Short communication

Inhibitory effect of 4-(2-aminoethyl)-benzenesulfonyl fluoride, a serine protease inhibitor, on PI3K inhibitor-induced CHOP expression

Toru Hosoi[1], Kanae Hyoda[2], Yasunobu Okuma[3], Yasuyuki Nomura[2, 4], Koichiro Ozawa[1]

[1]*Department of Pharmacotherapy Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3Kasumi, Minami-ku, Hiroshima 734-8551, Japan*

[2]*Department of Pharmacology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo 060-0812, Japan*

[3]*Department of Pharmacology, Faculty of Pharmaceutical Sciences, Chiba Institute of Sciences, Choshi, Chiba 288-0025, Japan*

[4]*Yokohama College of Pharmacy, Yokohama, Kanagawa 245-0066, Japan*

Toru Hosoi and Kanae Hyoda contributed equally to the present work.

Address correspondence to: Koichiro Ozawa, Department of Pharmacotherapy Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3Kasumi, Minami-ku, Hiroshima 734-8551, Japan

e-mail: ozawak@hiroshima-u.ac.jp

Acknowledgements: This research was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan and by Mochida Memorial Foundation for Medical and Pharmaceutical Research.

ABSTRACT

Endoplasmic reticulum stress contributes to several diseases such as neurodegenerative disorders and diabetes. In the previous report, we found that phosphatidylinositol 3-kinase (PI3K) down-regulation is important for inducing CHOP expression, an endoplasmic reticulum stress-induced transcription factor. In the present study, we investigated the effect of 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), a serine protease inhibitor, on PI3K inhibitor-induced CHOP expression. We found that AEBSF completely inhibited PI3K inhibitor-induced CHOP expression at both mRNA and protein levels. It is suggested that AEBSF is an important drug from a pharmacological point of view and the results may have important implications for understanding endoplasmic reticulum stress-related diseases.

Keywords

4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF); phosphatidylinositol 3-kinase (PI3K); CHOP; endoplasmic reticulum stress

1. **INTRODUCTION**

Recent evidence has suggested that neurodegenerative disorders and diabetes are involved in the disruption of endoplasmic reticulum function (Katayama et al., 1999, Y. Imai et al., 2001., Kaufman et al., 2002). In response to endoplasmic reticulum stress, unfolded proteins accumulate and aggregate in the endoplasmic reticulum, resulting in activation of the unfolded protein response (Mori et al., 2000). Several growth factors promote cell survival by activating phosphatidylinositide-3-OH kinase (PI3K) (Burgering et al., 1995, Dudek et al., 1997). One of the downstream targets of PI3K is a serine/threonine kinase, Akt/protein kinase B (PKB). Increasingly, evidence has suggested that the PI3K/Akt pathway is involved in endoplasmic reticulum stress. endoplasmic reticulum stress down-regulates Akt activation and insulin-like growth factor-1 (IGF-1) reduces endoplasmic reticulum stress-induced apoptosis in insulinoma cells (Srinivasan et al., 2005). We recently showed that Akt is inactivated by endoplasmic reticulum stress in the L929 fibroblast cell line (Hyoda et al., 2006). Moreover, we found that inactivation of PI3K induced expression of CHOP, a transcription factor known to be induced by endoplasmic reticulum stress (Hyoda et al., 2006). These observations suggest that the PI3K/Akt pathways play an important role in endoplasmic reticulum stress. Recently, serine protease has been suggested to be involved in endoplasmic reticulum stress related diseases (Ye et al., 2000; Okada et al., 2003). However, the signaling mechanisms of PI3K inactivation-induced CHOP expression are unclear. Thus, in the present study, we investigated the mechanisms of PI3K inactivation-induced CHOP expression. We found that serine protease-inhibitor, AEBSF (Kazmirowski et al., 1971), inhibited these processes.

2. **MATERIALS AND METHODS**

2.1. Materials and reagents

Tunicamycin and thapsigargin were obtained from Wako Pure Chemical Ltd. (Japan). 2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002), wortmannin and 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) were purchased from SIGMA (St. Louis, MO).

