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Instructions for use

1 **Alpha and beta adrenoceptors activate interleukin-6 transcription through different**

2 **pathways in cultured astrocytes from rat spinal cord**

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14

Abstract

16   In brain astrocytes, noradrenaline (NA) has been shown to up-regulate IL-6

17   production via β-adrenoceptors (ARs). However, the underlying intracellular mechanisms

18   for this regulation is not clear, and it remains unknown whether α-ARs are involved. In

19   this study, we investigated the AR-mediated regulation of IL-6 mRNA levels in the

20   cultured astrocytes from rat spinal cord. NA, the α_1-agonist phenylephrine, and the β-

21   agonist isoproterenol increased IL-6 mRNA levels. The phenylephrine-induced IL-6

22   increase was accompanied by an increase in ERK phosphorylation, and these effects were

23   blocked by inhibitors of PKC and ERK. The isoproterenol-induced IL-6 increase was

24   accompanied by an increase in CREB phosphorylation, and these effects were blocked by

25   a PKA inhibitor. Our results indicate that IL-6 increases by α_1- and β-ARs are mediated

26   via the PKC/ERK and cAMP/PKA/CREB pathways, respectively. Moreover, conditioned

27   medium collected from astrocytes treated with the α_2-AR agonist dexmedetomidine,

28   increased IL-6 mRNA in other astrocytes. In this study, we elucidate that α_1- and α_2-ARs,

29   in addition to β-ARs, promote IL-6 transcription through different pathways in spinal cord

30   astrocytes.

31

34

1. Introduction

Noradrenergic neurons project to most of regions of the central nervous system (CNS) [1]. Levels of noradrenaline (NA) in the CNS change under physiological and pathological conditions, such as ischemic stroke, neuropathic pain, and Alzheimer's disease [2–4]. Furthermore, inactivation of noradrenergic neurons enhances inflammation [5] and conversely, promotes recovery from ischemic brain injury [6], suggesting that NA is involved in the pathogenesis of the CNS diseases.

In the CNS, NA is released not only from synapses, but also from varicosities [7], and thus acts on astrocytes surrounding neurons. NA up-regulates the production of neurotrophins, growth factors, and proinflammatory cytokines such as interleukin-6 (IL-6) in astrocytes [8,9]. IL-6 has various roles besides enhancing inflammation, acts as a neurotrophic factor [10], and suppresses neuronal cell death [9]. Since the major source of IL-6 in the CNS is astrocytes [11], NA could affect physiological functions and disease pathogenesis by modulating IL-6 production via astrocytic adrenoceptors (ARs). There are two main groups of ARs, i.e., α and β, with 9 subtypes in total [12]; astrocytes express α_1-, α_2- and β-ARs [13]. In cultured cerebral astrocytes, NA increases IL-6 production via activation of β-ARs [14]. However, the intracellular mechanisms that underlie NA-induced IL-6 production in astrocytes are not fully explained. In addition, it is not clear whether α-ARs are involved in IL-6 production. It was reported that α-ARs were not involved in

54 NA-activated IL-6 transcription in astrocytes [9], conversely, another study showed that

55 NA-activated IL-6 production was inhibited by an α-AR antagonist [15]. In both studies,

56 only antagonists rather than both agonists and antagonists were used, and the expression

57 of AR-subtypes was not confirmed. Astrocytic α-ARs have functionally significant roles,

58 such as regulation of neuronal activity and blood flow [16–18]. Since these functions may

59 be related to astrocytic IL-6 production, it is important to elucidate the involvement of α-

60 ARs in IL-6 production.

61   In the spinal cord, the concentration of NA changes in response to ischemia,

62 neuropathic pain, and aging [4,19,20]. *In vivo* administration of IL-6 promotes axonal

63 sprouting and synapse formation, leading regeneration and functional recovery after

64 spinal cord injury [21]. Furthermore, astrocytes show regional differences in receptor

65 expression, transcriptional factor activity, and responses to inflammation [22–24].

66 Therefore, it is worth investigating the contribution of ARs to the regulation of IL-6 in

67 spinal cord astrocytes.

68   In this study, we identified AR-subtypes that participate in the transcriptional

69 regulation of IL-6 and investigated the intracellular signaling following the activation of

70 each AR-subtypes in the cultured astrocytes from rat spinal cord.

