수의학석사학위논문

에탄올이 아밀로이드 베타 과다생성을 유도하는 과정에서 eIF2α에 의한 PGE_2 조절의 역할

The Role of eIF2α-Upregulated PGE_2 Production in Ethanol-Induced Amyloid-β Overproduction

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서울대학교 대학원

수의학과 수의생명과학 전공

아흐메드

ABSTRACT

The Role of eIF2α-Upregulated PGE_2 Production in Ethanol-Induced Amyloid-β Overproduction

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Ethanol abuse aggravates dementia-associated cognitive defects through the progression of Alzheimer's disease (AD) pathophysiology. Beta secretase 1 (BACE1) is a key regulator of AD pathogenesis by controlling amyloid beta peptide (Aβ) accumulation. However, the exact mechanism by which ethanol triggers BACE1 still has not been elucidated. Endoplasmic reticulum

(ER) stress and neuroinflammation have been proposed in ethanol-exaggerated neurodegeneration. Thus, this study investigated the role of ER stress and PGE_2, a neuroinflammation mediator, in the signaling of ethanol-stimulated BACE1 expression and Aβ production. Using the human-derived neuroblastoma cell line SK-N-MC, the results show that ethanol up-regulated BACE1 expression in a dose-dependent manner. Ethanol stimulated reactive oxygen species (ROS), which induced ER stress markers, CHOP expression, and eIF2α phosphorylation. PBA (ER stress inhibitor) attenuated the ethanol-increased cyclooxygenase-2 (COX-2) expression and PGE_2 production. By using salubrinal (inhibitor of eIF2α dephosphorylation) and *EIF2A* siRNA, the results show that eIF2α phosphorylation mediated the ethanol-induced COX-2 expression. COX-2-induced BACE1 upregulation was abolished by NS-398 (selective COX-2 inhibitor). Furthermore, PF-04418948 (EP_2 receptor blocker) ameliorated the ethanol-stimulated Aβ secretion. Ethanol promoted PKA activation and CREB phosphorylation and their translocation into the nucleus; PKA activation and CREB phosphorylation are inhibited by PF-04418948. Moreover, pretreatment with 14-22 amide (PKA inhibitor) and *CREB1* siRNA transfection suppressed the ethanol-elevated BACE1 levels. In conclusion, ethanol-induced COX-2 expression increases PGE_2 production which elevates the BACE1 level through the EP_2 receptor-linked PKA /CREB pathway.

Keywords: Alzheimer's disease, Ethanol, Amyloidogenesis, Beta secretase 1 (BACE1), Eukaryotic initiation factor 2α (eIF2α), Prostaglandin E_2 (PGE_2)

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ABBREVIATIONS

Aβ	Amyloid beta
AD	Alzheimer's disease
APP	Amyloid precursor protein
BACE1	Beta-site APP-cleaving enzyme 1
ROS	Reactive oxygen species
ER	Endoplasmic reticulum
eIF2α	Eukaryotic initiation factor alpha
CHOP	C/EBP-homologous protein
PGE_2	Prostaglandin E_2
EP	Prostaglandin E receptor
COX	Cyclooxygenase
NSAID	Non-steroidal anti-inflammatory drugs
mPGES	Microsomal PGE_2 synthase
DMEM	Dulbecco Modified Eagle Medium
PBS	Phosphate buffered solution
FBS	Fetal bovine serum
CREB	cAMP response element-binding protein
PKA	Protein kinase A
MAPK	Mitogen-activated protein kinase

AraC	Cytosine arabinoside
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PVDF	Polyvinylidene fluoride
ATF4	Activating transcription factor 4
PI3K	Phosphatidylinositide 3-kinases
Akt	Protein kinase B

INTRODUCTION

Alzheimer's disease (AD), the most common form of dementia, is a progressive neurodegenerative disease (Kalaria et al., 2008). Although the exact cellular mechanism mediating the pathogenesis of AD is not fully understood, it has been found to be associated with the aggregation of amyloid-beta (Aβ) peptides. Beta-site APP-cleaving enzyme 1 (BACE1) is a rate-limiting enzyme for Aβ production through the proteolytic cleavage of amyloid precursor protein (APP) (Tanzi and Bertram, 2005; Zhang et al., 2011). Many studies have proposed that an increase in the BACE1 expression pattern could have an essential role in the progression of AD (Fukumoto et al., 2002; Yang et al., 2003; Zhao et al., 2007). Heavy alcohol (ethanol) consumption is one of the possible risk factors contributing to AD (Kapaki et al., 2005; Letenneur et al., 2004; Saunders et al., 1991; Venkataraman et al., 2016). A prior study suggested an association between alcohol abuse and a molecular mechanism in AD pathogenesis. Although chronic alcohol consumption results in elevated BACE1 levels (Anstey et al., 2009), the exact molecular mechanism by which ethanol induces BACE1

remain obscure.

Accumulation of misfolded proteins in the lumen of endoplasmic reticulum (ER) triggers a cellular response called ER stress (Hetz and Mollereau, 2014). ER stress is one of the assumed mechanisms that mediates the adverse effect of ethanol on neurons (Yang and Luo, 2015). In vivo experiments have revealed the ethanol-induced ER stress markers CHOP and eIF2α phosphorylation in the brain (Ke et al., 2011). Moreover, ER stress signaling was found to be activated in brain samples from AD patients (Hoozemans et al., 2009; Stutzbach et al., 2013). A positive correlation between Aβ extracellular oligomerization and induced ER stress markers also indicates the essential role of ER stress in AD (Placido et al., 2015). Moreover, it has been postulated that ER stress could promote the BACE1 expression mediated by eIF2α phosphorylation (Mouton-Liger et al., 2012). Because the contribution of ethanol-stimulated ER stress in AD pathogenesis has not been discussed in detail, further investigation is required.

Ethanol abuse has been shown to intensify prostaglandins and

overproduce inflammatory cytokines in the rat brain (Alfonso-Loeches et al., 2010) suggesting the involvement of

neuroinflammation in alcohol-induced neurodegeneration. The role of inflammation in AD progression has been shown by several observations including diminished Aβ deposition in the brain of an AD animal model after long-term NSAID treatment (Lim et al., 2000). In AD patients, high COX-2 expression and PGE_2 have been found in the brain and cerebrospinal fluid, respectively (Ho et al., 1999; Montine et al., 1999). Moreover, PGE_2 mediates COX-2 induced-Aβ accumulation (Kotilinek et al., 2008). The biological activities of PGE_2 are mediated by the four subtypes of the PGE_2 receptors (EP1-4 receptors) (Hoshino et al., 2007). Nevertheless, the role of EP receptors in ethanol stimulated Aβ production has not yet been investigated. Moreover, previous reports have revealed the integration of ER stress and inflammation in the pathogenesis of some diseases other than AD (Hung et al., 2004; Luo et al., 2016). The relation between ethanol-induced ER stress and stimulation of PGE_2 signaling has not been thoroughly investigated. Furthermore, on a cellular level, little attention has been given to ethanol β-amyloidogenic effects; therefore, more research is needed to clearly show the contribution of the ER stress and COX-2 associated pathways in the above effects. Such an investigation could explain how ethanol leads to AD progression. In this study, therefore, I aimed to investigate how ER stress and PGE2 signaling mediate ethanol-induced BACE1 and Aβ production.

MATERIALS AND METHODS

1. Materials

SK-N-MC human neuroblastoma cells were provided by the Korean cell line bank (Seoul, Republic of Korea). Fetal bovine serum (FBS) and serum replacement (SR) were obtained from HyClone (UT, USA) and Gibco (Grand Island, NY, USA), respectively. β-actin, β-tubulin, lamin A/C, COX-2, CREB-1, p-CREB (Ser113), CHOP, and cat-PKA antibodies were purchased from Santa Cruz biotechnology (Dallas, TX, USA). The eIF2α and p-eIF2α antibodies were obtained from Cell signaling technology, Inc. (Danvers, MA, USA). Aβ and BACE1 antibodies were acquired from Abcam (Cambridge, MA, USA). The EP_2 antibody was purchased from CusAb (College Park, MD, USA). Ethanol and the antibody for the C99 fragment of APP were obtained from EMD Millipore Copr. (Merck KGaA, Darmstadt, Germany). Secondary horse radish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse antibodies were supplied by Thermo Fisher (Waltham. MA, USA). Small interfering RNAs (siRNAs) for *CREB1*, *EIF2A* and non-targeting were purchased from Dharmacon (Lafayette, CO,

USA). Acetaldehyde (AA), parafilm, 4-phenyl butyric acid (PBA), N-acetylcysteine (NAC), prostaglandin E2 (PGE2), fomepizole, diallyl disulfide and sodium azide were acquired from Sigma Aldrich (St. Louis, MO, USA). All inhibitors used in this study, such as sodium azide, NAC, PBA, salubrinal, NS-398, 14-22 amide and PF-04418948, did not significantly affect the cell viability (Fig. 1).

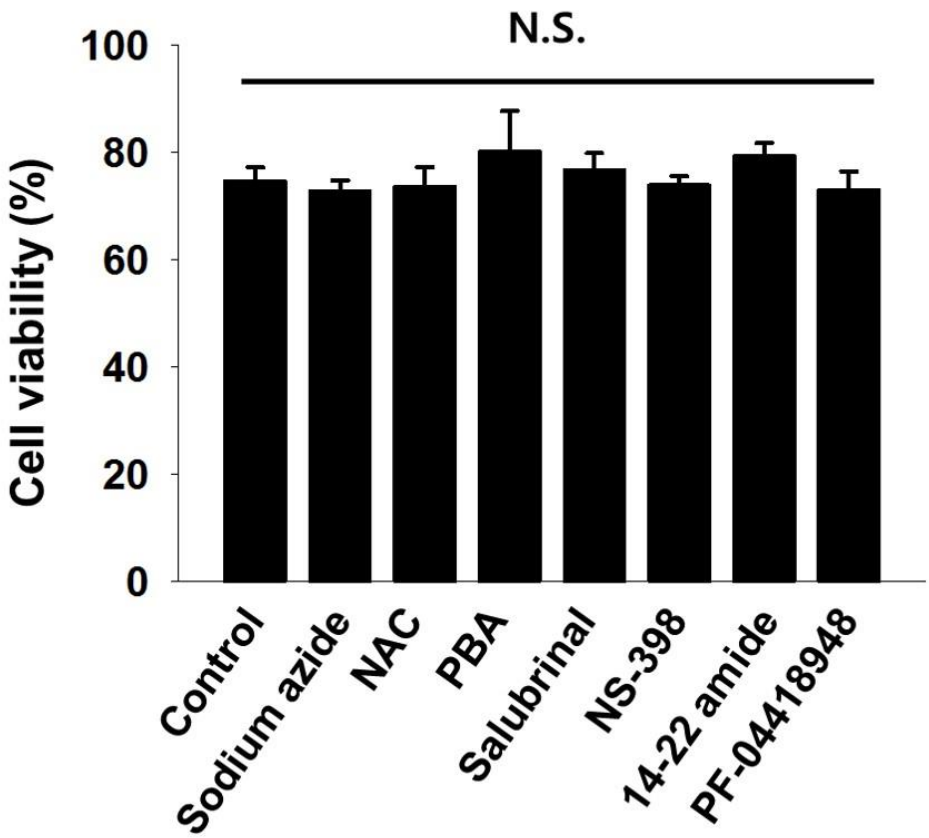


Figure 1. Effect of inhibitors on neuronal cell viability. Cells were treated with sodium azide (5 mM), NAC (5 mM), PBA (5 mM), Salubrinal (10 μM), NS-398 (50 μM), 14-22 amide (1 μM) and PF-04418948 (10 μM) for 24 h. Cell viability was measured by trypan blue exclusion assay. N.S. indicates no statistically significant.

2. Cell culture

Dishes (60 mm) were seeded with 5×10^5 SK-N-MC cells in 3 ml of culture medium consisting of 10% FBS and 1% antibiotic (penicillin and streptomycin)-antimycotic (amphotericin) mixture solution in Dulbecco's essential medium (DMEM; Gibco). Cells were incubated in a humid atmosphere at 37 ℃ and 5% CO_2 for 24 h, and then, the medium was exchanged with fresh medium. Subsequently, at 60% confluence, the culture medium was exchanged with DMEM (serum-free medium, 2 ml/60 mm dish) containing 1% SR and 1% antibiotic-antimycotic mixture to eliminate the effect of FBS and synchronize the cell cycle. Cells were incubated with the serum free medium for 12 h, and then conditioned medium was changed to another serum-free medium 30 min prior to treatment with reagents.

3. Primary culture of mouse hippocampal neurons

Hippocampus was isolated from prenatal mice (17-19 days) brain and gently minced using a sterile scalpel. Minced hippocampi were treated with trypsin (0.25%). 2.5×10^6 cells were plated at poly-D-lysine coated 35 mm dish in neurobasal plating media (neurobasal

media containing B27 supplement [1 ml/50 ml], 25 μM glutamate, 0.5 mM Glutamine, 1 mM HEPES, 10% Heat Inactivated Donor Horse Serum) and placed in an incubator 37 ℃ with 5% CO_2. After 24 h, growth media was changed to neurobasal feeding media (neurobasal media containing B27 Supplement [1 ml/50 ml], 0.5 mM Glutamine Solution, 0.5% antibiotics, 1 mM HEPES) and 5 μM of cytosine arabinoside (AraC, Sigma Aldrich). After incubation of 24 h, medium was changed to the neurobasal feeding media without AraC. All mice primary hippocampal culture protocols followed the National Institutes of Health Guidelines for the Humane Treatment of Animals. It was approved by the Institutional Animal Care and Use Committee of Seoul National University (SNU-151116-1).

4. Ethanol and AA exposure

Ethanol {2×10-1% (34 mM), 4×10-1% (69 mM), 8×10-1% (103 mM)} and AA {(3×10-4% (54 μM), 6×10-4% (107 μM), 12×10-4% (215 μM)} were added directly to the 2 mL of conditioned media in the dose-dependent experiment. In the other experiments, cells were exposed to 4×10^{-1}% ethanol and 6×10^{-4}% AA 30 min after treatment with the reagent. Dishes were directly sealed with a

thin layer of paraffin film to prevent the evaporation of the ethanol and AA.

5. Western blotting

Cells were collected after being washed twice with cold PBS and then pelleted by centrifugation at at 13,200 rpm for 5 min at 4 ℃. Afterward, cells were incubated for 30 min on ice with RIPA lysis buffer (Thermo Fisher) supplemented with proteinase and phosphatase inhibitor cocktail before centrifugation for at 13,200 rpm for 5 min at 4 ℃. The protein concentration was determined using a bicinchoninic acid (BCA) assay kit (Bio-Rad, Hercules, CA, USA). Samples containing 10-20 μg of protein were loaded into 10-15 % sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) for electrophoresis and transferred to a polyvinylidene fluoride (PVDF) membrane. Protein-containing membranes were washed with Tris-buffered saline containing 0.1% Tween-20 (TBST) solution and then blocked with 5% skim milk for 30 min. Blocked membranes were washed with TBST (3 times every 10 min), and incubated with primary antibody overnight at 4 ℃. Then, the

membranes were washed and incubated with HRP-conjugated secondary antibody at RT for 2 h. The western blotting bands were visualized by Chemiluminescence (Bio-Rad). Densitometric analysis was carried out with the Image J software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD, USA; http://rsb.info.nih. go.kr/ij/).

6. Real time polymerase chain reaction (real time-PCR)

RNA was extracted from SK-N-MC using a commercial RNA extraction kit (TaKaRa, Otsu, Japan). Using a reverse transcription kit (iNtRON Biotechnology, Seongnam, Republic of Korea), reverse transcription of 1 μg of the extracted RNA was carried out. Reverse transcription was performed at 45 ℃ for 60 min for cDNA synthesis and 95 ℃ for 5 min for RTase inactivation. With the forward and reverse primers for EP1-4 and β-actin, cDNA was amplified. Two microliters of the reverse transcription products were then amplified with the QuantiSpeed SYBR Kits (Life technologies, Gaithersburg, MD, USA). Real-time quantification of the RNA

targets was performed in a Rotor-Gene 6000 real-time thermal cycling system (Corbett Research; NSW, Australia). The primer sequences are described in supplementary Table 1. The Real-Time PCR was performed as follows: 15 min at 95 ℃ for DNA polymerase activation; 15 sec at 95 ℃ for denaturing; and 40 cycles of 15 sec at 94 ℃, 30 sec at 56 ℃, and 30 sec at 72 ℃. Data were collected during the extension step (30 sec at 72 ℃), and analysis was performed with the software provided. Following the real-time PCR, a melting curve analysis was conducted to verify the specificity and identity of the PCR products.