2.2. Cell culture

Mouse fibroblast L929 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal calf serum and antibiotics (100 units/ml penicillin G and 100 μg/ml streptomycin; GIBCO BRL) at 37°C in humidified 5% CO_2, 95% air. L929 cells were transferred to a serum-free medium before the Western blotting.

2.3. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated using TRI Reagent (Sigma-Aldrich, St. Louis, MO). RT-PCR was performed as described previously (Hosoi et al., 2002). Precisely, cDNA was synthesized from total RNA by reverse transcription using 100 U of Superscript Reverse Transcriptase (Invitrogen) and Oligo $(dt)_{12\text{-}18}$ primer (Invitrogen) in a 20 μl reaction mixture containing Superscript buffer (Invitrogen), 1 mM dNTP mix, 10 mM dithiothreitol (DTT), and 40 U of RNase inhibitor. Total RNA and Oligo $(dt)_{12\text{-}18}$ primer were incubated

at 70°C for 10 min prior to the reverse transcription. After incubation for 1 h at 42°C, RT reaction was terminated by denaturing the Reverse Transcriptase enzyme for 15 min at 70°C. For PCR amplification, 1.2 μl of cDNA was added to 12 μl of a reaction mix containing 0.2 μM of each primer, 0.2 μM of dNTP mix, 0.6 U of Taq polymerase, and reaction buffer. PCR was performed in a DNA Thermal Cycler (GeneAmp® PCR System 9700). The following primers were used: CHOP upstream, 5'-ccc tgc ctt tca cct tgg-3'; CHOP downstream, 5'-ccg ctc gtt ctc ctg ctc-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) upstream, 5'-aaa ccc atc acc atc ttc cag -3'; and GAPDH downstream, 5'-agg ggc cat cca cag tct tct-3'. The PCR products (10 μL) were resolved by electrophoresis in an 8% polyacrylamide gel in TBE buffer. The gels were stained with ethidium bromide and then photographed under ultraviolet light. cDNA for GAPDH and CHOP were amplified for 18 (94°C 1 min, 57°C 1 min, 72°C 1 min) and 20 (94°C 1 min, 60°C 1 min, 72°C 1 min) cycles, respectively, and these PCR reactions were run separately. These cycle numbers were chosen based on a preliminary study determining the linear range of amplification for each respective molecule. To compare the expression of mRNAs in the different experimental groups, the amount of mRNA in each structure studied was estimated as the ratio of CHOP/GAPDH.

2.4. Western blotting analysis

Western blotting was performed as described previously (Hosoi et al., 2004). Cells were washed with ice-cold PBS and lysed in a buffer containing 10 mM

HEPES-NaOH (pH 7.5), 150 mM NaCl, 1 mM EGTA, 1 mM Na_3VO_4, 10 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethyl sulfonyl floride (PMSF) and 1% NP-40 for 20 min. The lysates were centrifuged at 20,630 g for 20 min at 4°C, and the supernatants were collected. The samples were boiled with laemmli buffer for 3 min, fractionated by SDS-PAGE, and transferred at 4°C to nitrocellulose membranes. The membranes were incubated with anti-phospho-Akt (Ser473: Cell Signaling; 1:1,000), anti-Akt (Cell Signaling; 1:1,000), anti-phospho-eIF2α (Ser51: Cell Signaling; 1:1,000), anti-XBP-1 (Santa Cruz; 1:500) and anti-CHOP (Santa Cruz; 1:500) antibodies and then with anti-horseradish peroxidase-linked antibody. Peroxidase was detected by chemiluminescence using an ECL system (Amersham).

2.5. Statistics

Results were expressed as means ± S.E.M. Statistical analysis was performed with Student's *t*-test.

