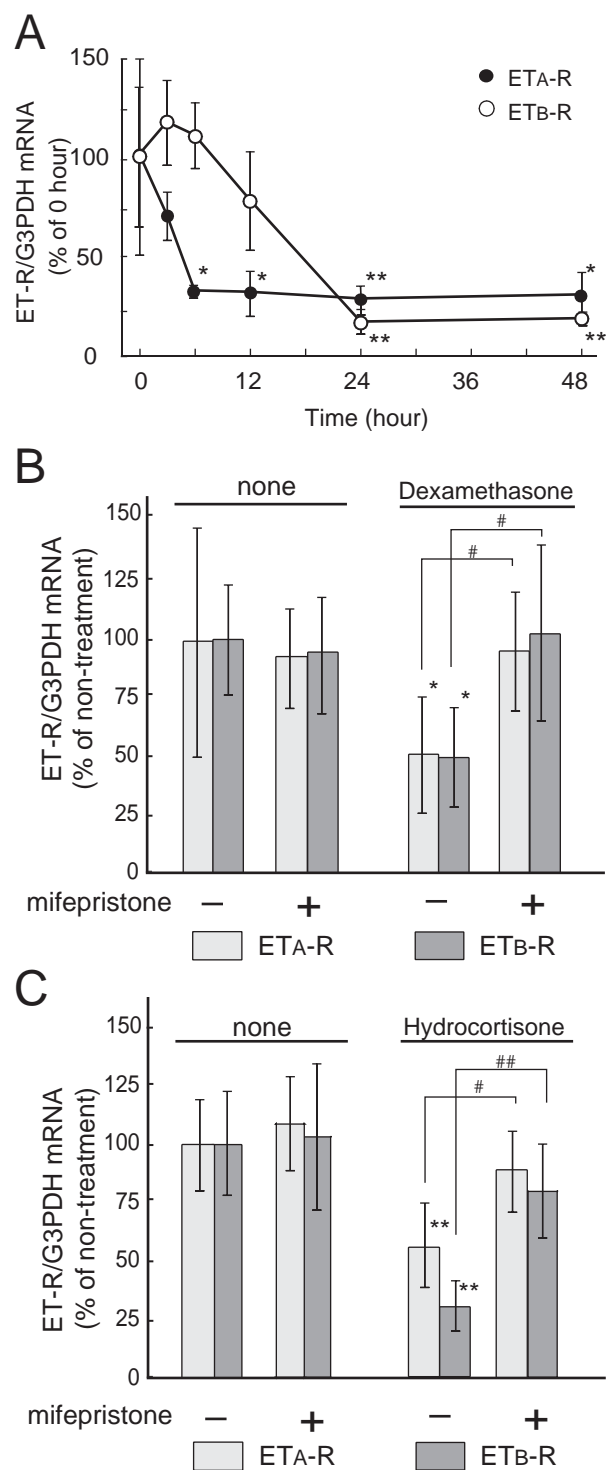


Figure 3

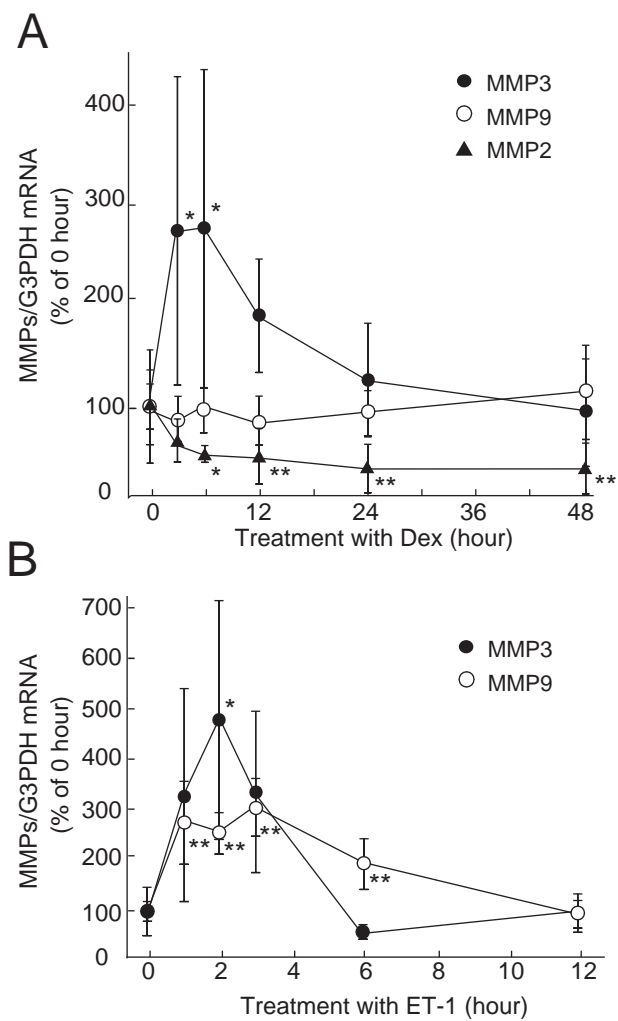