Species	Genes	Sense	Antisense
Human	*EP1*	5’ – AGCTTGTCGGTATCATGGTGG –3’	5’ – AAGAGGCGAAGCAGTTGGC –3’
	EP2	5’ – GAAACCTCTTCCCGAAAGGAAA –3’	5’ – GACTGAACGCATTAGTCTCAGAA –3’
	EP3	5’ – CGCCTCAACCACTCCTACAC –3’	5’ – GACACCGATCCGCAATCCTC –3'
	EP4	5’ – CCGGCGGTGATGTTCATCTT –3’	5’ – CCCACATACCAGCGTGTAGAA –3’
	BACE1	5’ – GGTCGTCCTTCCATGCTGAA –3’	5’ – TTCGCTTTTCCCTGGGATCG –3’
	β–actin	5’ – AACCGCGAGAAGAYGACCCACATCATGTTT –3’	5’ – AGCAGCCGTGGCCATCTCTTGCTCGAAGTC –3’
	COX2	5’ – GTTCCACCCGCAGTACAGAA –3’	5’ – TCCACAGCATCGATGTCACC –3’

Table 1. Primer sequences for RT–PCR amplification

7. Immunocytochemistry

Cells cultured in confocal dishes (Thermo Fisher) were fixed with 80% acetone in PBS for 10 min at −20 ℃ followed by washing (3 times) with PBS. Subsequently, cells were blocked with 5% FBS in PBS to reduce the nonspecific binding. Blocked cells were incubated with a 1:100 dilution of primary antibody overnight at 4 ℃ followed by washing three times with PBS. Cells were incubated for 1 h at RT with Alexa Fluor secondary antibody and PI (propidium iodide) and then washed three times with PBS. Images were obtained with a Fluoview^{TM} 300 confocal microscopy (Olympus, Japan).

8. Measurements of intracellular ROS levels

Determination of the intracellular ROS level was performed using CM−H_2DCFDA staining (DCF−DA, Life technology). Detached cells with 0.25% trypsin were counted using a Petroff−Hausser Counter. Next, cells (5×10^5) were incubated with 10 μm DCF−DA in PBS for 1 h at 30 ℃ in the dark followed by washing twice with PBS. Then, a 100 μl cell suspension was loaded into a 96−well black plate and measured with a luminometer (Victor, Perkin−Elmer, Waltham, MA,

USA).

9. Transfection of small interfering RNAs

Prior to ethanol exposure, specific siRNAs for *EIF2A* and *CREB1*, and a non-targeting siRNA (as a negative control) were transfected into SK-N-MC cells for 24 h with the TurboFect™ transfection Reagent (Thermo Fisher) according to the manufacturer's instructions. The concentration of each transfected siRNA was 25 nM.

10. Nuclear fractionation

Following collection, cells were suspended in nuclear fractionation buffer [1.5 mM KH2PO4, 2.5 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF, and 10 mg/ml leupeptin (pH 7.5)] by pipetting and then incubated on ice for 10 min. Lysates were centrifuged at 3,000 rpm/4 ℃ for 5 min, and the supernatant was collected as a non-nuclear fraction. The remaining pellet was incubated with RIPA

lysis buffer on ice for 20 min and then centrifuged at 15,000 rpm/4 ℃ for 30 min. The supernatant representing the nuclear

fraction was collected.

11. Determination of prostaglandin E_2 and Aβ concentration

Aβ and PGE_2 production levels in the cell culture media after ethanol exposure were estimated using specific Aβ and PGE_2 enzmyme-linked immunosorbent assay (ELISA) Kits obtained from Wako Chemical (Chuo-Ku, Osaka, Japan) and Cayman Chemical (Ann Arbor, MI, USA), respectively. Following the manufacturer's instructions, the PGE_2 concentration was determined.

12. Immunoprecipitation

To estimate the amount of Aβ in the media using a commercial co-immunoprecipitation kit (Thermo Fisher), Aβ specific antibody was immobilized and conjugated to agarose beads according to the manufacturer's instructions. After the SK-N-MC treatment, cell-conditioned media were collected and centrifuged at 3,000 rpm for 10 min at 4 ℃ to remove cell debris. Subsequently, a cocktail of

proteinase/phosphatase inhibitors was added to clean the media. The media were either stored at −80 ℃ or incubated with the agarose bead−conjugated Aβ specific antibody for 12 h at 4 ℃. The agarose beads were spun down by centrifugation at 1,000 rpm for 1 min at 4 ℃. The beads were washed six times with washing buffer, and the proteins were collected after incubation with elution buffer for 5 min. After that, the protein concentration was measured with the BCA assay, and the same amount of protein for the different samples was used to do the western blot analysis as previously mentioned.

13. Statistical analysis

All data shown in the results are showed as a mean ± standard error of mean (S.E.M Comparison between the treated and control groups were performed by Student's t−test for two group analysis. A p value < 0.05 was considered statistically significant.

RESULTS

1. Effect of ethanol on BACE1 expression and ER stress

Previous studies reported that the blood alcohol concentration (BAC) level in alcohol abuser may reaches and exceeds 0.4%. Therefore, I investigated the effect of various concentrations of ethanol (0.2, 0.4, and 0.8%) on BACE1 expression in neuronal cells. As shown in Figure 2a, the expression levels of BACE1 and the C99 fragment of APP were directly increased with the increment in the ethanol concentration. Relative quantitative qPCR was performed to evaluate the *BACE1* mRNA transcript level. *BACE1* mRNA expression increased in the ethanol (4×10^{-1}%) treated cells (Fig. 2b). To confirm this effect I determined the BACE1 expression in ethanol-treated mouse hippocampal neurons; ethanol-induced BACE1 expression in mouse hippocampal neurons (Fig. 2c). Moreover, an increase in the fluorescence intensity of BACE1 in

SK-N-MC cells treated with the same amount of ethanol as above was observed through immunofluorescence analysis (Fig. 2d). Based on the immunoprecipitation and ELISA results of the conditioned media, cells exposed to ethanol (4×10^{-1}%) exhibited increased Aβ secretion (Figs. 3a and 3b). In addition, I investigated the cytotoxic effect of ethanol on neuronal cell death. My data showed that the various concentrations of ethanol (0 – 8×10-1%) incubation for 24 h did not affect the cell viability (Fig. 4). It has been known that ethanol is mainly metabolized via three metabolic enzymes, such as catalase, alcohol dehydrogenase and cytochrome P450IIE1 (CYP) (Villalobos-Garcia and Hernandez-Munoz, 2017). To evaluate the effect of ethanol metabolism on BACE1 expression, cells were pretreated with inhibitors of ethanol metabolism, such as an alcohol dehydrogenase inhibitor fomepizole, a CYP inhibitor diallyl disulfide and a catalase inhibitor sodium azide. As shown in the figures 5a and 5b, pretreatment of fomepizole or diallyl disulfide did not affect the ethanol-induced BACE1 expression. Cells were pre-treated with sodium azide as a catalase inhibitor to evaluate the effect of ethanol metabolism on BACE1 expression. However, there was a significant decrease in the levels of BACE1 and C99 in

the sodium azide-pretreated cells (Fig. 6a). These findings indicate that catalase is a major metabolic enzyme involved in BACE1 expression induced by ethanol. To confirm the effect of ethanol metabolite acetaldehyde (AA) on BACE1 expression, I used different concentrations of AA (0 - 12×10-4%). BACE1 expression was induced in response to different AA concentrations (Fig. 6b). To examine whether ER stress contributes to the ethanol-induced BACE1 expression, I determined the response of CHOP and the phosphorylation of eIF2α to different ethanol concentrations. As shown in Figure 7a, ethanol-induced eIF2α phosphorylation and CHOP expression occurred in a dose-dependent manner. To investigate the potential mechanism that mediates the ethanol-induced ER stress, I investigated the effect of ethanol on the intra-cellular ROS production using the DCF-DA assay. As shown in the figure 7b, The DCF-DA result indicated that ethanol significantly induced intra-cellular ROS production. Hence, I pretreated the cells with NAC, a ROS scavenger, to determine the role of ROS in the ethanol-induced ER stress. My results showed that NAC pretreatment suppressed ethanol-induced p-eIF2α and CHOP, as well as BACE1 and C99 (Fig. 7c). To

investigate whether ER stress is up-stream of the ethanol-increased BACE1, I pretreated PBA, an ER stress inhibitor, prior to ethanol treatment. And, Iobserved that PBA pretreatment eliminated the ethanol-induced BACE1 and C99 levels caused by both ethanol and AA (Figs. 8a and 8b). Collectively, my findings suggest that ethanol stimulates ER stress via ROS production, which is involved in BACE1 expression.

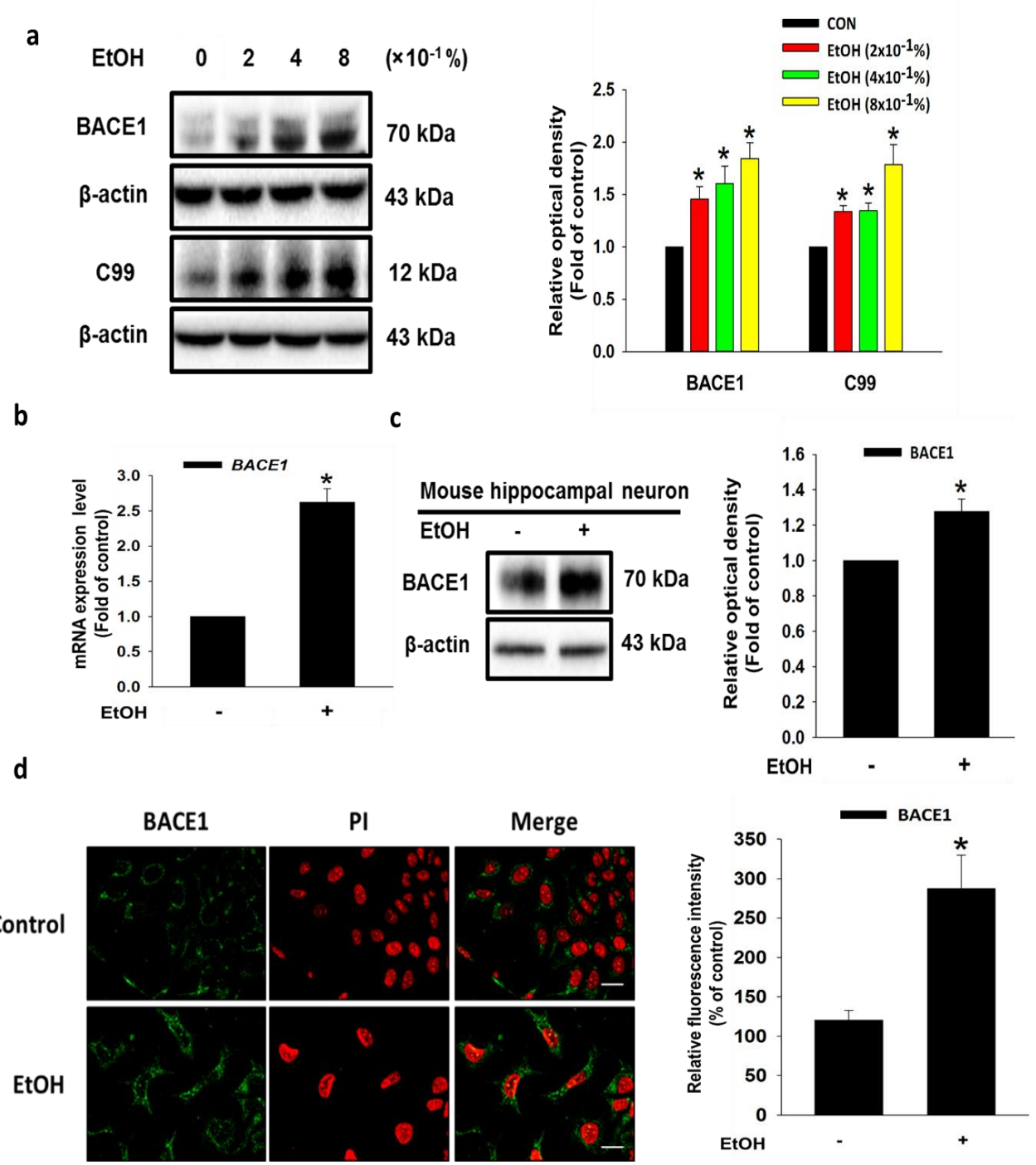
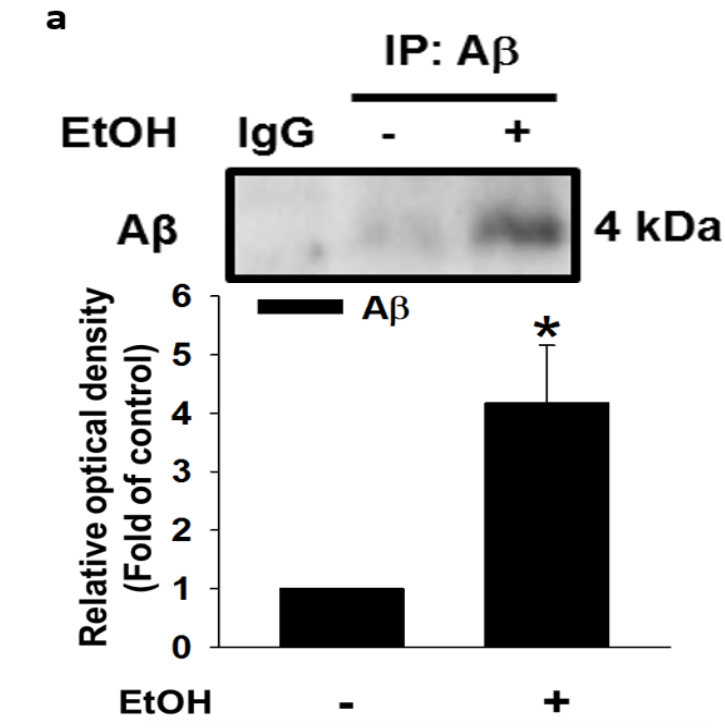

a
EtOH 0 2 4 8 (×10-1 %)
BACE1
β-actin
C99
β-actin
70 kDa
43 kDa
12 kDa
43 kDa
CON
EtOH (2x10-1%)
EtOH (4x10-1%)
EtOH (8x10-1%)
Relative optical density (Fold of control)
BACE1
C99
b
BACE1
mRNA expression level (Fold of control)
EtOH
c
Mouse hippocampal neuron
EtOH
BACE1
β-actin
70 kDa
43 kDa
Relative optical density (Fold of control)
d
BACE1
PI
Merge
Control
EtOH
Relative fluorescence intensity (% of control)

Figure 2. Effect of EtOH on BACE1 expression. (a) Cells incubated with ethanol concentrations (0 – 8×10^{-1}%) for 24 h. BACE1, C99, and β-actin expression were detected by western blotting. n=3. *p<0.05 versus control. (b) Cells were incubated with ethanol (4×10-1%). Quantitative analysis of BACE1 and β-actin mRNA expression estimated by real-time PCR. BACE1 mRNA expression was normalized by β-actin mRNA expression. n=3. *p<0.05 versus control. (c) Mouse hippocampal neurons were incubated with ethanol (4×10^{-1}%). BACE1 and β-actin expressions were detected by western blotting. n=3. *p<0.05 versus control. (d) Cells were immunostained with BACE1 and PI after incubation with ethanol (4×10-1%) for 24 h. Scale bars, 10 um (magnification, ×800).


a
IP: Aβ
EtOH
IgG
-
+
Aβ
4 kDa
Aβ
Relative optical density
(Fold of control)
0
1
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3
4
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EtOH
-
+

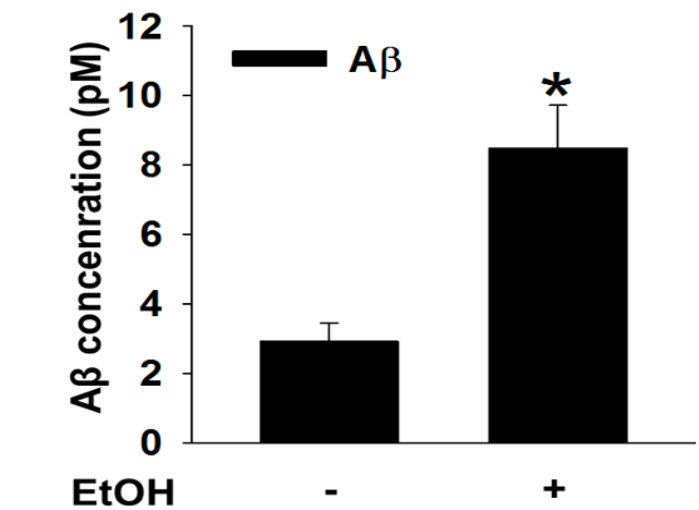

b
Aβ concenration (pM)
0
2
4
6
8
10
12
Aβ
*
EtOH
-
+

Figure 3. Effect of EtOH on Aβ secretion. (a) Cells were exposed to ethanol (4×10^{-1}%). Then, immunoprecipitated Aβ from the medium was analyzed by western blotting. n=3. (b) Cells were incubated with ethanol (4×10−1%). Aβ concentration in the medium was quantified using specific Aβ ELISA kit.