3. **RESULTS**

3.1. Effect of AEBSF on endoplasmic reticulum stress-induced CHOP expression

Endoplasmic reticulum stress has been shown to activate activating transcription factor 6 (ATF6). When unfolded proteins accumulate in the endoplasmic reticulum, full length ATF6 is cleaved to release its cytoplasmic domain, which enters the nucleus and acts as a transcription factor. ATF6 is processed by serine protease, site-1 protease (S1P) (Ye et al., 2000). The cytoplasmic domain of ATF6 has been reported to induce CHOP expression (Yoshida et al., 2000). AEBSF is a potent serine protease inhibitor, which acts by sulfonylation of the serine residue at the active site (Markwardt et al., 1974). Recently, AEBSF has been reported to inhibit activation of ATF6 by inhibiting S1P (Okada T., 2003). Thus, we examined whether AEBSF blocks endoplasmic reticulum stress-induced CHOP expression. The dosage used for AEBSF treatment was determined according to the previous reports (Okada et al., 2003; Nakanishi et al., 2005). L929 cells were treated with AEBSF 1 h before inducing endoplasmic reticulum stress. Endoplasmic reticulum stress was induced by tunicamycin or thapsigargin. As shown in figure 1A, we observed an inhibition of endoplasmic reticulum stress-induced CHOP expression in AEBSF-treated cells. These results suggest that serine proteases such as S1P are involved in endoplasmic reticulum stress-induced CHOP expression.

3.2. Effect of AEBSF on PI3K inhibitor-induced CHOP expression

In the previous study, we found that AKT activation is down-regulated in response to endoplasmic reticulum stress in the L929 cell line (Hyoda et al., 2006). Moreover,

treatment with PI3K inhibitor alone induced CHOP expression (Hyoda et al., 2006). However, the mechanism(s) of PI3K inhibitor-induced CHOP expression is unknown. Endoplasmic reticulum stress-induced CHOP expression has been reported to be mediated through PERK-eIF2α, IRE1-XBP1 and serine protease S1P-ATF6 pathway (Scheuner et al., 2001; Harding et al., 2000; Yoshida et al., 2000; Oyadomari et al., 2004). Thus, we examined the possible involvement of serine proteases in PI3K inhibitor-induced CHOP expression. AEBSF was treated 1 h before PI3K inhibitors (LY294002 and wortmannin) and then analyzed for CHOP expression. RT-PCR analysis was performed to investigate CHOP transcripts. We obtained liner relation for CHOP induction between 19 through 22 cycles of PCR (Fig1 B). Thus, we selected 20 cycles to investigate CHOP transcripts. We observed an increase in CHOP transcripts by PI3K inhibitors. This increase was completely inhibited by AEBSF (Fig. 1C,D). Protein levels of CHOP expression were also completely inhibited by AEBSF (Fig. 1D,E). PI3K inhibitor inhibited basal levels of phosphorylated AKT (Fig. 1D), indicating effective inhibition of PI3K-AKT pathway. On the other hand, we observed neither eIF2α phosphorylation nor spliced XBP1 translation in PI3K inhibitor-treated cells (Fig. 2). Therefore, these results suggest that serine protease is involved in PI3K inhibitor-induced CHOP expression.

4. **DISCUSSION**

PI3K is involved in many cellular events such as regulation of cell growth, proliferation, survival, differentiation, and cytoskeletal changes. One of the characteristic targets of PI3K is the protein kinase AKT. Increasing evidence has suggested that death-promoting stimuli lead to deactivation of AKT. *N*-methyl-D-aspartate excitotoxicity, vascular stroke, and nitric oxide and hydrogen peroxide lead to deactivation of AKT (Luo et al., 2003). Moreover, endoplasmic reticulum stress inhibits AKT activation (Srinivasan et al., 2005; Hyoda et al., 2006). Thus, deactivation of AKT is a physiologically important event in various disease states. We have reported that inhibition of PI3K, the activator of AKT, induces CHOP expression, an endoplasmic reticulum stress responsive apoptotic transcription factor (Hyoda et al., 2006). Endoplasmic reticulum stress-induced CHOP expression has been reported to be mediated through PERK-eIF2α, IRE1-XBP1 and serine protease S1P-ATF6 pathway (Scheuner et al., 2001; Harding et al., 2000; Yoshida et al., 2000; Oyadomari et al., 2004). However, the molecular mechanism(s) of PI3K inhibitor-induced CHOP expression are unknown. In the present study, we found for the first time that serine protease inhibitor AEBSF inhibited PI3K inhibitor-induced CHOP expression. On the other hand, we did not observe eIF2α activation nor XBP1 induction in PI3K inhibitor treated cells (Fig2). Therefore, PERK-eIF2α or IRE1-XBP1 pathways may not be involved in PI3K inhibitor-induced CHOP expression. Thus, these results suggest that PI3K/AKT deactivation-induced CHOP expression is predominantly mediated through AEBSF sensitive serine protease. More detailed pharmacological role of AEBSF in respect to endoplasmic reticulum stress will be needed to analyze in the future study.