71

72 **2. Materials and methods**

73

74 **2.1. Materials**

75   Antibodies against ERK1/2 (#4695S, 1:2500), phospho-ERK 1/2 (#9101S, 1:2500),

76 p38 (#9212S, 1:2000), phospho-p38 (#9211S, 1:1000), SAPK/JNK (#9252S, 1:2500),

77 phospho-SAPK/JNK (#9251S, 1:1500), STAT3 (#4904S, 1:4000), and phospho-STAT3

78 (#9145S, 1:2000) were purchased from Cell Signaling Technology (Danvers, MA, USA).

79 Antibody against CREB (#sc-377154, 1:500) and phospho-CREB (#sc-81486, 1:250) were

80 purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A peroxidase-

81 conjugated mouse anti-glyceraldehyde 3-phosphate dehydrogenase antibody (GAPDH;

82 #G9295, 1:50000), dexmedetomidine hydrochloride, atipamezole hydrochloride, and

83 isoproterenol hydrochloride were purchased from Sigma–Aldrich (St. Louis, MO, USA).

84 Phenylephrine hydrochloride, propranolol hydrochloride, forskolin, U0126, and SP600125

85 were purchased from FUJIFILM Wako Pure Chemical (Osaka, Japan). L-noradrenaline

86 bitartrate monohydrate and prazosin hydrochloride were purchased from Tokyo Chemical

87 Industry (Tokyo, Japan). H89 and bisindolylmaleimide II (BIM) were purchased from

88 Cayman Chemical (Ann Arbor, MI, USA).

89

90 ## 2.2. Animals

91   All animal care and experimental protocols were approved by the Committee on

92 Animal Experimentation, Graduate School of Veterinary Medicine, Hokkaido University

93 (No. 19-0009), which has been awarded Accreditation Status by the Association for

94 Assessment and Accreditation of Laboratory Animal Care (AAALAC) International.

95 Wistar rats were obtained from CLEA Japan (Tokyo, Japan). Male and female pups aged

96 3–5 days were used for primary astrocyte cultures.

97

98 ## 2.3. Primary cultures of spinal cord astrocytes

99   Primary cultures of spinal cord astrocytes were obtained as previously described

100 [25]. In brief, spinal cords were isolated from rat pups, minced, and incubated with papain

101 (10 U/ml) and DNase (0.1 mg/ml). Dissociated cells were suspended in Dulbecco's Modified

102 Eagle's Medium/Ham's F-12 containing 10% fetal bovine serum, 100 U/ml penicillin, and

103 0.1 mg/ml streptomycin. The cell suspension was seeded onto a poly-l-lysine-coated T75

104 flask. After 7–8 days, the flask was shaken at 250 rpm at 37°C for at least 12 h to remove

105 all cells except for astrocytes. Adherent cells were detached with trypsin and re-seeded

106 onto poly-l-lysine-coated 6- and 12-well plates at a density of 8.0×10^3 cells/cm^2. After 3

107 days, the cell culture had reached confluence and the medium was changed to serum-free

108 medium. Cell cultures were treated with NA or AR-agonists 1 h after the medium

109   exchange, and were treated with AR-antagonists or inhibitors immediately after the

110   exchange. After the given time (detailed in the figure legends and results section), the cell

111   culture was used for experiments.

112

113   **2.4. RNA extraction and real-time PCR analyses**

114   Total RNAs were extracted from cultured astrocytes using RNAiso Plus (Takara

115   Bio, Tokyo, Japan). To remove genomic DNA and synthesize cDNA, the RNA sample was

116   then incubated with qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan).

117   Real-time PCR was performed using Thunderbird SYBR qPCR Mix (TOYOBO), each

118   primer, and the cDNA reaction solution. The primers used were as follows: 5'- GAT TGT

119   ATG AAC AGC GAT GAT GC-3' (forward) and 5'- AGA AAC GGA ACT CCA GAA GAC C -

120   3' (reverse) for IL-6; 5'- GCA AGA GAG AGG CCC TCA G -3' (forward) and 5'- TGT GAG

121   GGA GAT GCT CAG TG -3' (reverse) for GAPDH. Thermal cycles were performed using

122   Eco Real Time PCR System (Illumina, CA, USA). Cycling conditions were 95°C for 1 min

123   (for initial denaturation), followed by 40 cycles of denaturation (95°C, 15 s), annealing and

124   extension (61°C, 45 s). RNAs without reverse transcription were used as a negative control

125   to examine DNA contamination and these were not amplified by real-time PCR. Melt curve

126   analysis confirmed that the obtained amplicon was only the one expected in each reaction.