Figure 4

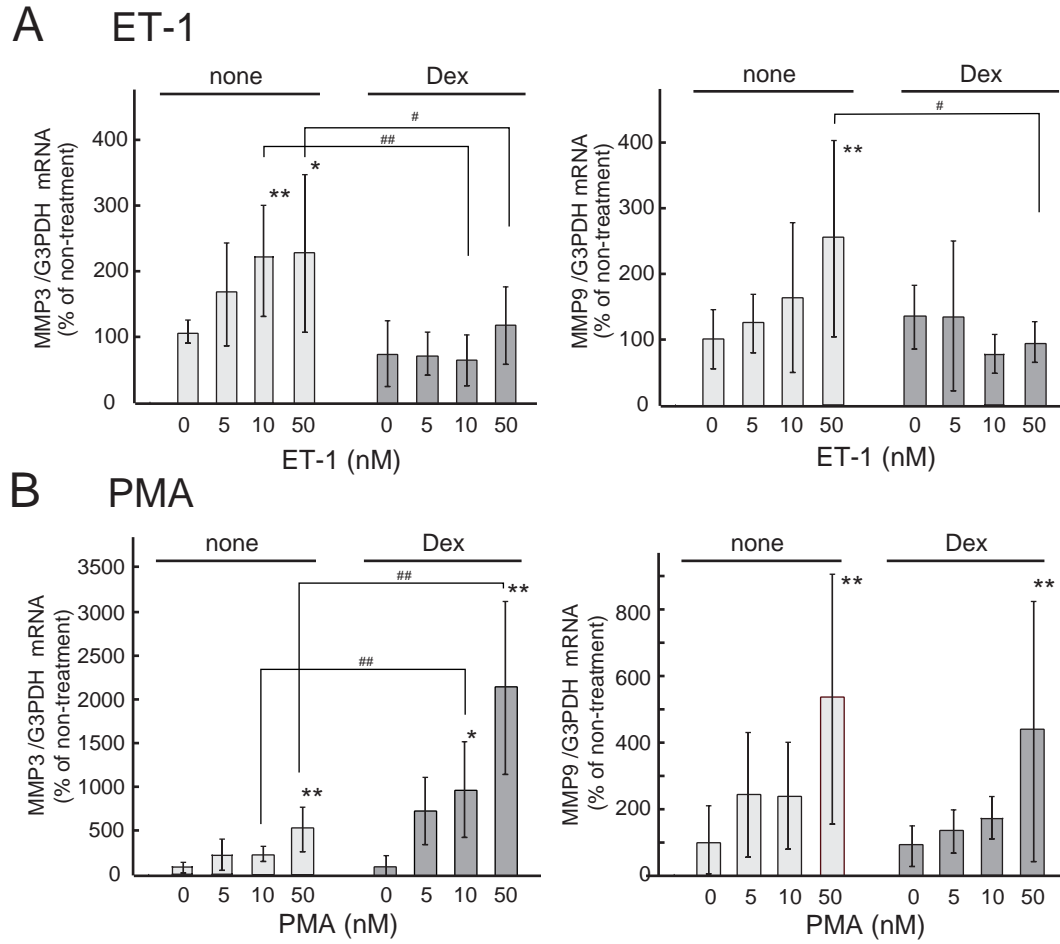


Figure 5