Serine protease has been reported to be involved in the initiation of DNA damage-induced apoptosis (de Bruin et al., 2003). Moreover, serine protease mediates apoptosis-like cell death and phagocytosis under caspase-inhibiting conditions (Egger et al., 2003). We observed complete inhibition of PI3K deactivation-induced CHOP expression by serine protease inhibitor. CHOP is an apoptotic transcription factor, and is induced by endoplasmic reticulum stress. Thus, it is possible that PI3K deactivation-induced CHOP expression may result in cell death. As CHOP induction may mediate serine protease, it is suggested that serine protease is involved in these processes. Cerebral deposition of amyloid β-protein (Aβ) is a critical feature of Alzheimer's disease, and Aβ has been reported to induce endoplasmic reticulum stress (Nakagawa et al., 2000). Interestingly, AEBSF has been shown to inhibit Aβ production in neural cells (Citron et al., 1996). It is suggested that AEBSF is an important drug from a pharmacological point of view and the results may have important implications for understanding endoplasmic reticulum stress-related diseases.

FIGURE LEGEND

Fig. 1. AEBSF suppressed PI3K inhibitor-induced CHOP expression.

L929 cells were pretreated with AEBSF (300 μM) for 1 h and then stimulated with LY294002 (LY, 20-50 μM), wortmannin (wt, 500 nM), tunicamycin (Tm, 3 μg/mL) and thapsigargin (Tg, 3 μM) for the indicated times, respectively. **A.** CHOP was detected by Western blotting. **B.** Liner relation for CHOP induction between 19 through 22 cycles of the RT-PCR. **C.** RT-PCR analysis was performed using specific primers for CHOP and GAPDH. **D.** The amounts of CHOP mRNA are expressed as the ratio of densitometric measurements of the samples to the corresponding GAPDH internal control. Values are presented as means ± S.E.M. for three separate experiments. **E.** CHOP, phospho (Ser473)-Akt and Akt were detected by Western blotting. **F.** Densitometric analysis was performed using image analyzing software. The expression levels were expressed as fold intensity compared with the control treated group. Values are presented as means ± S.E.M. (n=3 per group). $*P < 0.05$, $**P < 0.01$ (statistically significant difference between LY and LY+AEBSF)

Fig2. PI3K inhibitor did not affect eIF2α phosphorylation or XBP-1 translation.

L929 cells were treated with LY294002 (LY, 20-50 μM), wortmannin (wt, 500 nM) and thapsigargin (Tg, 3 μM) for the indicated times, respectively. **A.** Phospho-eIF2α (Ser51), phospho-Akt (Ser473) and Akt were detected by Western blotting. **B.** Spliced XBP-1, phospho-Akt (Ser473) and Akt were detected by Western blotting.

REFERENCES

de Bruin E.C., Meersma D., de Wilde J., den Otter I., Schipper E.M., Medema J.P., Peltenburg L.T., 2003. A serine protease is involved in the initiation of DNA damage-induced apoptosis. Cell Death Differ. 10, 1204-1212.

Burgering B.M., Coffer P.J., 1995. Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. Nature 376, 599-602.

Citron M., Diehl T.S., Capell A., Haass C., Teplow D.B., Selkoe D.J., 1996. Inhibition of amyloid β-protein production in neural cells by the serine protease inhibitor AEBSF. Neuron 17, 171-179.

Dudek H., Datta S.R., Franke T.F., Birnbaum M.J., Yao R., Cooper G.M., Segal R.A., Kaplan D.R., Greenberg M.E., 1997. Regulation of neuronal survival by the serine-threonine protein kinase Akt. Science 275, 661-665.