127   The expression levels of the IL-6 relative to GAPDH were calculated by the $\Delta\Delta$Cq method

128 and were expressed as relative to the control, which was arbitrarily set to a value of "1.0".

129

130 **2.5. Western blotting**

131   Astrocytes were lysed in RIPA buffer containing a protease inhibitor cocktail

132 (nacalai tesque, Kyoto, Japan). The samples were separated by 10% SDS-PAGE and

133 transferred to polyvinylidene difluoride membranes (Millipore, CA, USA). The membranes

134 were blocked with 5% skimmed milk and then incubated with a primary antibody at 4°C

135 for at least 12 h. Thereafter, the membranes were incubated for 1 h at room temperature

136 with a horseradish peroxidase-conjugated secondary antibody (GE Healthcare, Little

137 Chalfont, UK). Antibody binding was visualized by ECL Prime (GE Healthcare). Band

138 intensities were measured using ImageJ software (National Institutes of Health) and

139 expressed as relative to the control, which was arbitrarily set to a value of "1.0".

140

141 **2.6. Preparation and treatments of astrocyte conditioned medium**

142   Agonists or antagonists were treated to the primary cultures of astrocytes in the

143 manner described above. Following agonist treatments for 30 min, cells were refreshed

144 with serum-free medium. After 24 h incubation, the medium was collected from each

145 treated well. RNA extraction was performed at the same time as the medium was collected.

146 The medium was centrifuged at 300 × g to remove dead cells and the supernatant was

147   used as astrocyte conditioned medium (ACM). The drug-treated astrocytes to produce

148   ACM were defined as ACM-donor cells. The ACM was transferred to another primary

149   culture of astrocytes, which was defined as ACM-recipient cells. The ACM-recipient cells

150   were incubated for 3 h followed by RNA extraction.

151

152   **2.7. Data analysis**

153   Data are expressed as means ± S.E.M (n = number of independent measurements).

154   Statistical comparisons between two groups were made using the unpaired Student's t-

155   test. For multiple comparisons, one-way ANOVA followed by the Dunnett's test was used.

156   A value of $p < 0.05$ was considered as a statistically significant level. All statistical analysis

157   was performed with Ekuseru-Toukei 2008 (Social Survey Research Information Co., Ltd.,

158   Tokyo, Japan).

159

160   ## 3. Results

161

162   ### 3.1. Effects of AR-agonists and antagonists on IL-6 mRNA levels

163   We have previously confirmed the expression of α_1-, α_2- and β-ARs by RT-PCR

164   analysis in the cultured astrocytes from rat spinal cord [26]. Treatment of astrocytes with

165   NA (1 or 10 μM) increased the IL-6 mRNA level (Fig. 1A), which reached a peak 1 h after

166   treatment and remained significantly higher than the untreated control for up to 6 h. The

167   treatment of either NA or the β-agonist isoproterenol for 3 h increased IL-6 mRNA level in

168   a concentration-dependent manner (Fig. 1B). The maximum increase in the IL-6 mRNA

169   level was higher in response to NA than that it was to isoproterenol.

170   In addition to isoproterenol (1 μM), the IL-6 mRNA level was increased by the α_1-

171   agonist phenylephrine (1 μM) but not by the α_2-agonist dexmedetomidine (1 μM) (Fig. 1C).

172   The NA-induced increase in the IL-6 mRNA level was inhibited by the α_1-antagonist

173   prazosin (1 μM), the α_2-antagonist atipamezole (10 μM), and the β-antagonist propranolol

174   (10 μM) (Fig. 1D). Co-treatment with prazosin and propranolol abolished the NA-induced

175   increase in the IL-6 mRNA level. Additional treatment with atipamezole did not cause

176   further inhibition. None of the antagonists alone had any effect on IL-6 mRNA levels (Fig.

177   1E).