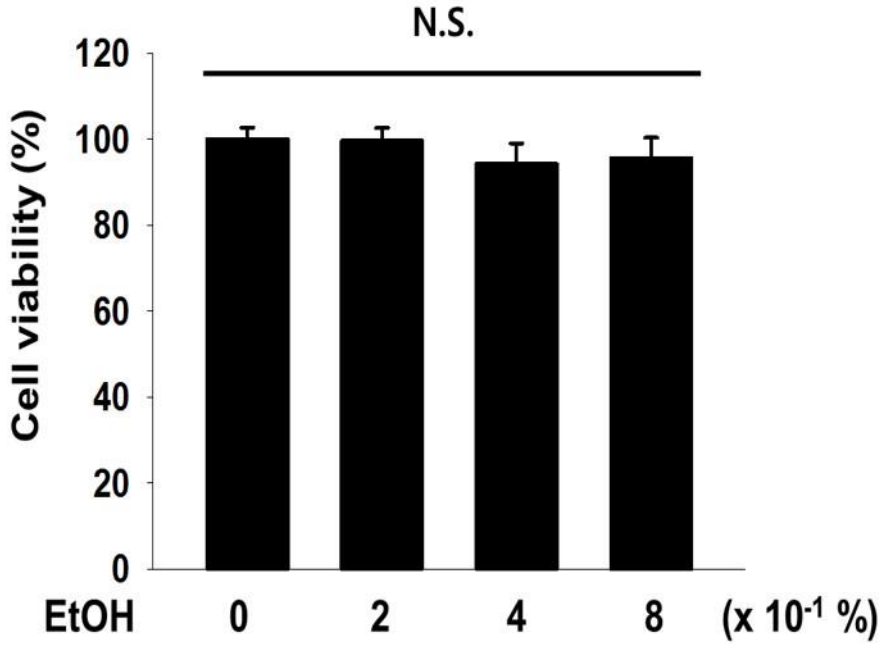


Figure 4. Effect of the various concentrations of ethanol on cell viability. Cells were incubated with ethanol (0 – 8×10−1%) for 24 h. The cell viability was measured by trypan blue exclusion assay. n=4. N.S. indicates no statistically significant.

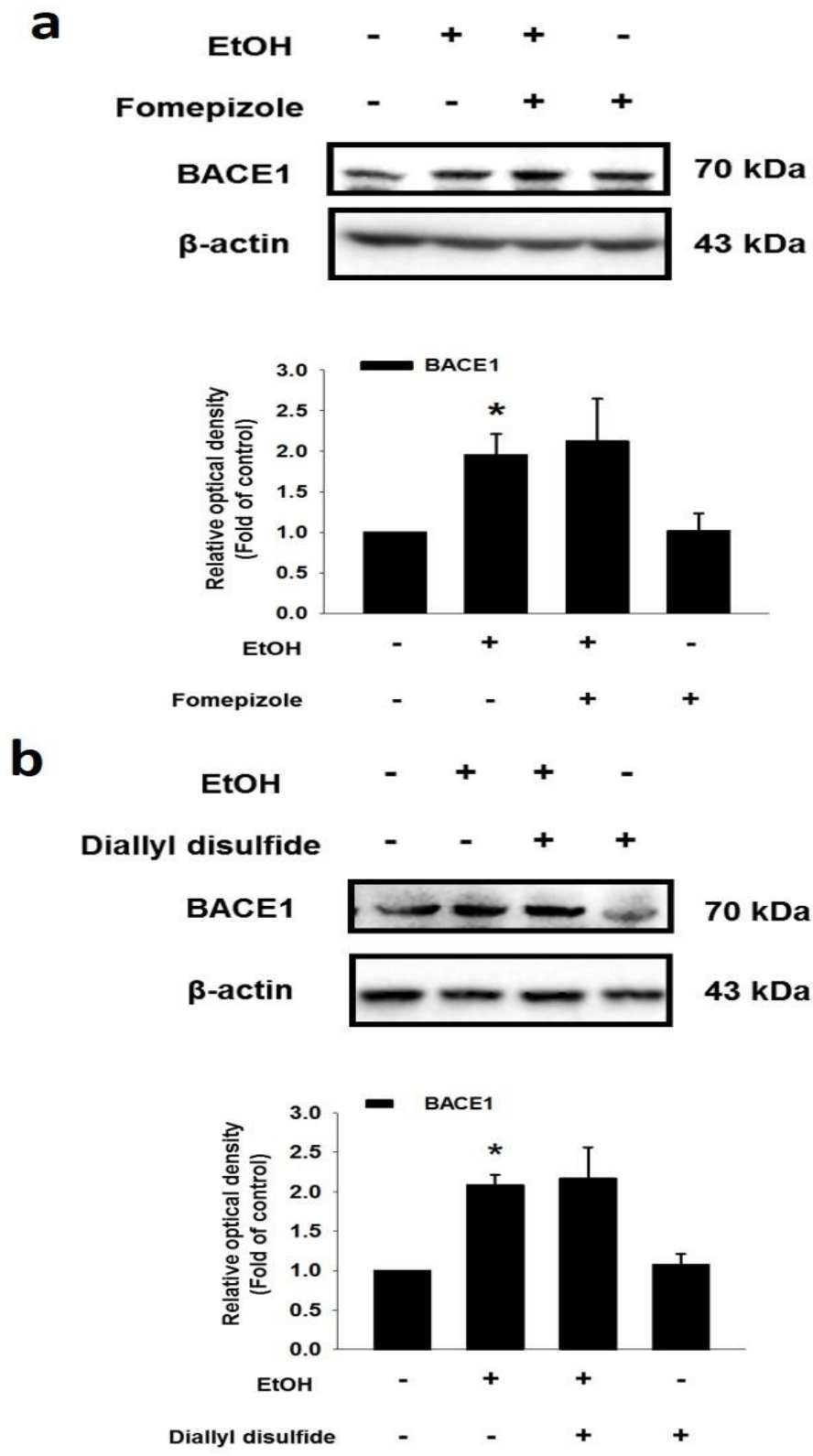

a
EtOH - + + -
Fomepizole - - + +
BACE1
70 kDa
β-actin
43 kDa
BACE1
Relative optical density (Fold of control)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
EtOH - + + -
Fomepizole - - + +
b
EtOH - + + -
Diallyl disulfide - - + +
BACE1
70 kDa
β-actin
43 kDa
BACE1
Relative optical density (Fold of control)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
EtOH - + + -
Diallyl disulfide - - + +

Figure 5. Role of alcohol dehydrogenase and CYP in ethanol-induced BACE1 expression. (a, b) Cells were pretreated with fomepizole (2 μM) or diallyl disulfide (10 μM) for 30 min prior to ethanol treatment (4×10-1%) for 24 h. BACE1, C99 and β-actin expressions were analyzed by western blotting. Blot images are representative. n=3. *p<0.05 versus vehicle-treated control.

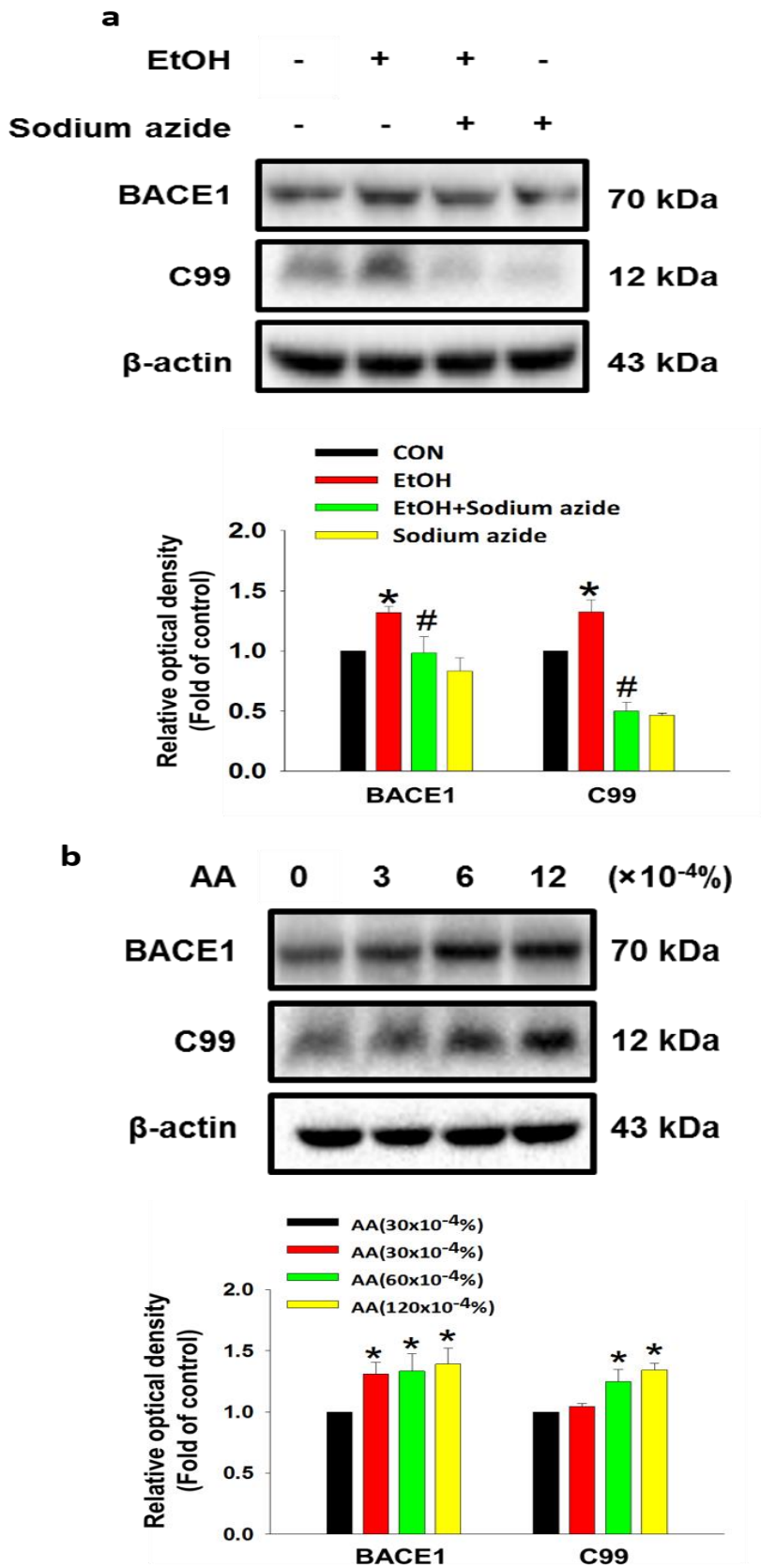

a
EtOH - + + -
Sodium azide - - + +
BACE1
C99
β-actin
70 kDa
12 kDa
43 kDa
CON
EtOH
EtOH+Sodium azide
Sodium azide
Relative optical density
(Fold of control)
2.0
1.5
1.0
0.5
0.0
BACE1
C99
b
AA 0 3 6 12 (×10-4%)
AA(30x10-4%)
AA(30x10-4%)
AA(60x10-4%)
AA(120x10-4%)

Figure 6. Role of catalase enzyme and acetaldehyde in ethanol-induced BACE1 expression. (**a**) Sodium azide (5 mM) was treated 30 min before ethanol (4×10^{-1}%) exposure. BACE1, C99, and β-actin expression in SK-N-MC were detected by western blotting. n=3. *p<0.05 versus vehicle-treated control. # p<0.05 versus 4×10^{-1}% ethanol treatment. (**b**) Cells were incubated with AA concentrations (0 – 12×10^{-4}%) for 24 h followed by western blotting of BACE1, C99, and β-actin. n=3. *p<0.05 versus vehicle-treated control.

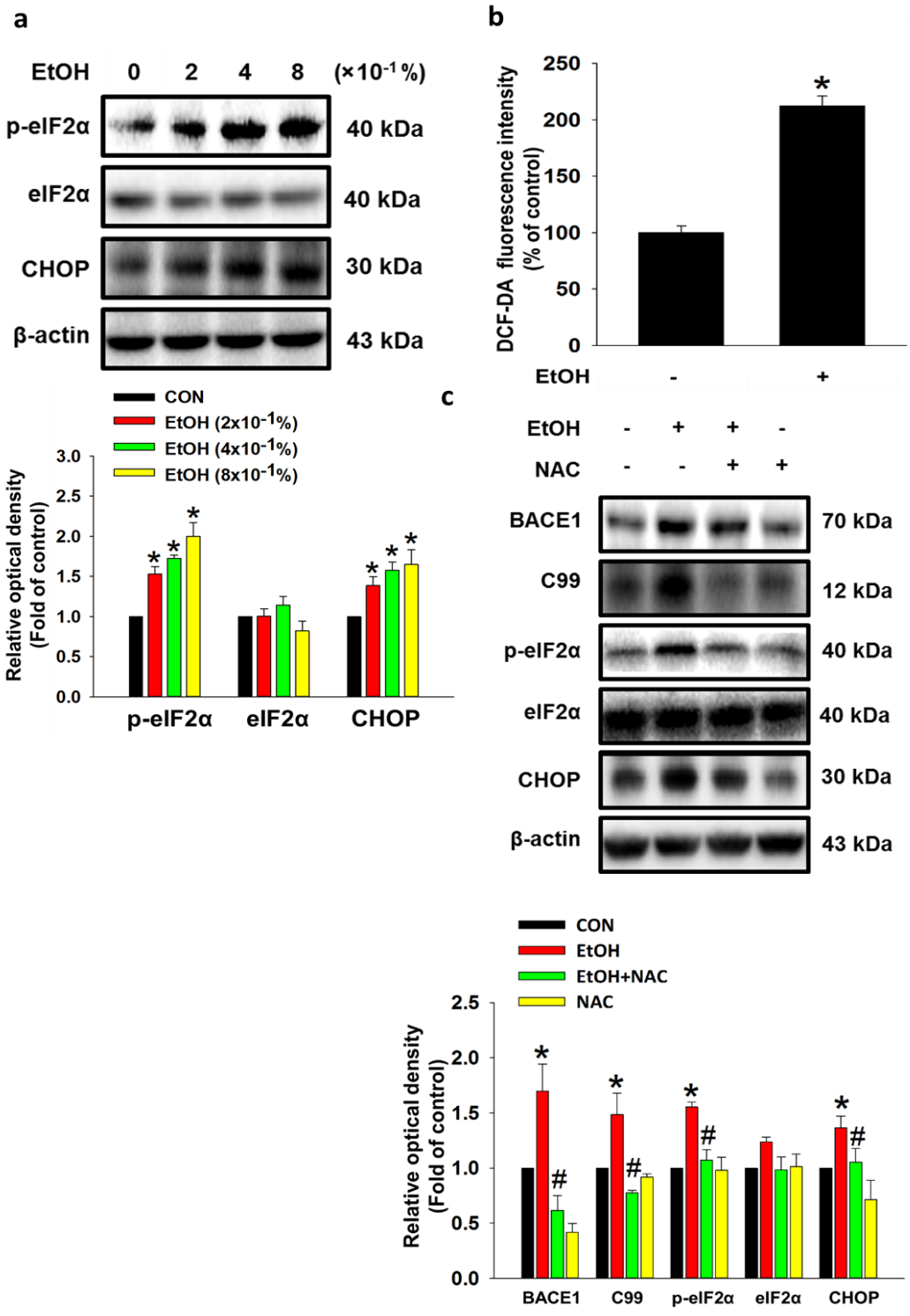
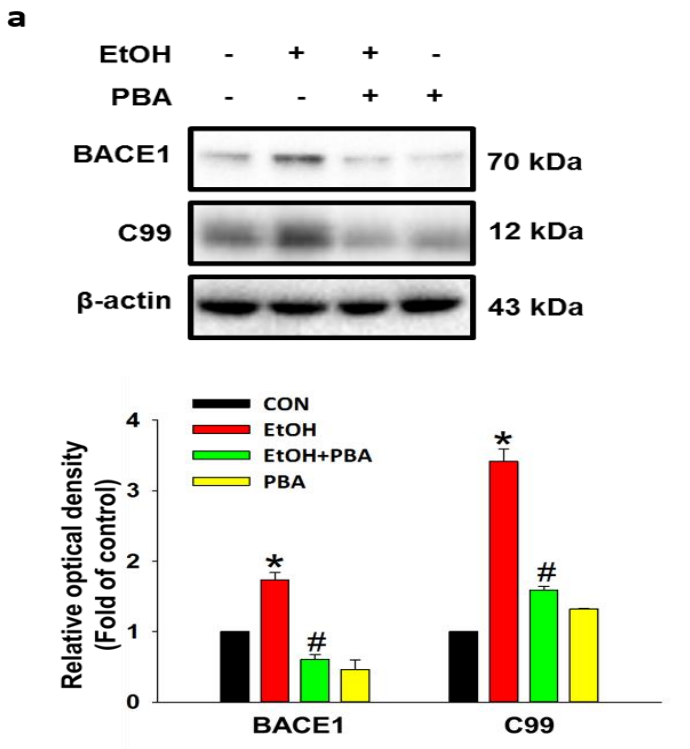