Egger L., Schneider J., Rheme C., Tapernoux M., Hacki J., Borner C., 2003. Serine proteases mediate apoptosis-like cell death and phagocytosis under caspase-inhibiting conditions. Cell Death Differ. 10, 1188-1203.

Harding H.P., Novoa I., Zhang Y., Zeng H., Wek R., Schapira M., Ron D., 2000. Regulated translation initiation controls stress-induced gene expression in mammalian cells. Mol Cell. 6, 1099-1108.

Hosoi T., Kawagishi T., Okuma Y., Tanaka J., Nomura Y., 2002. Brain stem is a direct target for leptin's action in the central nervous system. Endocrinology 143, 3498-3504.

Hosoi T., Suzuki S., Nomura J., Ono A., Okuma Y., Akira S., Nomura Y., 2004. Bacterial DNA induced iNOS expression through MyD88-p38 MAP kinase in mouse primary

cultured glial cells. Mol. Brain Res. 124, 159-164.

Hyoda K., Hosoi T., Horie N., Okuma Y., Ozawa K., Nomura Y., 2006. PI3K-Akt inactivation induced CHOP expression in endoplasmic reticulum-stressed cells. Biochem Biophys Res Commun. 340, 286-290.

Imai Y., Soda M., Inoue H., Hattori N., Mizuno Y., Takahashi R., 2001. An unfolded putative transmembrane polypeptide, which can lead to endoplasmic reticulum stress, is a substrate of Parkin. Cell 105, 891-902.

Katayama T., Imaizumi K., Sato N., Miyoshi K., Kudo T., Hitomi J., Morihara T., Yoneda T., Gomi F., Mori Y., Nakano Y., Takeda J., Tsuda T., Itoyama Y., Murayama O., Takashima A., St. George-Hyslop P., Takeda M., Tohyama M., 1999. Presenilin-1 mutations downregulate the signalling pathway of the unfolded-protein response, Nat. Cell Biol. 1, 479-485.

Kaufman R.J., 2002. Orchestrating the unfolded protein response in health and disease. J. Clin. Invest. 110, 1389-98.

Kazmirowski H., 1971. Synthese antiproteolytisch wirksamer Derivate des Benzolsulfofluorids. Pharmazie 26, 390–394.

Luo H.R., Hattori H., Hossain M.A., Hester L., Huang Y., Lee-Kwon W., Donowitz M., Nagata E., Snyder S.H., 2003. Akt as a mediator of cell death. Proc. Natl. Acad. Sci. U S A. 100, 11712-11717.

Markwardt F., Drawert J., Walsmann P., 1974. Synthetic low molecular weight inhibitors of serum kallikrein. Biochem. Pharmacol. 23, 2247–2256

Mori K., 2000. Tripartite management of unfolded proteins in the endoplasmic reticulum.

Cell 101, 451-454.

Nakagawa T., Zhu H., Morishima N., Li E., Xu J., Yankner B.A., Yuan J., 2000. Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-β. Nature 403, 98-103.

Nakanishi K., Sudo T., Morishima N., 2005. Endoplasmic reticulum stress signaling transmitted by ATF6 mediates apoptosis during muscle development. J. Cell Biol. 169, 555-560.

Okada T., Haze K., Nadanaka S., Yoshida H., Seidah N.G., Hirano Y., Sato R., Negishi M., Mori K., 2003. A serine protease inhibitor prevents endoplasmic reticulum stress-induced cleavage but not transport of the membrane-bound transcription factor ATF6. J. Biol. Chem. 278, 31024-31032.

Oyadomari S., Mori M., 2004. Roles of CHOP/GADD153 in endoplasmic reticulum stress. Cell Death Differ. 11, 381-389.

Scheuner D., Song B., McEwen E., Liu C., Laybutt R., Gillespie P., Saunders T., Bonner-Weir S., Kaufman R.J., 2001. Translational control is required for the unfolded protein response and in vivo glucose homeostasis. Mol. Cell. 7, 1165-1176.