178

3.2. Mechanisms of increase in IL-6 mRNA via β-ARs

In general, β-ARs coupled to Gs proteins activate the cAMP/protein kinase A (PKA) pathway. The isoproterenol-induced increase in the IL-6 mRNA level was inhibited by the PKA inhibitor H89 (5 μM), but not by the protein kinase C (PKC) inhibitor BIM (5 μM) (Fig. 2A). The adenylyl cyclase activator forskolin (10 μM) also increased the IL-6 mRNA level (Fig. 2B). PKA activates cAMP response element binding protein (CREB), which regulates the expression of a variety of genes [27]. Isoproterenol increased CREB phosphorylation 30 min after treatment (Fig. 2C). In the presence of propranolol or H89, isoproterenol did not increase CREB phosphorylation. Forskolin also increased CREB phosphorylation. These results suggest that the cAMP/PKA/CREB pathway is involved in the increase in IL-6 mRNA level following β-AR activation.

3.3. Mechanisms of increase in IL-6 mRNA via α_1-ARs

α_1-ARs coupled to Gq proteins activate the Ca^{2+}/PKC pathway, which increases mitogen-activated protein kinases (MAPKs) phosphorylation [28]. Therefore, we investigated whether PKC and/or MAPKs participates in the transcriptional activation of IL-6 following α_1-AR activation. The phenylephrine-induced increase in the IL-6 mRNA level was inhibited by BIM, but not by H89 (Fig. 3A). Phenylephrine did not affect CREB phosphorylation (Fig. 3B). The MAPK-ERK (extracellular signal-regulated kinase) kinase

198   (MEK)/ERK inhibitor U0126 (10 μM) abolished the phenylephrine-induced increase in the

199   IL-6 mRNA level (Fig. 3C). Phenylephrine increased ERK phosphorylation (Fig. 3D). In

200   the presence of prazosin, BIM, or U0126, phenylephrine did not increase ERK

201   phosphorylation. On the other hand, phenylephrine did not affect c-jun N-terminal kinase

202   (JNK) or p38 phosphorylation (Fig. 3E, F). These results suggest that the PKC/ERK

203   pathway is involved in the increase in IL-6 mRNA level following α_1-AR activation.

204

205   **3.4. Effects of MAPKs or PKA inhibitor on NA-induced transcriptional activation of IL-6**

206   NA increased ERK and JNK phosphorylation but not p38, and these increases

207   were inhibited by prazosin (Fig. 3D, E, F). Therefore, we investigated whether ERK or

208   JNK phosphorylation affects the NA-induced increase in the IL-6 mRNA levels. U0126

209   and the JNK inhibitor SP600125 (10 μM) suppressed the NA-induced ERK and JNK

210   phosphorylation (Fig. 4A, B). Furthermore, U0126 and SP600125 abolished the NA-

211   induced IL-6 increase (Fig. 4C). SP600125 alone did not have any effect on IL-6 mRNA

212   level (Fig. 4D). Moreover, H89 abolished the NA-induced IL-6 increase (Fig. 4E). Since IL-

213   6 transcription is activated by STAT3 phosphorylation [29], we investigated the effect of

214   AR-agonists on it. NA, phenylephrine, and isoproterenol had no effect on STAT3

215   phosphorylation (Fig. 4F). These results suggest that both cAMP/PKA and ERK and/or

216   JNK pathways are involved in the NA-induced increase in the IL-6 mRNA level.

217

3.5. Effects of AR-agonists on IL-6 mRNA levels via astrocyte conditioned medium.

218

219   We tested the long-term or indirect effects on IL-6 transcription following AR-

220 activation. The treatment of NA or AR-agonists for 24 h did not have any effect on IL-6

221 mRNA levels (Fig. 5A). Then, we examined whether some factors released by AR-

222 activation are involved in IL-6 transcription. The 3 h treatment of astrocyte conditioned

223 medium (ACM), derived from the ACM-donor cells treated with NA or dexmedetomidine

224 but not phenylephrine and isoproterenol, increased IL-6 mRNA levels in the ACM-

225 recipient cells (Fig. 5B, C). The effect of dexmedetomidine was abolished by the treatment

226 of atipamezole for the ACM-donor cells (Fig 5D). However, there were no changes in IL-6

227 mRNA levels in the ACM-donor cells (Fig. 5E). These results suggest that α_2-ARs increase

228 IL-6 mRNA level via some factors released into the extracellular medium.

229

4. Discussion

230

231   In this study, we elucidate that α_1- and α_2-ARs, in addition to β-ARs, are involved

232   in the transcriptional activation of IL-6 in astrocytes. Moreover, we showed that AR-

233   mediated activation of IL-6 transcription involves CREB and MAPKs. Astrocytic ARs are

234   likely to play a role in physiological and/or pathophysiological functions in the CNS by

235   regulating the production of IL-6.