a
EtOH 0 2 4 8 (×10-1 %)
p-eIF2α
eIF2α
CHOP
β-actin
40 kDa
40 kDa
30 kDa
43 kDa
CON
EtOH (2x10-1%)
EtOH (4x10-1%)
EtOH (8x10-1%)
Relative optical density (Fold of control)
p-eIF2α
eIF2α
CHOP
b
DCF-DA fluorescence intensity (% of control)
EtOH - +
c
EtOH - + + -
NAC - - + +
BACE1
C99
p-eIF2α
eIF2α
CHOP
β-actin
70 kDa
12 kDa
40 kDa
40 kDa
30 kDa
43 kDa
CON
EtOH
EtOH+NAC
NAC
Relative optical density (Fold of control)
BACE1
C99
p-eIF2α
eIF2α
CHOP

Figure 7. EtOH-induced ROS generation stimulates BACE-1 expression and ER stress. (**a**) CHOP, eIF2α, p-eIF2α and β-actin expressions were analyzed by western blotting. *n=3*. **p*<0.05 versus control. (**b**) Intracellular ROS was determined after treatment of ethanol (4×10^{-1}%). *n=3*. **p*<0.05 versus control. (**c**) Cells were pretreated with NAC (5 mM) for 30 min then exposed to ethanol 4×10^{-1}% for 24 h. BACE1, C99, CHOP, eIF2α, p-eIF2α and β-actin were detected by western blotting. *n=3*. **p*<0.05 versus vehicle-treated control. # *p*<0.05 versus 4×10^{-1}% ethanol treatment.


a
EtOH - + + -
PBA - - + +
BACE1
C99
β-actin
70 kDa
12 kDa
43 kDa
CON
EtOH
EtOH+PBA
PBA
Relative optical density
(Fold of control)
0
1
2
3
4
*
#
BACE1
C99

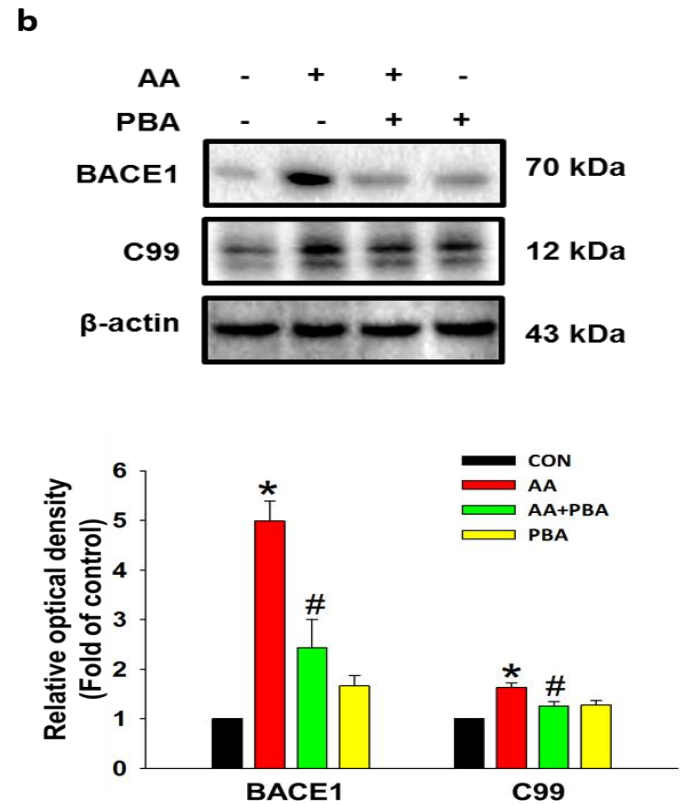

b
AA - + + -
PBA - - + +
BACE1
C99
β-actin
70 kDa
12 kDa
43 kDa
CON
AA
AA+PBA
PBA
Relative optical density
(Fold of control)
0
1
2
3
4
5
6
*
#
BACE1
C99

Figure 8. EtOH-induced ER stress stimulates BACE-1 expression. (**a,b**) Detection of BACE1, and C99 in PBA (5mM) pretreated-cells by western blotting. $n=3$. $*p<0.05$ versus vehicle-treated control. # $p<0.05$ versus 4×10^{-1}% ethanol treatment and 6×10^{-4}% AA treatment, in **a** and **b**, respectively.

2. Involvement of ER stress in ethanol up-regulated COX-2

To explore whether ethanol induces COX-2 in neurons, I performed a concentration-response experiment. My results show that COX-2 expression was elevated in correlation to the ethanol concentration (Fig. 9a). Consistent with this result, the qPCR result showed an increase in the *COX-2* mRNA levels in the ethanol treated cells compared with that of the non-treated control cells (Fig. 9b). Moreover, ethanol (4×10^{-1}%) significantly promoted COX-2 expression within 12 h (Fig. 9c). To clarify whether ethanol-induced COX-2 expression is related to ER stress, I investigated the effect of PBA on the ethanol-induced COX-2 expression. My findings show that PBA ameliorated the ethanol-induced COX-2 expression and PGE_2 production (Figs. 10a and 10b). Moreover, transfection of *EIF2A* siRNA blocked COX-2 expression in ethanol treated cells (Fig. 11a). I confirmed that transfection of EIF2A siRNA significantly inhibited mRNA expression of EIF2A (Fig. 11b). As further verification, my data

also showed that ethanol-induced COX-2 expression and eIF2α phosphorylation were augmented in cells treated with salubrinal, an eIF2α dephosphorylation inhibitor (Figs. 12a and 12b). In addition, confirmed that ethanol-induced PGE2 production was potentiated by salubrinal (Fig. 12c). Cells incubated with NS-398, a selective COX-2 inhibitor, prior to ethanol exposure produced a low amount of PGE2 compared to the ethanol-treated cells without NS-398 (Fig. 13a). To determine whether COX-2 has a role in ethanol-induced BACE1, I treated cells with NS-398 followed by AA and ethanol exposure. The western blot results showed a significant decrease in BACE1 expression in the NS-398 pretreated cells compared to AA or ethanol-exposed cells without NS-398 (Figs. 13b and 13c). In addition, I confirmed that NS-398 pretreatment abolished BACE1 expression induced by ethanol in mouse hippocampal neuron (Fig. 13d). As an alternative investigation, I treated cells with various concentrations of PGE2 to detect the relation between the PGE2-produced from COX-2 overexpression and the ethanol-evoked BACE1. PGE2 increased the BACE1 and C99 expression in a dose-dependent manner (Fig. 14a). To verify this result, I investigated the effect of PGE2 on BACE1 expression

at the single cell level using a confocal microscope. As shown in the figure 14b, BACE1 expression was increased in the PGE2-treated cells. Taken together, my findings suggest that ethanol-induced eIF2α phosphorylation stimulates COX-2 expression and PGE2 production, which is associated with ethanol-induced BACE1 expression.

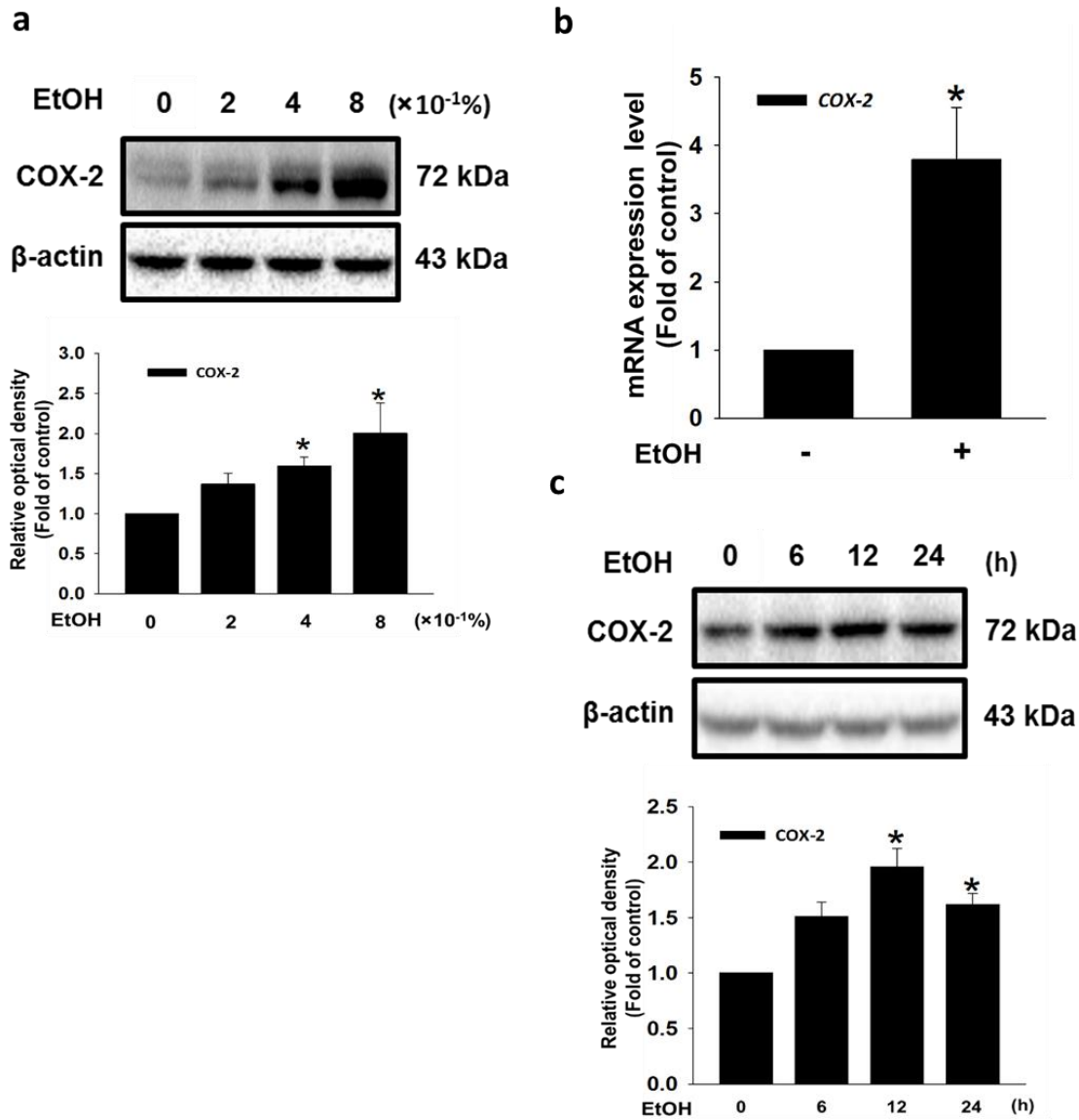
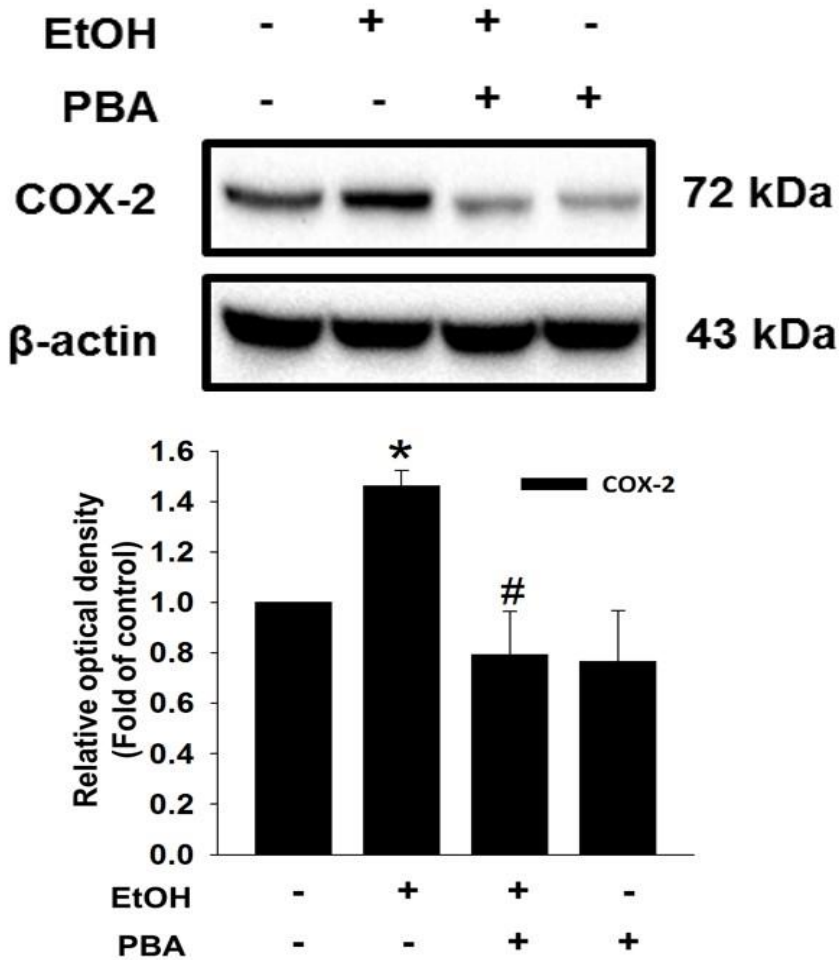


Figure 9. Effect of EtOH on COX-2 expression. (a,c) COX-2 expression was detected by immunoblotting using specific antibody. (b) COX-2 and β-actin mRNA levels determined by RT-PCR and β-actin mRNA used as internal control.