Srinivasan S., Ohsugi M., Liu Z., Fatrai S., Bernal-Mizrachi E., Permutt M.A., 2005. Endoplasmic reticulum stress-induced apoptosis is partly mediated by reduced insulin signaling through phosphatidylinositol 3-kinase/Akt and increased glycogen synthase kinase-3beta in mouse insulinoma cells. Diabetes 54, 968-975.

Ye J., Rawson R. B., Komuro R., Chen X., Dave U.P., Prywes R., Brown M.S., Goldstein J.L. 2000. ER stress induces cleavage of membrane-bound ATF6 by the same proteases

that process SREBPs. Mol. Cell 6, 1355-1364.

Yoshida H., Okada T., Haze K., Yanagi H., Yura T., Negishi M., Mori K. 2000. ATF6 activated by proteolysis binds in the presence of NF-Y (CBF) directly to the cis-acting element responsible for the mammalian unfolded protein response. Mol Cell Biol. 20, 6755-6767.

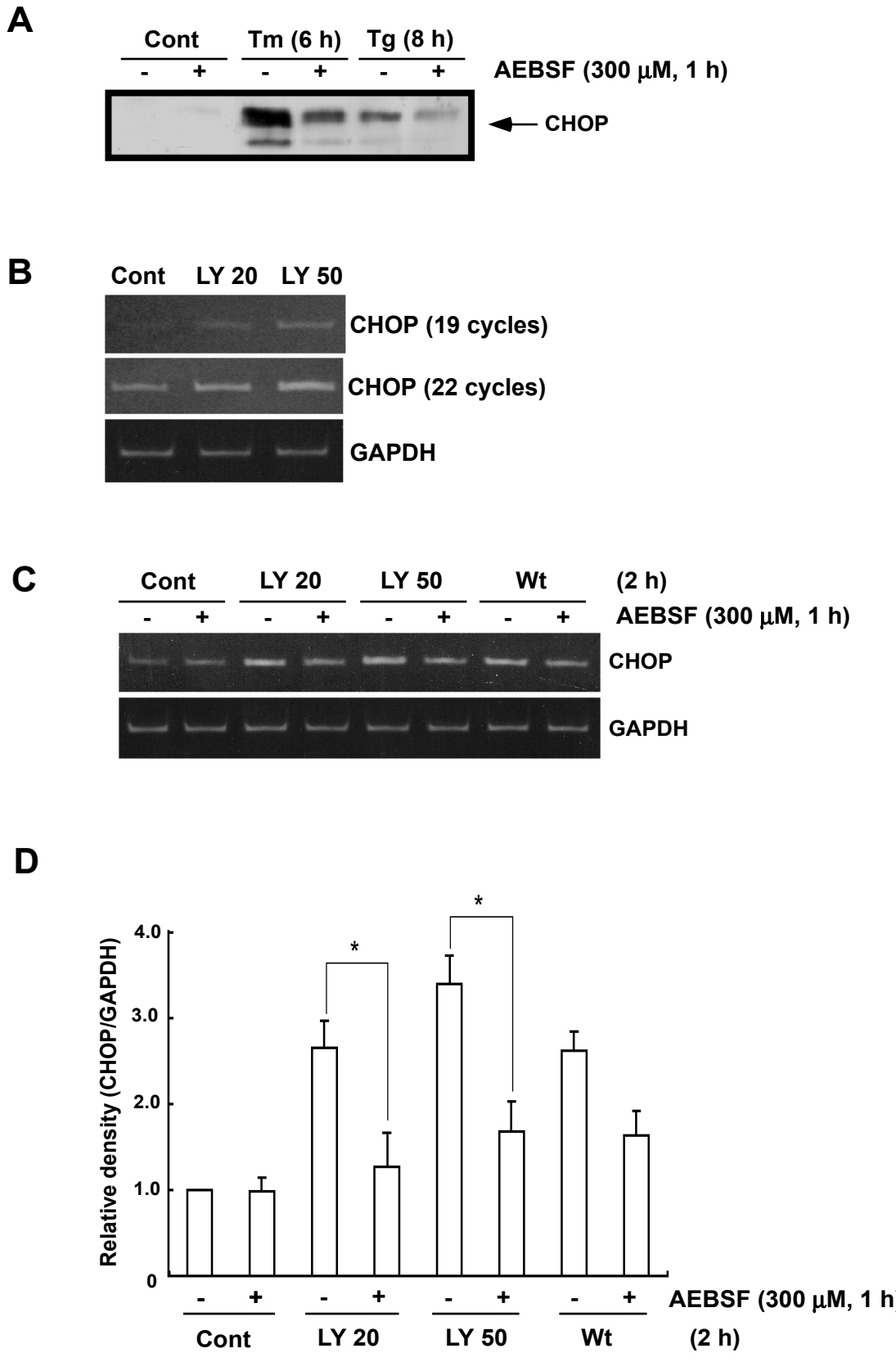
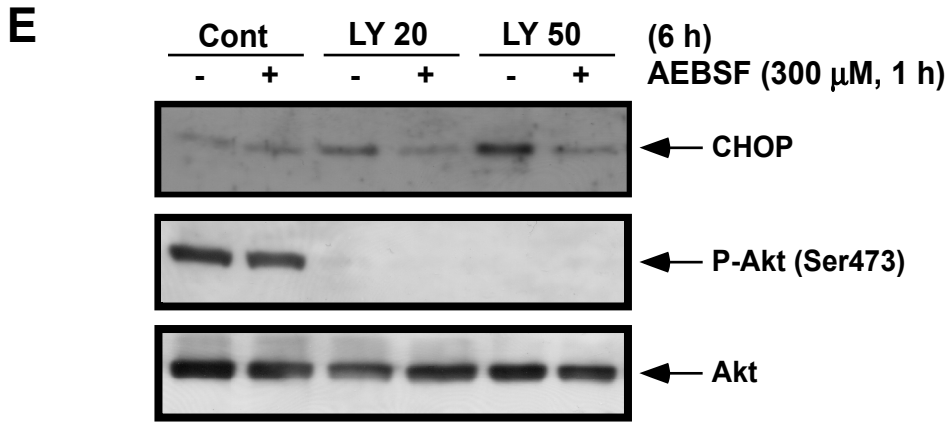