236   NA activated the transcription of IL-6 in spinal cord astrocytes. In this study, NA

237   concentration-dependently increased IL-6 transcription at concentrations of 10 nM to 1

238   μM. The physiological concentration of NA in cerebrospinal fluid is 1 nM to 100 nM [2,30].

239   Therefore, physiological concentrations of NA are sufficient to induce IL-6 production by

240   astrocytes. In addition, since NA levels increase in ischemia and neuropathic pain [2,4]

241   and decrease in Alzheimer's disease [3], IL-6 production by NA in astrocytes is likely to

242   change under pathological conditions.

243   The maximum effect of NA on IL-6 mRNA transcription was higher than that of

244   isoproterenol. NA has a higher affinity for α-ARs than β-ARs [31]. The effect of NA on IL-

245   6 transcription was suppressed by both α_1- and β-antagonists, and the α_1-agonist

246   phenylephrine also activated the IL-6 transcription. These results indicate that α_1-ARs

247   are involved in the transcriptional activation of IL-6 in spinal cord astrocytes. On the other

248   hand, in cultured cerebral astrocytes, NA activates IL-6 transcription, which is inhibited

14

249　by antagonists for β-ARs, but not for α-ARs [9]. Moreover, α_1-AR activation stimulates

250　ERK phosphorylation in cultured spinal cord astrocytes [32], but not in cerebral astrocytes

251　[33]. Astrocytes show regional differences in receptor expression, transcriptional factor

252　activity, and responses to inflammation [22–24]. Therefore, these astrocyte heterogeneities

253　may be involved in the discrepancy in the involvement of α_1-ARs between spinal and

254　cerebral astrocytes.

255　　　　The α_2-antagonist atipamezole suppressed the effect of NA on IL-6, whereas the

256　treatment of the α_2-agonist dexmedetomidine for 3 h showed no effect. Furthermore, the

257　effect of NA on IL-6 transcription was completely suppressed by co-treatment with α_1- and

258　β-AR antagonists, indicating that NA activates IL-6 transcription mainly via α_1- and β-

259　ARs. One of the possible explanations for this discrepancy between the agonist's and

260　antagonist's effects is that the mechanisms involved in the activation of one receptor

261　subtype by a specific agonist are different from the mechanisms involved in the activation

262　of multiple receptor subtypes by NA. For example, β_1- and α_{2A}, or α_{1A}- and α_{1B}-ARs form

263　receptor-heterodimers [34,35]. When only one receptor from the heterodimer is activated,

264　the intracellular signaling of the heterodimer is different to the signaling seen when both

265　receptors in the heterodimer are activated [36,37]. This could provide an explanation for

266　why NA-increased JNK phosphorylation was suppressed by prazosin, while exerting no

267　effect of phenylephrine on the JNK phosphorylation. Further investigations are needed to

268   reveal these mechanisms.

269   Transcription of IL-6 is promoted by binding of transcription factors, such as AP1,

270   CREB, and NF-κB, to specific DNA sequences and p300/CBP [38,39]. In addition,

271   activation of MAPKs signaling can lead to AP1 activation [40]. In this study,

272   phenylephrine activated ERK and IL-6 transcription, both of which were suppressed by

273   the PKC and ERK inhibitor. Phenylephrine also did not affect CREB, JNK, and p38

274   phosphorylation. These results indicate that α_1-ARs activate IL-6 transcription via the

275   PKC/ERK pathway in spinal cord astrocytes. Meanwhile, isoproterenol activated CREB

276   and IL-6 transcription, both of which were suppressed by the PKA inhibitor. The adenylate

277   cyclase activator forskolin mimicked the action of isoproterenol. These results indicate

278   that β-ARs activate IL-6 transcription via the cAMP/PKA/CREB pathway in spinal cord

279   astrocytes. As in this study, the effects of β-ARs on IL-6 secretion or transcription are

280   mediated via the cAMP/PKA pathway in cerebral astrocytes [9,14]. Moreover, the NA-

281   activated transcription of IL-6 was inhibited by both the ERK inhibitor and the JNK

282   inhibitor. The phosphorylation of ERK or JNK activates common factors, such as c-jun and

283   Elk [41], which may be also involved in IL-6 transcription. NA-activated transcription of

284   IL-6 was also inhibited by the PKA inhibitor. Therefore, the pathway of NA-activated IL-

285   6 transcription is likely to include the pathways activated by phenylephrine and