a


EtOH - + + -
PBA - - + +
COX-2
72 kDa
β-actin
43 kDa
Relative optical density (Fold of control)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
#
COX-2
EtOH - + + -
PBA - - + +

b

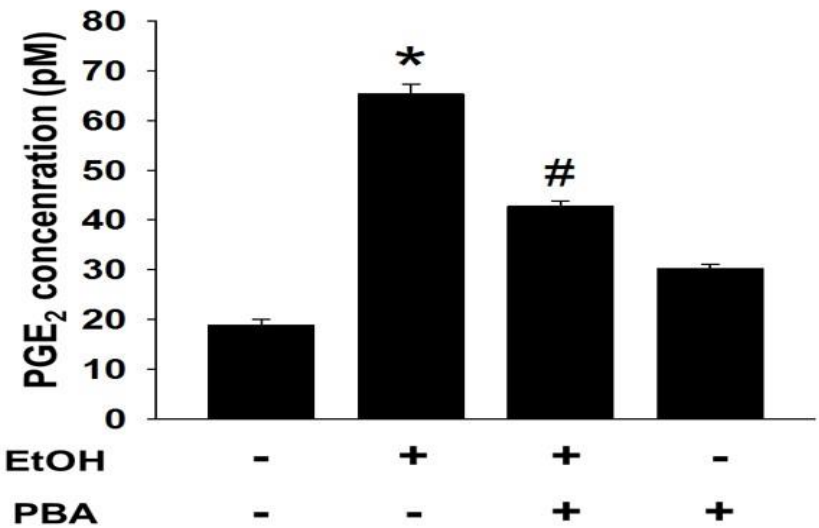
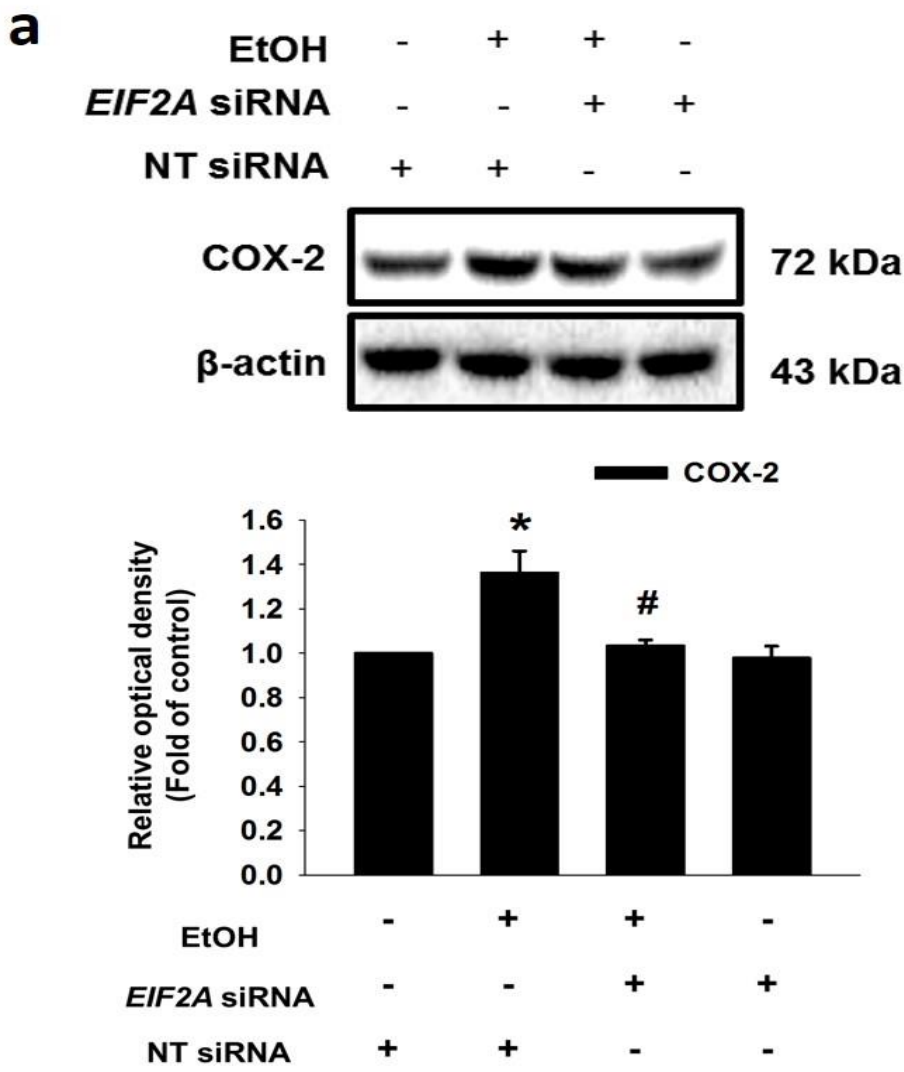

PGE2 concenration (pM)
80
70
60
50
40
30
20
10
0
*
#
EtOH - + + -
PBA - - + +

figure 10. Effect of EtOH-induced ER stress on COX-2 expression. (**a** and **b**) PBA was treated 30 min before ethanol (4×10^{-1}%) exposure. COX-2 expression estimated by western blotting and secretion of PGE_2 in cell medium was quantified using specific PGE_2 ELISA kit.


a
EtOH - + + -
EIF2A siRNA - - + +
NT siRNA + + - -
COX-2
72 kDa
β-actin
43 kDa
COX-2
Relative optical density (Fold of control)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
#
EtOH - + + -
EIF2A siRNA - - + +
NT siRNA + + - -

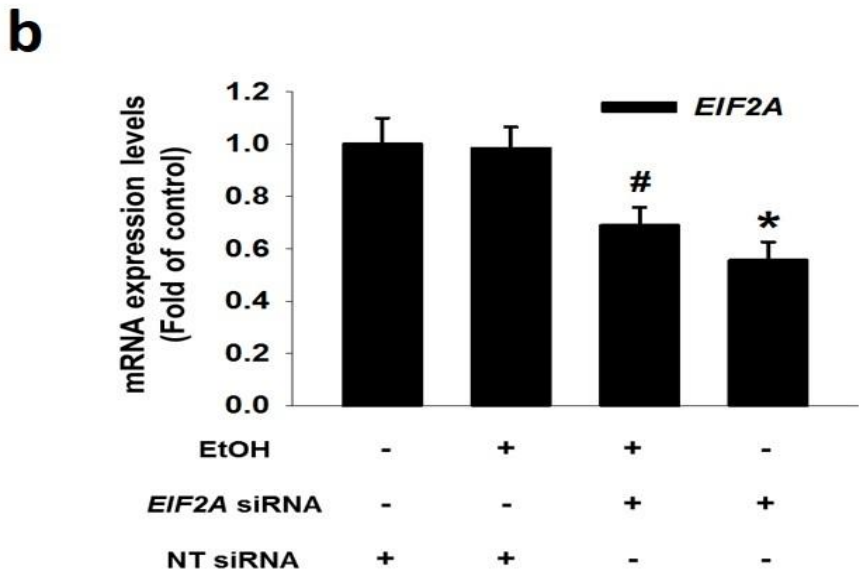

b
EIF2A
mRNA expression levels (Fold of control)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
#
*
EtOH - + + -
EIF2A siRNA - - + +
NT siRNA + + - -

Figure 11. Effect of EIF2A siRNA transfection on EtOH-induced COX-2 expression. (**a**) Cells were transfected with EIF2A siRNA and non-targeting (NT) siRNA for 24 h prior to 4×10^{-1}% ethanol treatment for 12 h. COX-2, and β-actin were analyzed by western blotting. (**b**) SK-N-MCs were transfected with EIF2A siRNA for 24 h prior to ethanol (4×10-1%) treatment. EIF2A and ACTB mRNA expression were analyzed by qPCR, respectively. n=3. *p<0.05 versus NT siRNA-treated control,. #p<0.05 versus 4×10-1% ethanol treatment.

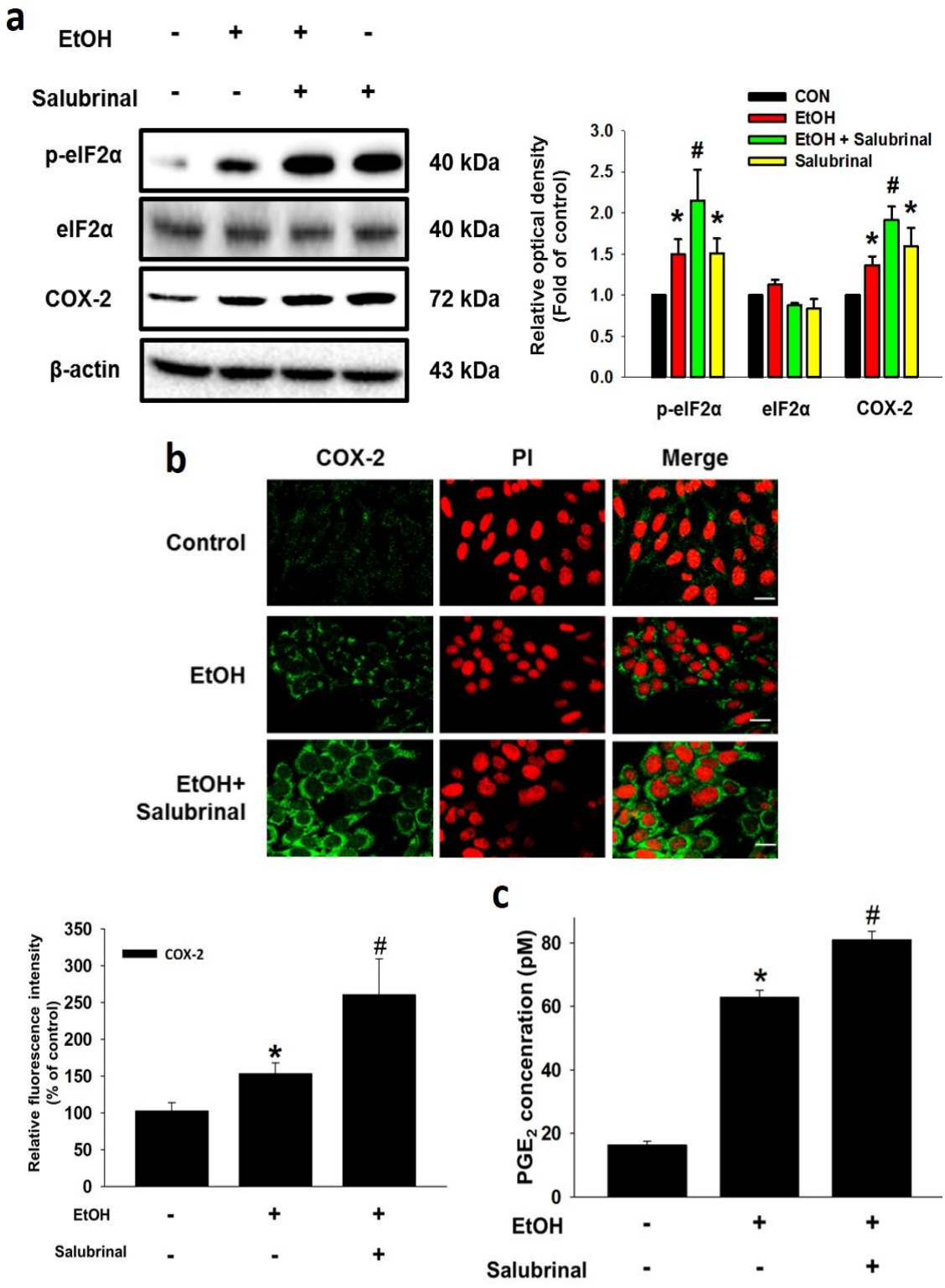


Figure 12. Effect of EtOH-induced eIF2α phosphorylation on COX-2 expression.

(**a** and **b**) Cells were pretreated with salubrinal (10 μM) for 30 min before ethanol treatment for 12 h. COX-2 expression was determined by immunoblotting and visualized by COX-2 immunostaining. **(c)** Secreted PGE_2 in cells medium was estimated by specific PGE_2 ELISA kit. n=3. Western blotting data represents a three independent experiment. *$p<0.05$ versus vehicle-treated control, # $p<0.05$ versus 4×10^{-1}% ethanol treatment.

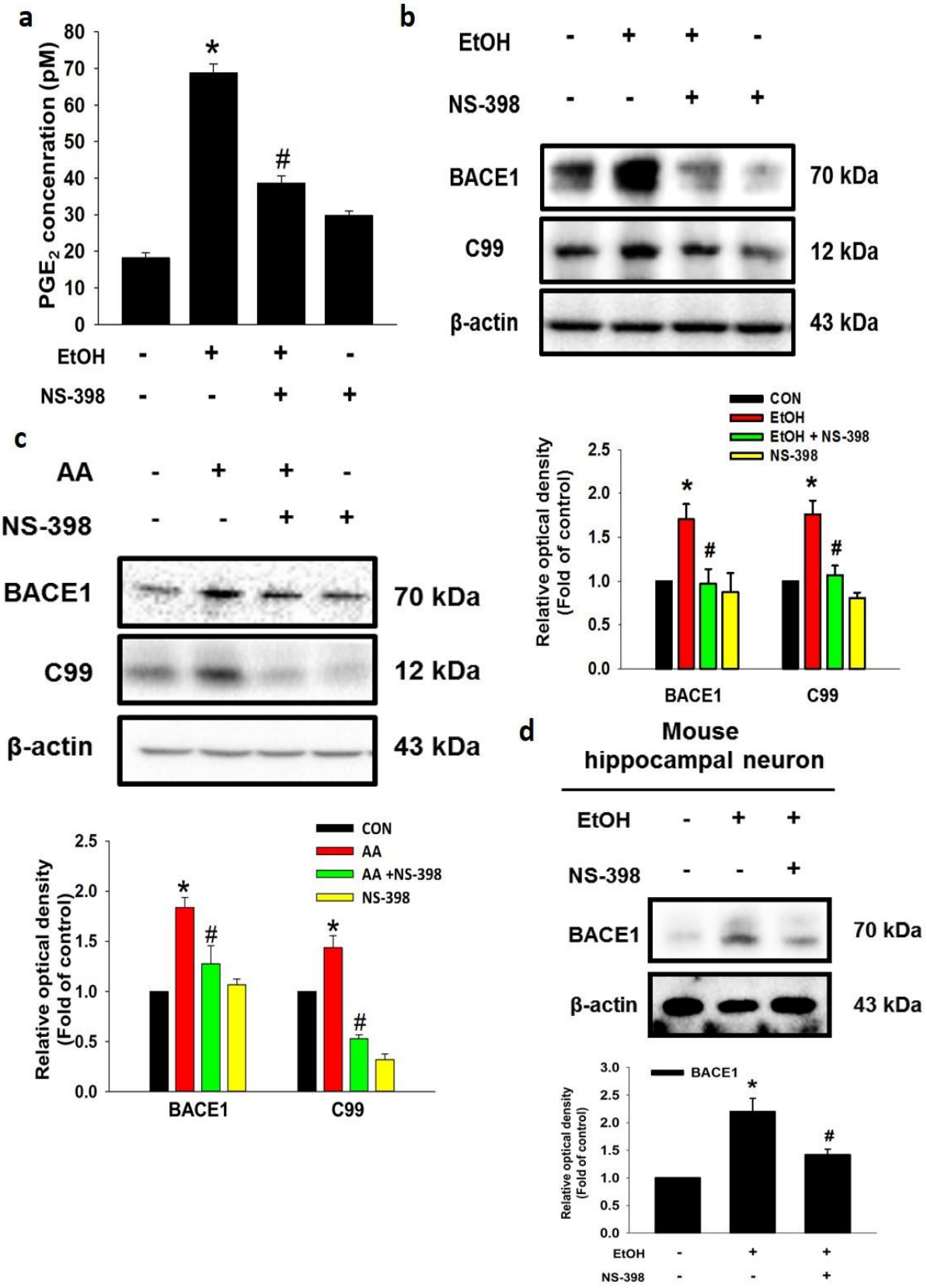

a
PGE2 concenration (pM)
EtOH
NS-398
b
EtOH
NS-398
BACE1
C99
β-actin
70 kDa
12 kDa
43 kDa
CON
EtOH
EtOH + NS-398
NS-398
Relative optical density (Fold of control)
BACE1
C99
c
AA
NS-398
BACE1
C99
β-actin
70 kDa
12 kDa
43 kDa
CON
AA
AA +NS-398
NS-398
Relative optical density (Fold of control)
BACE1
C99
d
Mouse hippocampal neuron
EtOH
NS-398
BACE1
β-actin
70 kDa
43 kDa
BACE1
Relative optical density (Fold of control)
EtOH
NS-398

Figure 13. Effect of EtOH-induced COX-2 on BACE1 expression. (a) Cells were pretreated with NS-398 (50 μM) for 30 min before 4×10-1% ethanol exposure for 12 h. Secreted PGE2 in cells medium was estimated by specific PGE2 ELISA kit. n=3. *p<0.05 versus vehicle-treated control, # p<0.05 versus 4×10-1% ethanol treatment. **(b and c)** Cells were incubated in NS-398 (50 μM) for 30 min before ethanol (4×10-1%) or AA (6×10-4%) treatment for 24 h. and BACE1, C99, and β-actin expressions were determined by western blotting. n=3. *p<0.05 versus vehicle-treated control. #p<0.05 versus 6×10-4% AA. **(d)** Mouse hippocampal neurons were treated pretreated with NS-398 (50 μM) for 30 min before 4×10^{-1}% ethanol exposure for 24 h. BACE1 and β-actin expression were detected by western blotting. *p<0.05 versus vehicle-treated control. # p<0.05 versus 4×10^{-1}% ethanol treatment.