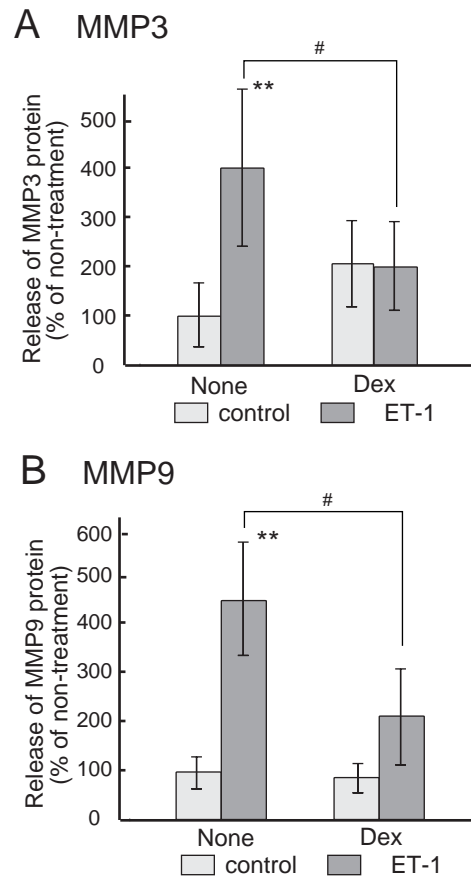


Figure 6

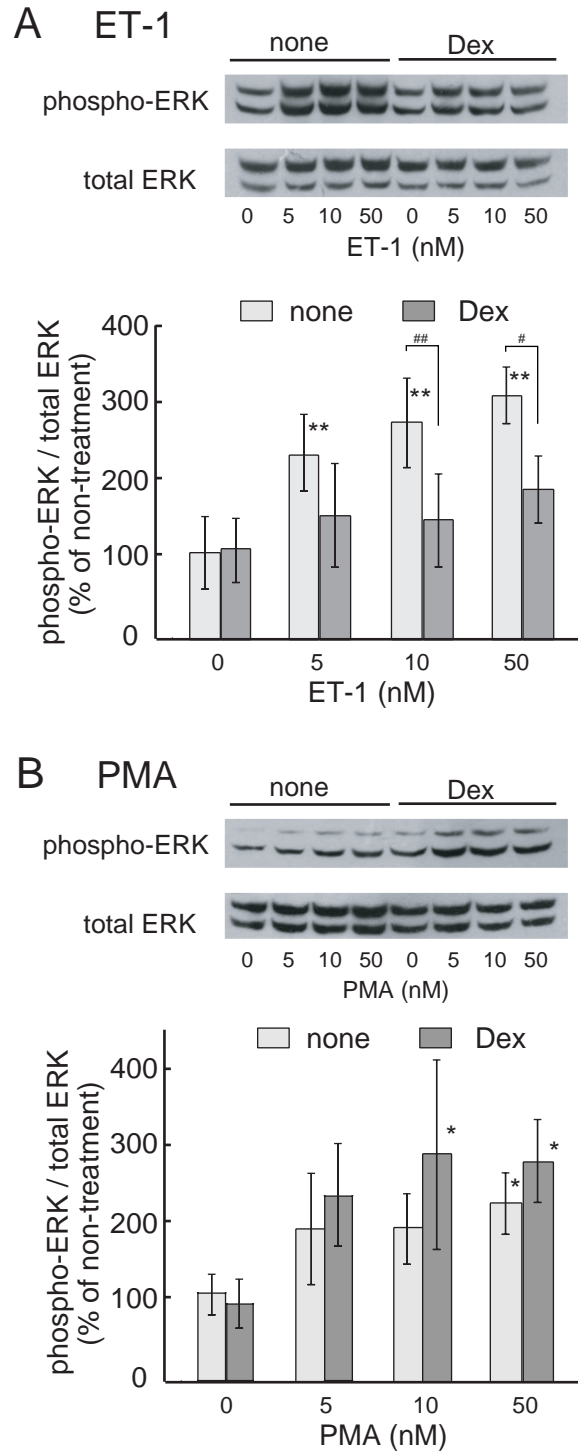


Figure 7