286   isoproterenol. In addition to these pathways, STAT3 phosphorylation activates IL-6

287 transcription in astrocytes [29], but NA, phenylephrine, and isoproterenol had no effect on

288 STAT3 phosphorylation in this study. Therefore, the STAT3 pathway is likely not involved

289 in AR-induced IL-6 transcriptional activation in astrocytes. Furthermore, in contrast to

290 this study, it has been shown that isoproterenol suppresses lipopolysaccharide (LPS)-

291 induced IL-6 promoter activities via β_2-ARs in astrocytes [42]. LPS-induced IL-6

292 transcriptional activation is mediated via the TLR4-NF-κB pathway [43,44], and the

293 activation of β-ARs attenuates NF-κB activity by increasing IκBα gene expression and

294 protein levels [45]. In the presence of LPS, β-ARs may suppress IL-6 transcription by

295 potent suppressing NF-κB. Therefore, it is likely that the activation of β-ARs shows

296 bidirectional effects on IL-6 transcription via the CREB and NF-κB pathways.

297   ACM derived from NA- or dexmedetomidine-treated astrocytes increased IL-6

298 mRNA levels in the ACM-recipient cells. It is possible that factor(s), released into the

299 extracellular medium by activation of α_2-ARs, may promote IL-6 transcription in

300 neighboring and distant astrocytes. Activation of astrocytic α_2-ARs increases

301 accumulation of glutamine, as a precursor of neurotoxic glutamate [46], and activation of

302 metabotropic glutamate receptors enhances the release or transcription of IL-6 in

303 astrocytes [47,48]. In addition, the effect of released factor(s) may disappear within a few

304 hours, because there were no changes in IL-6 mRNA levels in the ACM-donor cells.

305 Further studies are needed to elucidate the indirect pathway of IL-6 transcriptional

306   activation via the α_2-ARs.

307   Intraperitoneal injection of lipopolysaccharide to mice increases IL-6 mRNA levels

308   several-fold in brain astrocytes *in vivo* [49]. The IL-6 increases in responses to NA or AR-

309   agonists in this study were similar in magnitude to these *in vivo* studies. Besides immune

310   responses in the CNS, IL-6 acts as a neurotrophic factor [10], induces the differentiation

311   of neural stem cells [50], and promotes vasculogenesis during the brain development [51].

312   Therefore, under physiological conditions, NA may play a beneficial role in the CNS

313   functions by promoting IL-6 release from astrocytes. Conversely, treatment of spinal cord-

314   injured rats with anti-IL-6 neutralizing antibodies reduces the area of injury [52]. Under

315   pathological conditions, NA may act on astrocytes to promote IL-6 production and delay

316   recovery. Further studies are needed to reveal whether astrocytic ARs regulate IL-6

317   production *in vivo* and how astrocytic IL-6 acts under physiological and pathological

318   conditions.

319

320   **5. Conclusion**

321   In this study, we elucidated that NA promotes the transcriptional activation of IL-

322   6 in spinal cord astrocytes, and α_1- and α_2-ARs, in addition to ß-ARs, are involved. IL-6

323   increase by α_1- and ß-ARs are mediated via the PKC/ERK and cAMP/PKA/CREB pathways,

324   respectively. Moreover, astrocyte conditioned medium collected from cells treated with the

325   α_2-AR agonist dexmedetomidine, increased IL-6 mRNA in other astrocytes. Based on these

326   results, we suggest a role for NA in the CNS homeostasis and pathogenesis via modulation

327   of cytokine production by astrocytic ARs.

328

CRediT authorship contribution statement

Kohei Morimoto: Conceptualization, Data curation, Formal analysis, Investigation, Visualization, Writing – original draft. **Ryota Eguchi:** Funding acquisition, Writing - review & editing. **Taisuke Kitano:** Investigation, Writing - review & editing. **Ken-ich Otsuguro:** Conceptualization, Supervision, Funding acquisition, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figure legends

Figure 1. Effects of NA, AR-agonists, and AR-antagonists on IL-6 mRNA levels in cultured astrocytes