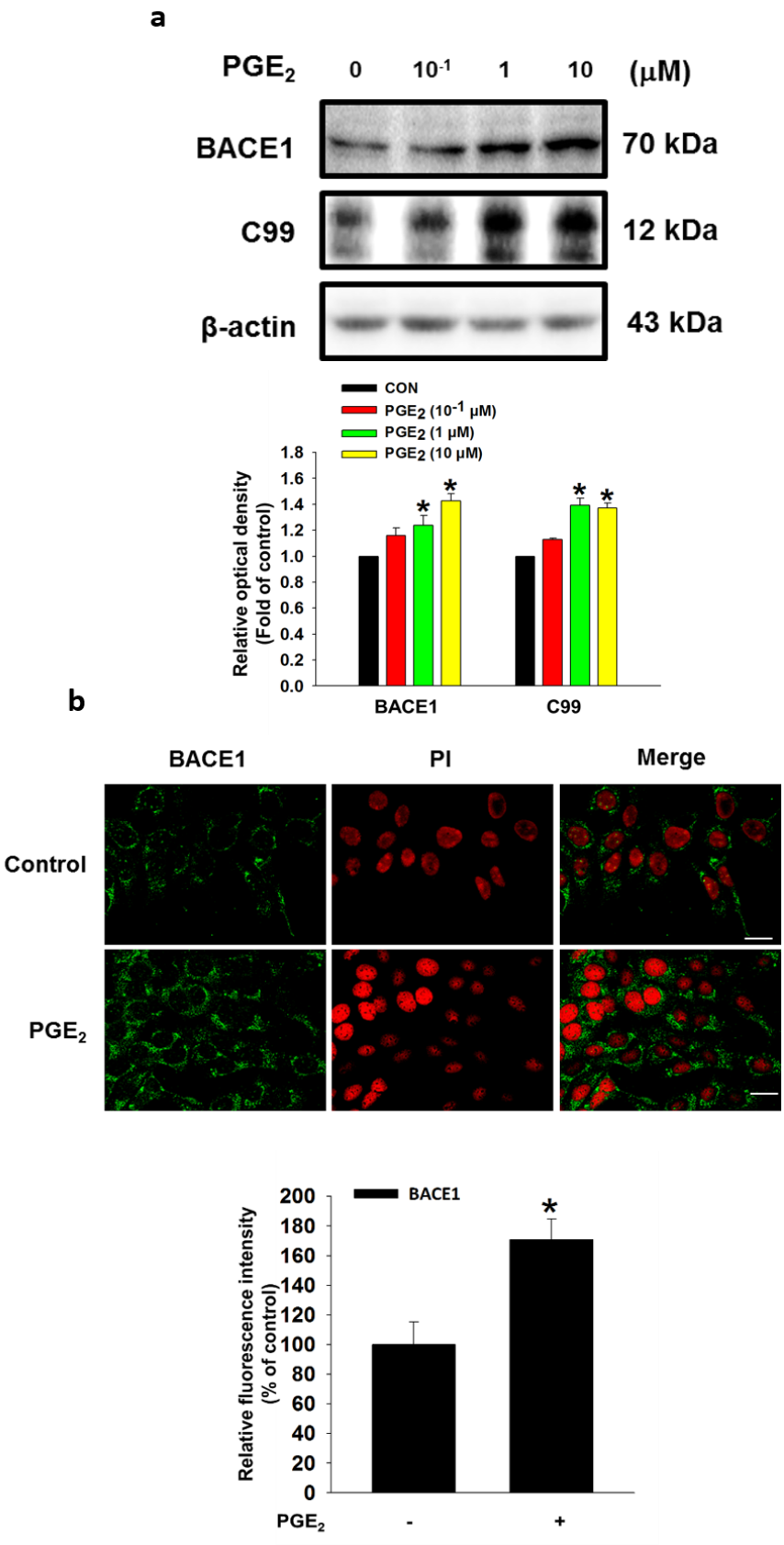

a
PGE2 0 10-1 1 10 (μM)
BACE1
70 kDa
C99
12 kDa
β-actin
43 kDa
CON
PGE2 (10-1 μM)
PGE2 (1 μM)
PGE2 (10 μM)
Relative optical density (Fold of control)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*
BACE1
C99
b
BACE1
PI
Merge
Control
PGE2
BACE1
Relative fluorescence intensity (% of control)
200
180
160
140
120
100
80
60
40
20
0
*
PGE2
-
+

Figure 14. Effect of PGE_2 on BACE1 expression. (**a**) Detection of BACE1, C99, and β-actin expression by immunoblotting in lysate of SK-N-MCs which were treated with PGE2 concentrations (0 - 10 μM). n=3. *p<0.05 versus vehicle-treated control. (**b**) Confocal imaging of BACE1 immunostained cells. Scale bars, 10 um (magnification, ×800)

3. Involvement of EP_2 receptor signaling in ethanol-induced Aβ production

Next, I tested whether PGE_2 receptors are involved in the increased BACE1 by ethanol. First, I determined the effect of ethanol on the expression of PGE_2 receptors. My PCR results show that *EP2* and *EP3* are mainly expressed in SK-N-MC (Fig. 15a). With qPCR, I determined the effect of ethanol on the receptor expression of both subtypes shown in the Figure 15b. *EP2* mRNA was overexpressed in the ethanol treated cell. In agreement, immunoblotting showed an increased EP_2 expression in response to increasing ethanol concentrations (Fig. 15c). Additionally, immunofluorescence staining showed an increase in the fluorescence intensity of EP_2 in the ethanol treated cells (Fig. 15d). Next, to determine how ethanol induces the EP_2 expression, I treated the cells with PBA for 30 min prior to ethanol and AA exposure. My results show that PBA blocked the ethanol-induced up-regulation of the EP_2 receptor at the transcription and translation levels (Figs. 16a and 126). Moreover, pre-treatment

with 4-PBA attenuated the AA-induced EP_2 expression (Fig. 16c). Next, my results show that nuclear translocation and phosphorylation of CREB-1 at the Ser113 residue were induced in the ethanol treated cells in a time-dependent manner (Figs. 17a and 17b). I observed that the phosphorylation of CREB-1 induced by ethanol was abolished by NS-398 pretreatment (Fig. 17c). The cells were pre-treated with 14-22 amide, a PKA inhibitor, and western blotting showed a more decreased expression level of both BACE1 and phosphorylated CREB-1 (Fig. 18a). In agreement with the above results, BACE1 expression in cells transfected with *CREB1* siRNA before ethanol exposure was much lower than that in the ethanol treated cells, and it is similar to the expression in the NT siRNA transfected control (Fig. 18b). I pretreated cells with PF-04418948, a selective EP_2 antagonist. The western blot results showed the suppression of both ethanol-induced CREB-1 phosphorylation at Ser113 residue and catalytic PKA expressions. Interestingly, cells pre-treated with PF-04418948 showed BACE1 expression levels similar to that in the control group, which was never exposed to ethanol (Fig. 19a). And, PF-04418948 pretreatment decreased the ethanol-increased fluorescence

intensity of catalytic PKA in the nuclear region (Fig. 19b). Moreover, ethanol-induced secretion of Aβ in the media was reduced in the PF-04418948 pre-treated cells (Fig. 19c). The results suggest that the PGE2-induced EP-2 activation leads to BACE1 expression and Aβ production through the PKA/CREB-1 pathway.

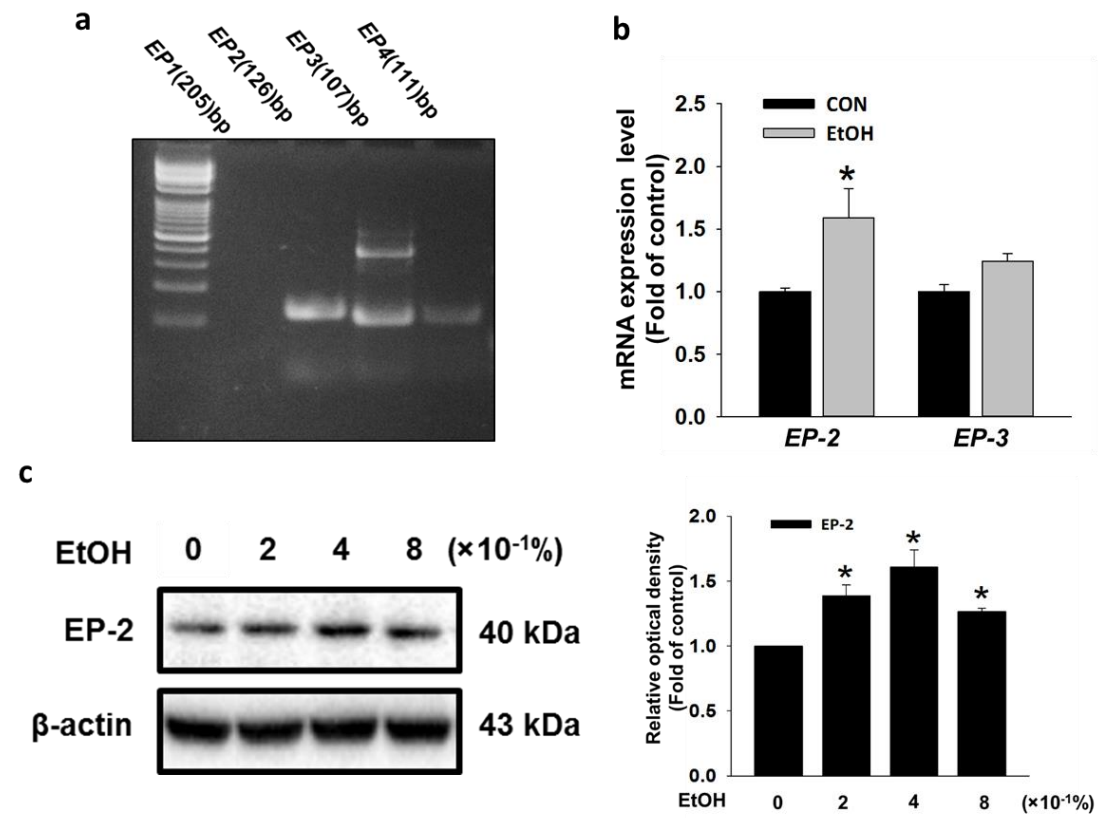

a
EP1(205)bp
EP2(126)bp
EP3(107)bp
EP4(111)bp
b
mRNA expression level (Fold of control)
CON
EtOH
EP-2
EP-3
c
EtOH 0 2 4 8 (×10-1%)
EP-2
40 kDa
β-actin
43 kDa
Relative optical density (Fold of control)
EP-2
d
EP-2
PI
Merge
Control
EtOH
Relative fluorescence intensity (% of control)
EP-2

Figure 15. Effect of EtOH on PGE_2 receptors expression. (**a**) Harboring of EP isotypes in SK-N-MC. EP1, EP2, EP3 and EP4 mRNA expression were detected by PCR. (**b**) Cells were treated with ethanol (4×10^{-1}%) for 24 h. *EP2*, *EP3*, and *β-actin* mRNA levels were analyzed by quantitative RT-PCR. *β-actin* mRNA served as internal control. n=3. *p<0.05 versus vehicle-treated control. (**c**) SK-N-MCs were incubated in ethanol concentrations (0 – 8×10^{-1}%). EP_2 expression was detected by western blotting. n=3. *p<0.05 versus vehicle-treated control. (**d**) Cells were treated with ethanol (4×10^{-1}%) and immunostained with EP_2 and PI. Scale bars, 10 um (magnification, ×800).

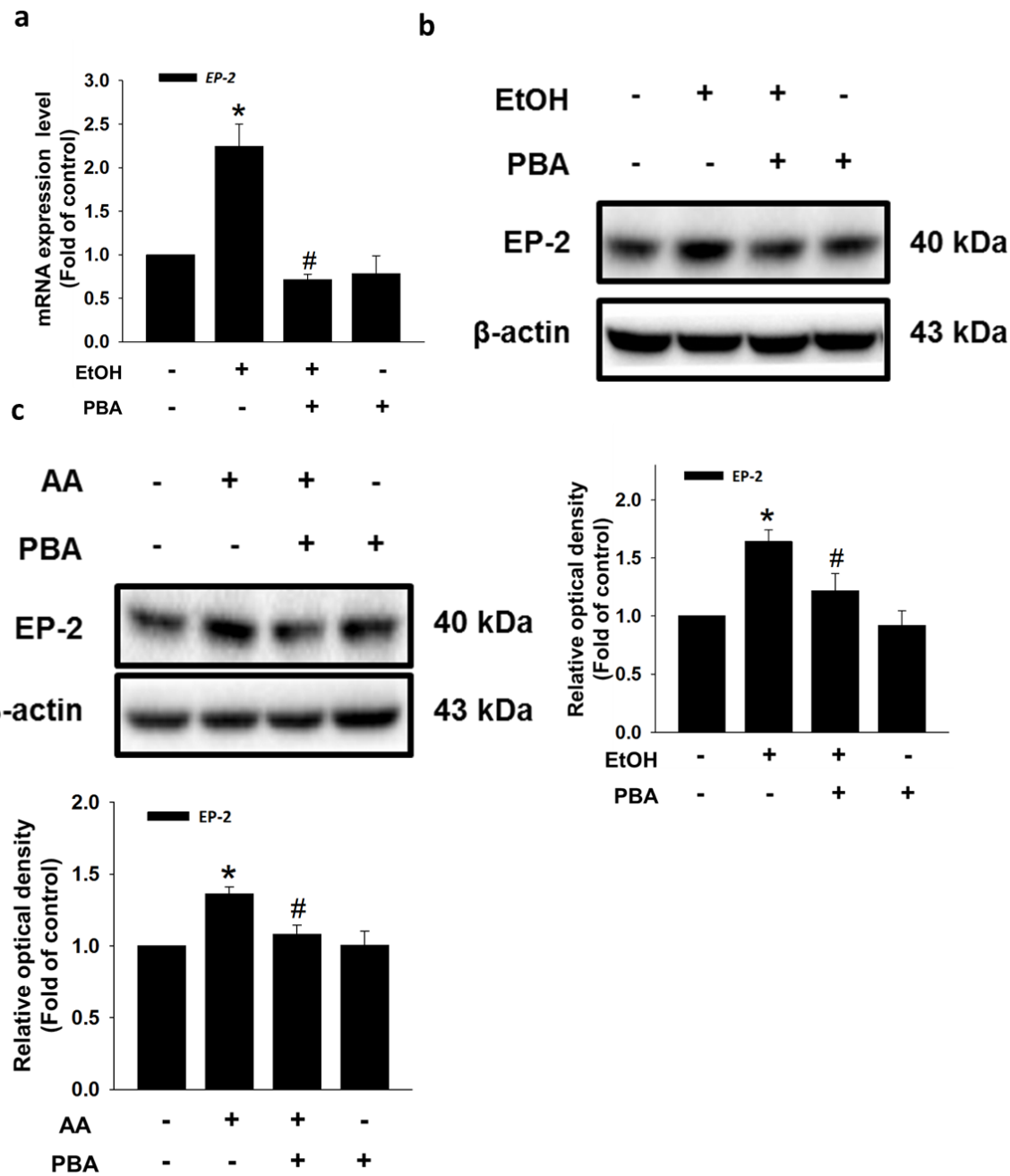

a
EP-2
mRNA expression level (Fold of control)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
#
EtOH - + + -
PBA - - + +
b
EtOH - + + -
PBA - - + +
EP-2
40 kDa
β-actin
43 kDa
c
AA - + + -
PBA - - + +
EP-2
40 kDa
β-actin
43 kDa
Relative optical density (Fold of control)
EP-2
2.0
1.5
1.0
0.5
0.0
*
#
EtOH - + + -
PBA - - + +
Relative optical density (Fold of control)
EP-2
2.0
1.5
1.0
0.5
0.0
*
#
AA - + + -
PBA - - + +

Figure 16. Role of EtOH-induced ER stress signaling in PGE_2 receptors expression. (**a** and **b**) Cells were treated with PBA (5 mM) for 30 min before exposing to ethanol (4×10^{-1}%). EP2, β-actin mRNA levels and EP_2 protein expression were analyzed by quantitative RT-PCR and immunoblotting, respectively. n=3. *p<0.05 versus vehicle-treated control. # p<0.05 versus 4×10^{-1}% ethanol treatment. (**c**) PBA (5 mM) pretreatment for 30 min before AA exposure (6×10^{-4}%). EP_2 expression was analyzed by western blotting using specific antibody. n=3. *p<0.05 versus vehicle-treated control. # p<0.05 versus 6×10^{-4}% AA treatment.