(A) IL-6 mRNA levels in astrocytes treated with NA (1 and 10 μM) for 1, 3, 6, and 12 h. $*p < 0.05$, $**p < 0.01$ vs. time-matched control (Dunnett's test), n = 6. (B) IL-6 mRNA levels in astrocytes treated with NA or the ß-agonist isoproterenol (ISO) (1 nM-10 μM) for 3 h. $*p < 0.05$, $**p < 0.01$ vs. control (Dunnett's test), n = 6. (C) IL-6 mRNA levels in astrocytes treated with the α_1-agonist phenylephrine (PHE, 1 μM), the α_2-agonist dexmedetomidine (DEX, 1 μM), and ISO (1 μM) for 3 h. $**p < 0.01$ vs. control (Dunnett's test), n = 6. (D) IL-6 mRNA levels in astrocytes treated with NA (1 μM) in the presence or absence of the α_1-antagonist prazosin (PRAZ, 1 μM), the α_2-antagonist atipamezole (ATIP, 10 μM), and the ß-antagonist propranolol (PROP, 10 μM) for 3 h. $\#\#p < 0.01$ vs. NA alone (Dunnett's test), n = 6. (E) IL-6 mRNA levels in astrocytes treated with PRAZ, ATIP, and PROP for 4 h. n = 6. Data are presented as means ± S.E.M. The mRNA levels of cytokines were expressed as relative to the control, which was arbitrarily set to a value of "1.0".

Figure 2. Intracellular mechanisms of transcriptional activation of IL-6 via ß-ARs

(A, B) IL-6 mRNA levels in astrocytes treated with the ß-agonist isoproterenol (1 μM), the

554   adenylyl cyclase activator forskolin (FSK, 10 μM) in the presence or absence of the PKA

555   inhibitor H89 (5 μM) and the PKC inhibitor BIM (5 μM) for 3 h. #p < 0.05 (Dunnett's test),

556   **p < 0.01 (unpaired Student's t-test), n.s.: not significant, n = 6. (C) The protein expression

557   levels of phosphorylated and total CREB were quantified and representative blots are

558   shown. GAPDH was used as a loading control. Astrocytes were treated with isoproterenol

559   and FSK in the presence or absence of the β-antagonist propranolol (PROP, 10 μM) and

560   H89 for 30 min. **p < 0.01 vs. control (Dunnett's test), n = 6. Data are presented as means

561   ± S.E.M.

562

563   **Figure 3. Intracellular mechanisms of transcriptional activation of IL-6 via α₁-ARs**

564   (A, C) IL-6 mRNA levels in astrocytes treated with the α₁-agonist phenylephrine (1 μM) in

565   the presence or absence of the PKC inhibitor BIM (5 μM), the PKA inhibitor H89 (5 μM),

566   and the MEK/ERK inhibitor U0126 (10 μM) for 3 h. ##p < 0.01 (Dunnett's test), **p < 0.01

567   (unpaired Student's t-test), n.s.: not significant, n = 6. (B, D-F) The protein expression

568   levels of phosphorylated and total CREB (B), ERK (D), JNK (E), and p38 (F) were

569   quantified and representative blots are shown. GAPDH was used as a loading control.

570   Astrocytes were treated with phenylephrine (PHE), or NA (1 μM) in the presence or

571   absence of the α₁-antagonist prazosin (PRAZ, 1 μM), BIM and U0126 for 30 min (B) or 10

572   min (D-F). *p < 0.05, **p < 0.01 vs. control (Dunnett's test), n = 6. Data are presented as

32

573   means ± S.E.M.

574

575   **Figure 4. Effects of ERK, JNK, and PKA inhibitor on NA-induced transcriptional**

576   **activation of IL-6.**

577   (A, B, F) The protein expression levels of phosphorylated and total ERK (A), JNK (B), and

578   STAT3 (F) were quantified and representative blots are shown. GAPDH was used as a

579   loading control. Astrocytes were treated with NA (1 μM), the α_1-agonist phenylephrine

580   (PHE, 1 μM), and the β-agonist isoproterenol (ISO, 1 μM) in the presence or absence of the

581   MEK/ERK inhibitor U0126 (10 μM) and the JNK inhibitor SP600125 (SP, 10 μM) for 10

582   min (A, B) or 30 min (F). #$p < 0.05$, ##$p < 0.01$ vs. NA alone (Dunnett's test), n = 6. (C, E)

583   IL-6 mRNA levels in astrocytes treated with NA in the presence or absence of U0126, SP

584   and the PKA inhibitor H89 (5 μM) for 3 h. ##$p < 0.01$ vs. NA alone (Dunnett's test), n = 6.