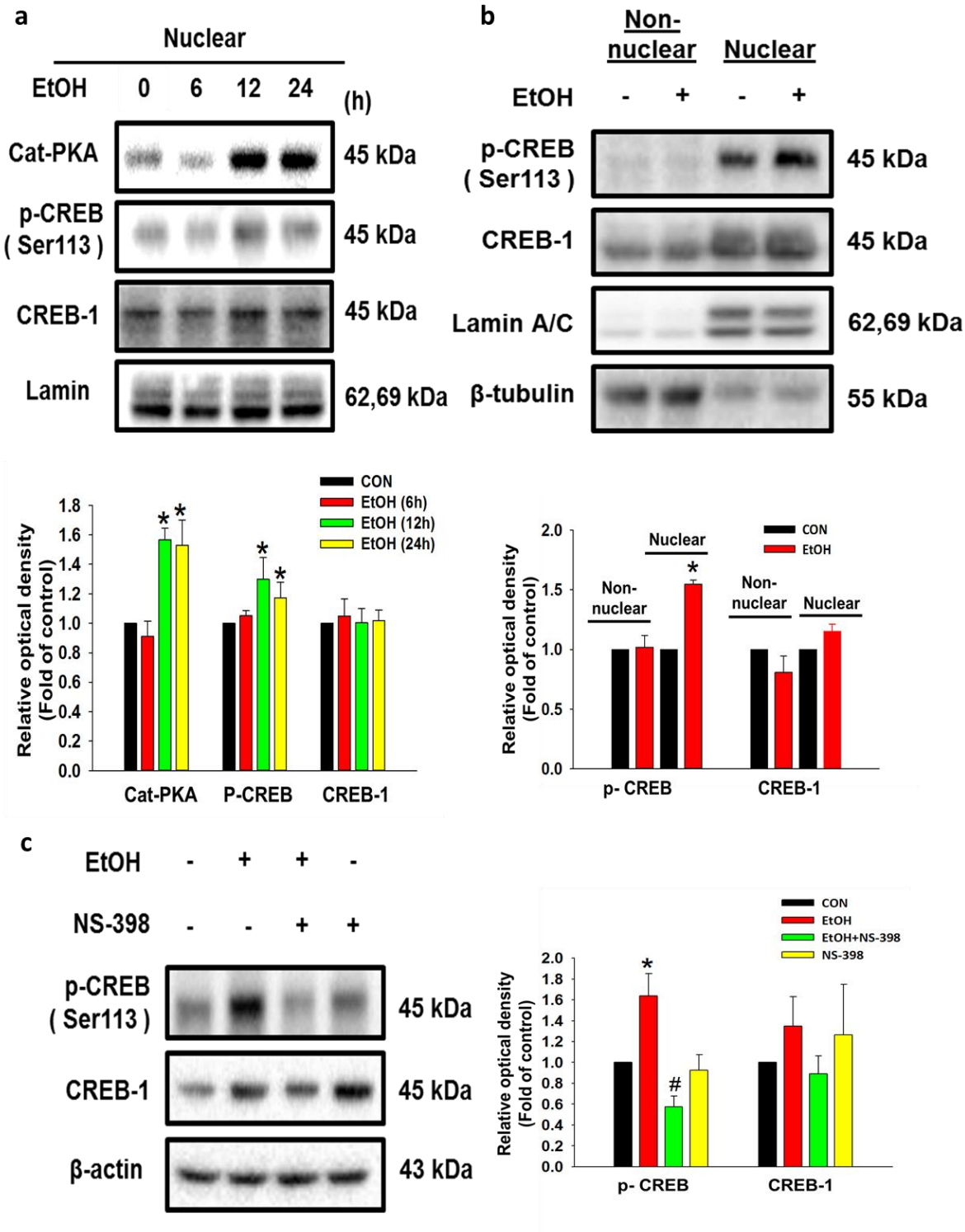

a
Nuclear
EtOH 0 6 12 24 (h)
Cat-PKA
p-CREB (Ser113)
CREB-1
Lamin
45 kDa
45 kDa
45 kDa
62,69 kDa
b
Non-nuclear
Nuclear
EtOH - + - +
p-CREB (Ser113)
CREB-1
Lamin A/C
β-tubulin
45 kDa
45 kDa
62,69 kDa
55 kDa
CON
EtOH (6h)
EtOH (12h)
EtOH (24h)
Relative optical density (Fold of control)
Cat-PKA
P-CREB
CREB-1
CON
EtOH
Nuclear
Non-nuclear
p- CREB
CREB-1
c
EtOH - + + -
NS-398 - - + +
p-CREB (Ser113)
CREB-1
β-actin
45 kDa
45 kDa
43 kDa
CON
EtOH
EtOH+NS-398
NS-398
p- CREB
CREB-1

Figure 17. Effect of EtOH-induced COX-2 on CREB phosphorylation. (**a**) SK-N-MCs were exposed to (4×10^{-1}%) ethanol for (0 – 24) h. Nuclear Cat-PKA, CREB and p-CREB (Ser113) expression were determined by western blotting. n=3. *p<0.05 versus control. (**b**) Cells were treated with ethanol (4×10^{-1}%). Non-nuclear and nuclear expression of CREB-1 and p-CREB (Ser113) were determined by western blotting. (**c**) SK-N-MCs were incubated in NS-398 (50 μM) for 30 min before ethanol exposure (4×10^{-1}%). CREB and p-CREB (Ser113) expression were determined by western blotting. n=3. *p<0.05 versus vehicle-treated control. #p<0.05 versus 4×10^{-1}% ethanol treatment.

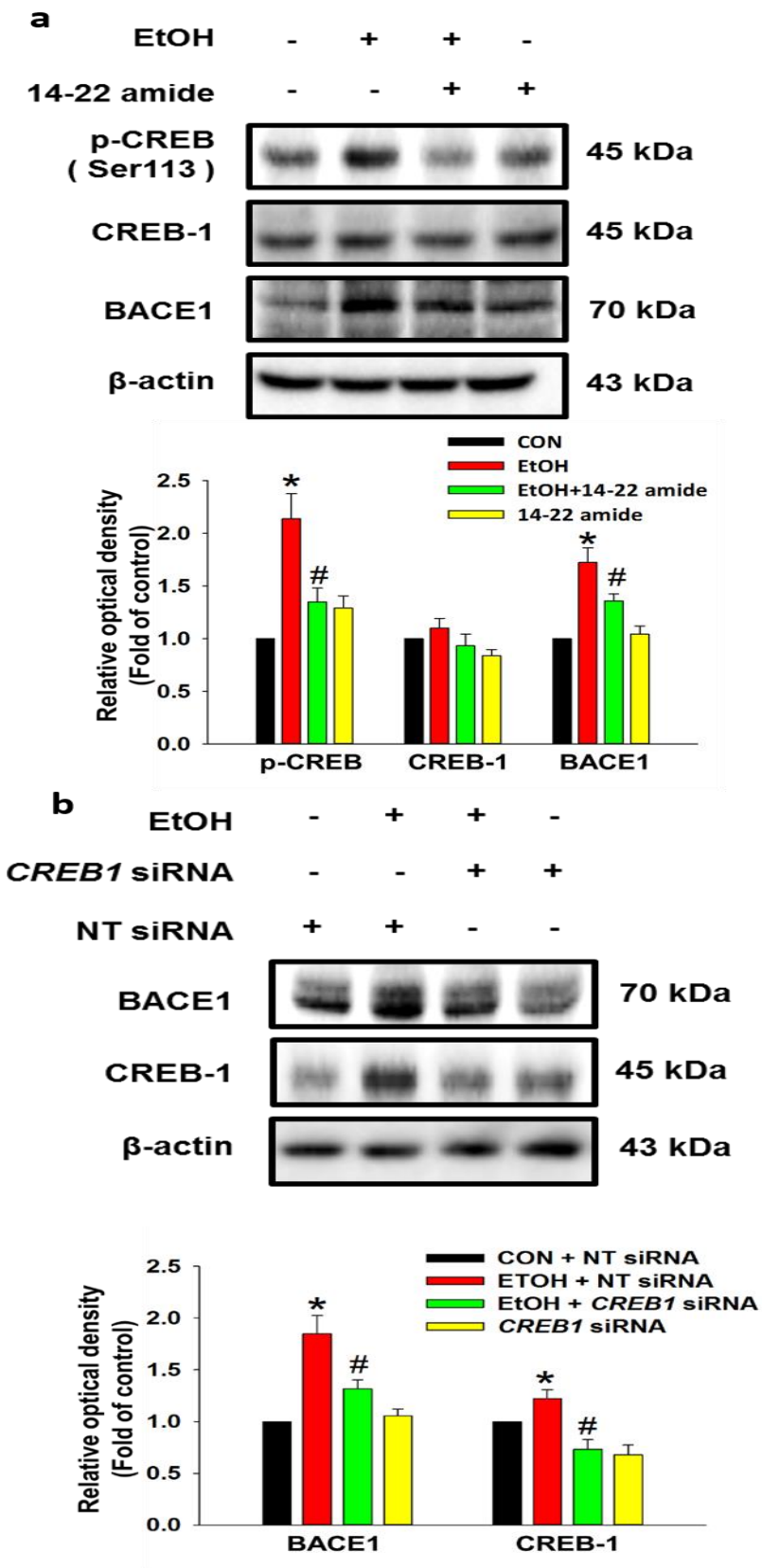

a
EtOH - + + -
14-22 amide - - + +
p-CREB (Ser113)
CREB-1
BACE1
β-actin
45 kDa
45 kDa
70 kDa
43 kDa
CON
EtOH
EtOH+14-22 amide
14-22 amide
Relative optical density (Fold of control)
2.5
2.0
1.5
1.0
0.5
0.0
*
#
p-CREB
CREB-1
BACE1
b
EtOH - + + -
CREB1 siRNA - - + +
NT siRNA + + - -
BACE1
CREB-1
β-actin
70 kDa
45 kDa
43 kDa
CON + NT siRNA
ETOH + NT siRNA
EtOH + CREB1 siRNA
CREB1 siRNA
Relative optical density (Fold of control)
BACE1
CREB-1

Figure 18 Effect of EtOH-induced CREB phosphorylation on BACE1 expression. (**a**) SK-N-MCs were pretreated of 14-22 amide (1 μM) for 30 min before ethanol (4×10^{-1}%) exposure. CREB-1, p-CREB (Ser113) and BACE1 were analyzed by western blotting. (**b**) Detection of BACE1 expression by western blotting in lysate of cells which were transfected with *CREB1* siRNA for 24 h prior to ethanol exposure for 24 h.

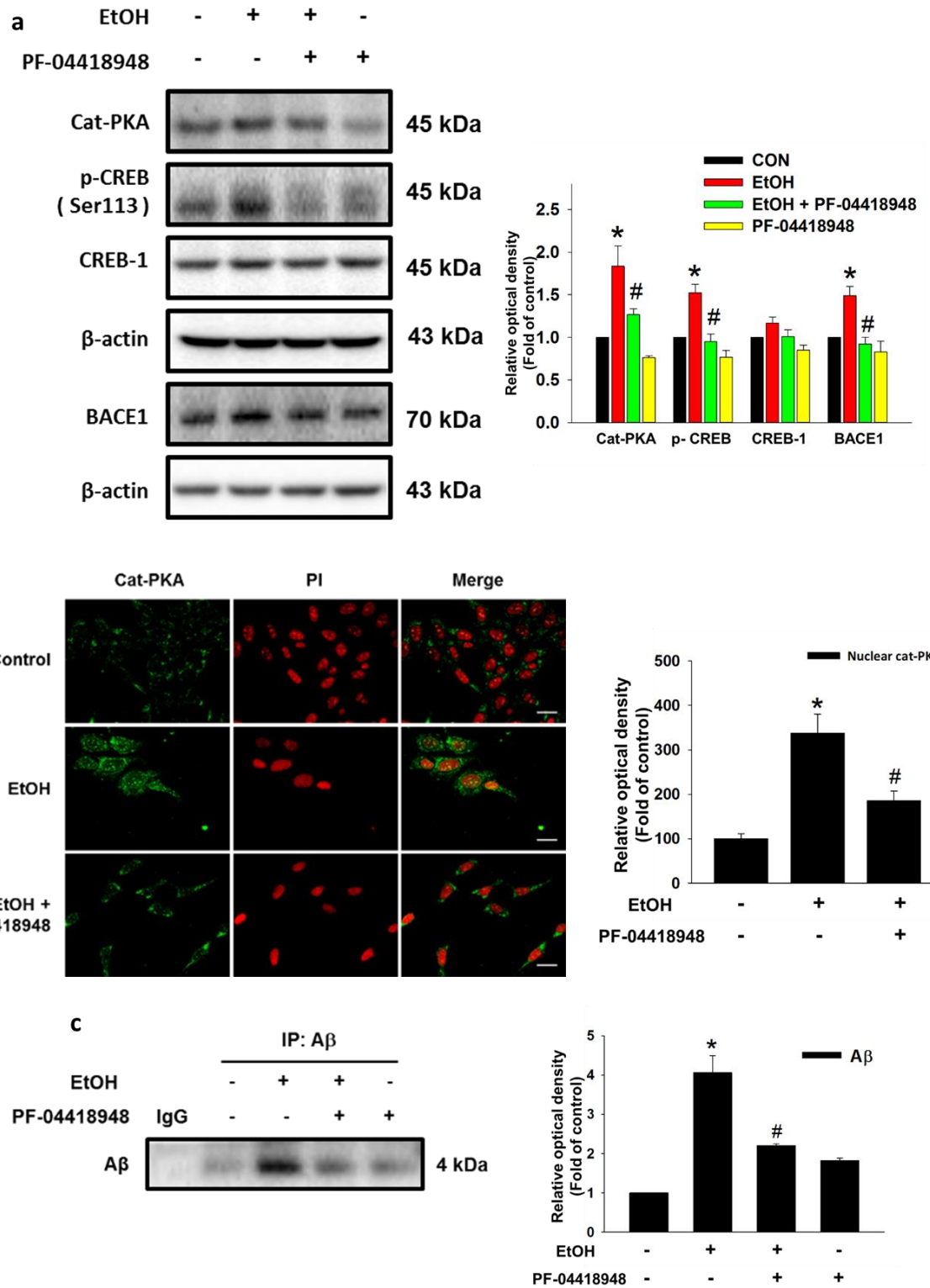

a
EtOH - + + -
PF-04418948 - - + +
Cat-PKA
p-CREB (Ser113)
CREB-1
β-actin
BACE1
β-actin
45 kDa
45 kDa
45 kDa
43 kDa
70 kDa
43 kDa
CON
EtOH
EtOH + PF-04418948
PF-04418948
Relative optical density (Fold of control)
Cat-PKA
p- CREB
CREB-1
BACE1
b
Cat-PKA
PI
Merge
Control
EtOH
EtOH + PF-04418948
Nuclear cat-PKA
Relative optical density (Fold of control)
EtOH - + +
PF-04418948 - - +
c
IP: Aβ
EtOH - + + -
PF-04418948 IgG - - + +
Aβ
4 kDa
Aβ
Relative optical density (Fold of control)
EtOH - + + -
PF-04418948 - - + +

Figure 19. EtOH-induced PGE_2 stimulates Amyloid beta secretion via PKA/CREB/BACE1 pathway. (**a**) Cells were incubated with PF-04418948 (10 μM) for 30 min prior to 4×10^{-1}% ethanol treatment. Cat-PKA, CREB, p-CREB (Ser113) and BACE1 expression were determined by western blotting. (**b**) SK-N-MCs were immunostained with Cat-PKA. Scale bars, 10 um (magnification, ×800). (**c**) Aβ was immunoprecipitated from cells medium and detected by western blotting. Western blot images represent a three independent experiment. $*p<0.05$ versus vehicle-treated control. # $p<0.05$ versus 4×10^{-1}% ethanol treatment.