585   (D) IL-6 mRNA levels in astrocytes treated with SP600125 for 4 h. n = 6. Data are

586   presented as means ± S.E.M.

587

588   **Figure 5. Effects of AR-agonists on IL-6 mRNA levels via astrocyte conditioned medium.**

589   (A) IL-6 mRNA levels in astrocytes treated with NA (1 μM), the α_1-agonist phenylephrine

590   (PHE, 1 μM), the α_2-agonist dexmedetomidine (DEX, 1μM), and the β-agonist isoproterenol

591   (1 μM) for 24 h, n = 6. (B) Schematic depiction of astrocyte conditioned medium (ACM)

592 transfer experiment. (C, D) IL-6 mRNA levels in astrocytes incubated with ACM derived

593 from astrocytes treated with NA, PHE, DEX and ISO in the presence or absence of α_2-

594 antagonist atipamezole (ATIP, 1 μM) for 3 h. *$p < 0.05$, **$p < 0.01$ vs. control (C, Dunnett's

595 test), #$p < 0.05$, ##$p < 0.01$ vs. DEX alone (D, Dunnett's test), n = 6. (E) IL-6 mRNA levels

596 in astrocytes treated with DEX in the presence or absence of ATIP for 30 min and

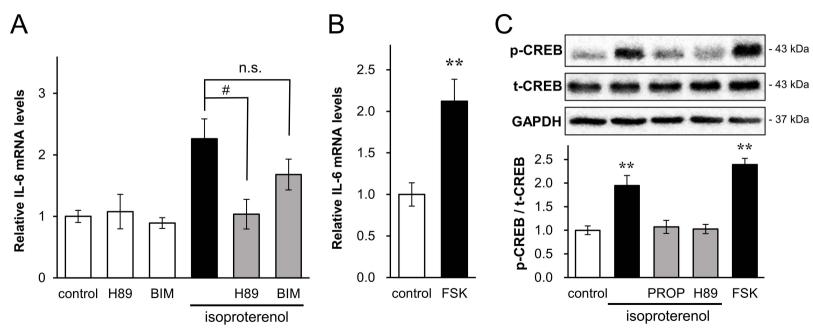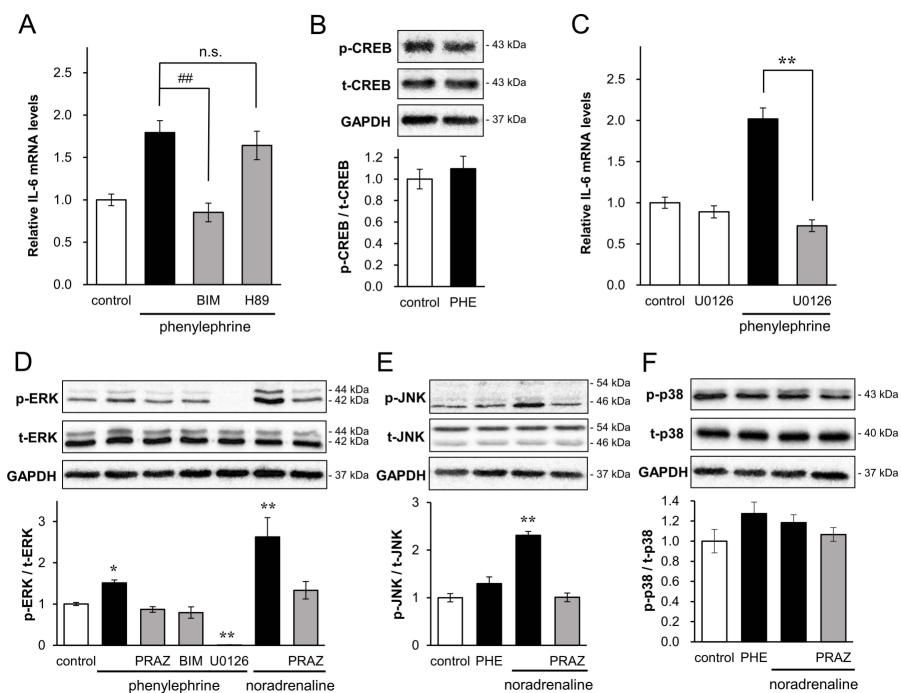597 incubated for 24 h following medium exchange. n = 6. Data are presented as means ±

598 S.E.M.

Astrocyte

Fig.1

Fig. 2

Fig. 3

Fig. 4

Fig. 5