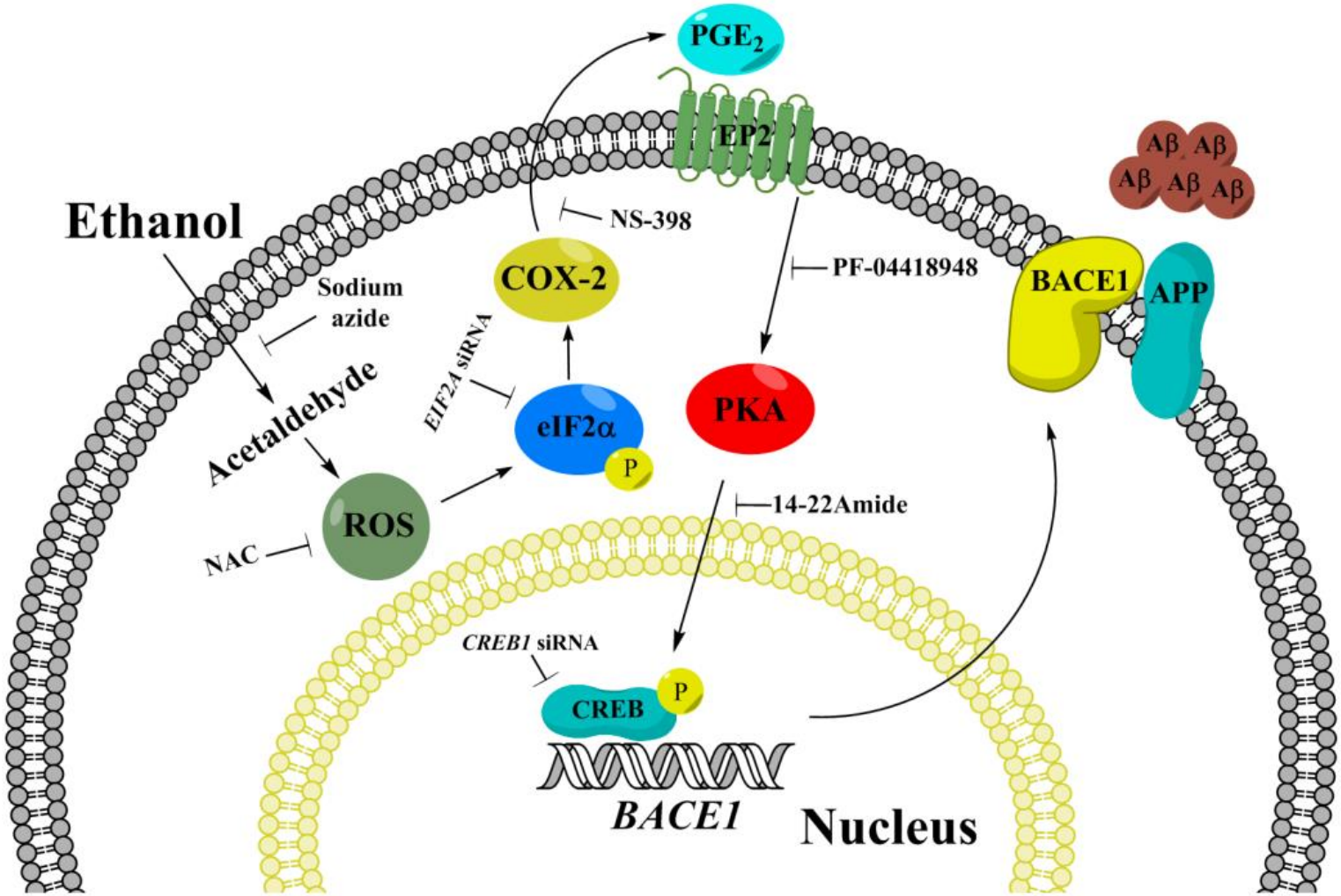

PGE2
EP2
Aβ Aβ Aβ Aβ Aβ
Ethanol
NS-398
COX-2
PF-04418948
BACE1
APP
Sodium azide
EIF2A siRNA
eIF2α
P
PKA
Acetaldehyde
ROS
NAC
14-22Amide
CREB1 siRNA
CREB
P
BACE1
Nucleus

Figure 20. Schematic model illustrates the molecular mechanisms involved in ethanol-induced BACE1. Ethanol evokes ROS generation which leads to eIF2α phosphorylation. Then, eIF2α phosphorylation stimulates COX-2 expression. Induced COX-2 stimulates PGE_2 production. PGE_2 binds with EP_2 receptor leads to PKA activation and CREB phosphorylation. Finally, activated CREB triggers BACE1 expression and Aβ secretion.

DISCUSSION

Previous animal and clinical studies have shown that ethanol-up-regulated BACE1 levels lead to dementia (Anstey et al., 2009; Kim et al., 2011). In contrast, other experimental investigations have shown that ethanol reduces Aβ accumulation suggesting that ethanol at low concentrations might have a neuroprotective effect in AD (Ormeno et al., 2013; Parodi et al., 2015). Although previous study reported that chronic alcohol consumption stimulates Aβ-producing enzyme expressions and tau accumulation (Gendron et al., 2008; Kim et al., 2011), there are few evidences showing the influence of alcohol on the Aβ accumulation in the human brain. A previous report showed the increased Aβ (1-42) level in the cerebral spinal fluid of patients with Wernicke-Korsakoff syndrome, alcohol-associated dementia (Matsushita et al., 2008). In My approach, I tested a range of ethanol concentrations (0.2, 0.4, and 0.8%) and showed that the up-regulation of BACE1 is ethanol concentration dependent; thus, I used an ethanol concentration of 0.4%. According to the BAC level, 0.4% is a potentially harmful concentration for a first-time drinker while for those who have been drinking alcohol

for many years, even if their BAC reaches and exceeds this level, they normally survive (Deitrich and Erwin, 1996; Kouzoukas et al., 2013). Although, this level is unnoticeable in alcohol abusers, along these lines, heavy alcohol drinking contributes to the onset of AD. Furthermore, my results show that alcohol metabolism has a potential role in ethanol-induced BACE1. It is well established that AA is the primary ethanol metabolic product (Deitrich et al., 2006) in the brain which mediates the detrimental ethanol effect by inducing ROS production (D'Addario et al., 2008). Moreover, acetaldehyde produced from ethanol metabolism elsewhere other than the brain, for example, in the liver, has limited ability to reach neurons (Deitrich et al., 2006). Therefore, AA detected in the brain is mainly produced from ethanol metabolism in the brain, and 0.1-0.2% ethanol in the blood penetrates the blood brain barrier (Chumakova et al., 2000; Hernandez et al., 2016). In agreement, my result confirmed that AA at these low concentrations increases the BACE1 level in the same manner as the ethanol concentrations. Moreover, inhibition of ethanol metabolism by catalase abolished the BACE1 expression induced by the ethanol treatment. My findings suggest that ethanol-produced AA stimulates BACE1 expression.

Ethanol-induced neurodegeneration is correlated with oxidative stress (Ke et al., 2011; Yang and Luo, 2015). My study revealed that blocking ethanol-stimulated ROS alleviated the ethanol-potentiated phosphorylation of eIF2α and expressions of CHOP and BACE1. ROS-induced oxidative stress is strongly interrelated with AD pathogenesis through altered BACE1 expression (Lee et al., 2016). Ethanol-produced ROS triggers the accumulation of improperly folded proteins in the ER which lead to the induction of ER stress (Chen et al., 2008). Cells treated with an oxidative agent showed that oxidative stress-induced BACE1 is mediated by eIF2α phosphorylation (Devi and Ohno, 2014; Mouton-Liger et al., 2012). Due to long length and particular AUGs of 5′ UTR in BACE1mRNA, BACE1 translation is constitutively minimized under basal condition, and is activated by eIF2α phosphorylation (Lammich et al., 2004; O'Connor et al., 2008). It has been known that the eIF2α can be phosphorylated by four different kinases: the Heme-regulated eukaryotic initiation factor eIF2α kinase (HRI), the double-stranded RNA-activated protein kinase (PKR), the PKR-like endoplasmic reticulum-related kinase (PERK), the general control nonderepressible-2 kinase (GCN2) (Dever, 2002). In my study,

ethanol stimulates oxidative and ER stress which can activate PKR and PERK leading to eIF2α phosphorylation, respectively (Li et al., 2010; Liu et al., 2013). Therefore, my findings indicate that ethanol-induced eIF2α phosphorylation can be a key factor stimulating BACE1 translation. Furthermore, inhibition of eIF2α kinases improves AD-associated memory deficit (Ma et al., 2013). Moreover, I showed that attenuation of ethanol-induced ER stress ameliorated COX-2 expression as well as stimulated PGE2 secretion. Long-term ethanol treatment induced transcription factor TNFα and up-regulated COX-2 expression in astrocytes (Valles et al., 2004). PGE2 is formed by the action of microsomal PGE2 synthase (mPGES), COX-1 and COX-2. However, COX-2 is responsible for the production of the main part of PGE2 in neurons and astrocytes (Peri et al., 1995; Smith et al., 1991). Induction of ER stress using tunicamycin resulted in PGE2 overproduction mediated by COX-2 (Hosoi et al., 2013; Hung et al., 2004). I clearly showed the direct effect of ethanol-induced ER stress on the regulation of COX-2 expression. Previous works demonstrated induction of ER stress signaling stimulated COX-2 expression through MAPK, NF-κB, eIF2α/ATF4 pathway (Hung et al., 2004;

Luo et al., 2016; Rasheed and Haqqi, 2012). In this study, ethanol-enhanced eIF2α phosphorylation up-regulates COX-2 expression. PERK activation, an eIF2α kinase, mediates ER stress-stimulated neuroinflammation (Meares et al., 2014). Meanwhile, my data showed salubrinal enhanced ethanol-induced eIF2α phosphorylation and COX-2 expression, whereas PBA pretreatment abolished ethanol-induced COX-2 and BACE1 expressions. Salubrinal inhibits the dephosphorylation of eIF2α (Boyce et al., 2005). And, PBA interacts with hydrophobic domain of unfolded and misfolded proteins, which increases the chance for correct folding (Ozcan et al., 2009; Ozcan et al., 2006). Those findings suggest that opposite effects of ER stress inhibitors on the ethanol-induced COX-2 expression in this study may be due to the different action mechanism of inhibitors. In addition, a previous report demonstrated that salubrinal reduced neuronal cell apoptosis and ER stress by enhancement of eIFα/ATF4/CHOP signaling down-regulated by chronic ER stress in hippocampal neuron (Kim et al., 2014). Conversely, another previous report showed that salubrinal augmented the free fatty acid-induced ER stress in pancreatic β-cell (Cnop et al., 2007). Although salubrinal has been known as an

ER stress inhibitor, present and previous findings suggest that the effect of salubrinal on the ER stress can be dependent on cell types, experimental condition and cellular microenvironment. Moreover, selective inhibition of COX-2 has been found to block the PGE2-induced Aβ aggregation and Aβ-induced memory suppression in AD (Kotilinek et al., 2008; Wang et al., 2014). My results show that the ethanol-induced BACE1 was attenuated by blocking COX-2 indicating that the ethanol-elevated COX-2 is part of the ethanol-induced ER stress leading to increased BACE1 levels.

In vitro and in vivo experiments have revealed that PGE2-induced Aβ secretion is associated with specific G-protein coupled receptors (EP-1 to -4), particularly, the EP-2 and EP-4 receptor subtypes (Hoshino et al., 2007). In an AD animal model, deletion of the EP-2 receptor counteracts the aggravation of AD (Liang et al., 2005). Another recent study showed the essential role of EP-3 downstream signals which intensify the cognitive deficits in AD (Maingret et al., 2017). Concerning EP receptors, my results showed that ethanol significantly up-regulates EP-2 receptor compared to the EP-3 receptor. Compatible with my observation, increased G protein activity levels were observed in the alcohol-

dependent brain (Jope et al., 1998). However, further investigation is needed to elucidate how ethanol affects the regulation of the EP receptors. My findings suggest that ER stress affects the ethanol-elevated EP-2 levels. Furthermore, I showed that ethanol enhances CREB phosphorylation by PKA activation eventually leading to increased BACE1 levels. Previous reports have emphasized that ethanol promotes adenylyl cyclase activity and subsequently cAMP accumulation which substantially mediates ethanol-enhanced PKA activation and nuclear translocation (Dohrman et al., 2002; Kumar et al., 2012). Ethanol has a regulatory effect on CREB phosphorylation which is mediated by PKA (Asher et al., 2002). Moreover, another investigator has shown the involvement of both the PI3K/AKT and PKA/CREB signaling pathways in the regulation of BACE1 expression and Aβ production stimulated by interleukin1β-induced PGE2 (Wang et al., 2014). The EP-2 receptor regulates PGE2-induced Aβ secretion through a signaling cascade associated with cAMP (Hoshino et al., 2007). Compatible with the pathway above; my study showed that the selective blocking of the EP-2 receptor alleviated the ethanol-stimulated CREB/PKA pathway and associated BACE1. Collectively, these

findings link ethanol-induced PGE2 signaling through the EP-2 receptor with the stimulation of BACE1 and Aβ secretion.

In conclusion, I demonstrated that ethanol-induced ER stress has a regulatory effect on the EP-2-associated PKA/CREB pathway by COX-2-mediated PGE2 production leading to BACE1 expression and Aβ production (Fig. 20). My findings present the detailed mechanism how ethanol-induced ER stress regulates BACE1 expression and Aβ production. Moreover, I also suggest the substantial role of the EP-2 receptor in ethanol-induced AD progression.

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국 문 초 록

에탄올이 아밀로이드 베타 과다생성을 유도하는 과정에서 eIF2α에 의한 PGE_2 조절의 역할

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아흐메드

에탄올 남용은 알츠하이머 병의 병태생리학적 진행에 따라 치매와 연관된 인지장애를 악화시킨다. 베타 분비효소 1 (BACE1)은 아밀로이드 베타 축적을 조절하며 알츠하이머 병 발병의 중요 조절인자이다. 그러나 에탄올이 어떻게 BACE1을 활성화시키는지 정확한 기전은 밝혀져 있지 않다. 한편 소포체 스트레스(ER stress)와 신경세포의 염증은 에탄올에 의한 신경퇴행의 요인으로 보고되고 있다. 이 연구의 목적은 에탄올에 의한 BACE1의 발현과 아밀로이드 베타 생산의 신호전달 과정 중에서

소포체 스트레스와 신경세포염증의 매개체인 PGE_2의 역할을 밝히는 것이다.

사람 유래의 뇌종양세포주인 SK-N-MC를 이용하여 실험한 결과 에탄올의 양에 따라 BACE1의 발현이 증가되었다. 또한 에탄올에 의해 소포체 스트레스의 표지자인 활성산소종(ROS)의 생성과, CHOP의 발현, eIF2α의 인산화가 촉진되었다. 에탄올에 의해 증가된 COX-2 발현과 PGE_2의 생성은 소포체 스트레스의 억제제인 PBA에 의해 감소되었다. eIF2α 인산화의 억제제인 salubrinal과 eIF2α siRNA를 사용한 결과 에탄올에 의한 COX-2 발현이 감소되었다. COX-2에 의한 BACE1의 활성화는 선택적 COX-2 억제제인 NS-398에 의해서 저해되었다. EP-2 수용체의 억제제인 PF-04418948는 에탄올에 의한 아밀로이드 베타 분비를 감소시켰다. 에탄올은 PKA의 활성화와 CREB의 인산화 및 핵 속으로의 이동을 촉진시켰고, 이는 PF-04418948에 의해 억제되었다. PKA 억제제인 14-22 amide나 CREB1 siRNA를 처리하면 에탄올에 의한 BACE1의 발현량 증가가 억제되었다.

결론적으로 에탄올은 COX-2 발현을 유도하여 PGE_2의 생산을 증가시키고, 증가된 PGE2는 EP-2 수용체에 연결된 PKA/CREB 경로를 통해 BACE1의 양을 증가시킨다.

주요어: 알츠하이머 병, 에탄올, Amyloidogenesis, 베타 분비효소 1 (BACE1), Eukaryotic initiation factor 2α (eIF2α), Prostaglandin E_2 (PGE_2)

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