Fig. 1 (A-D) Hosoi T et al



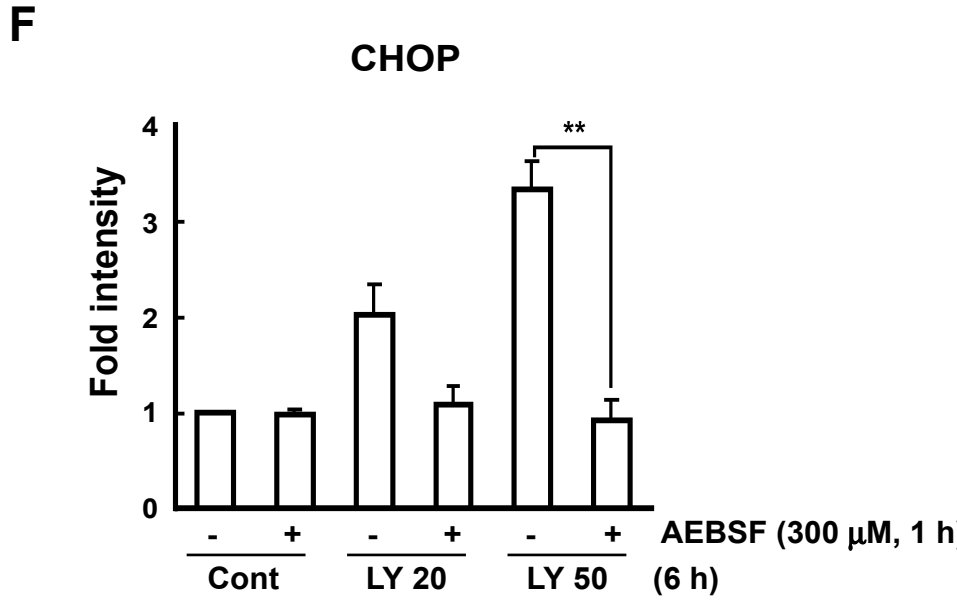


Fig. 1 (EF) Hosoi T et al

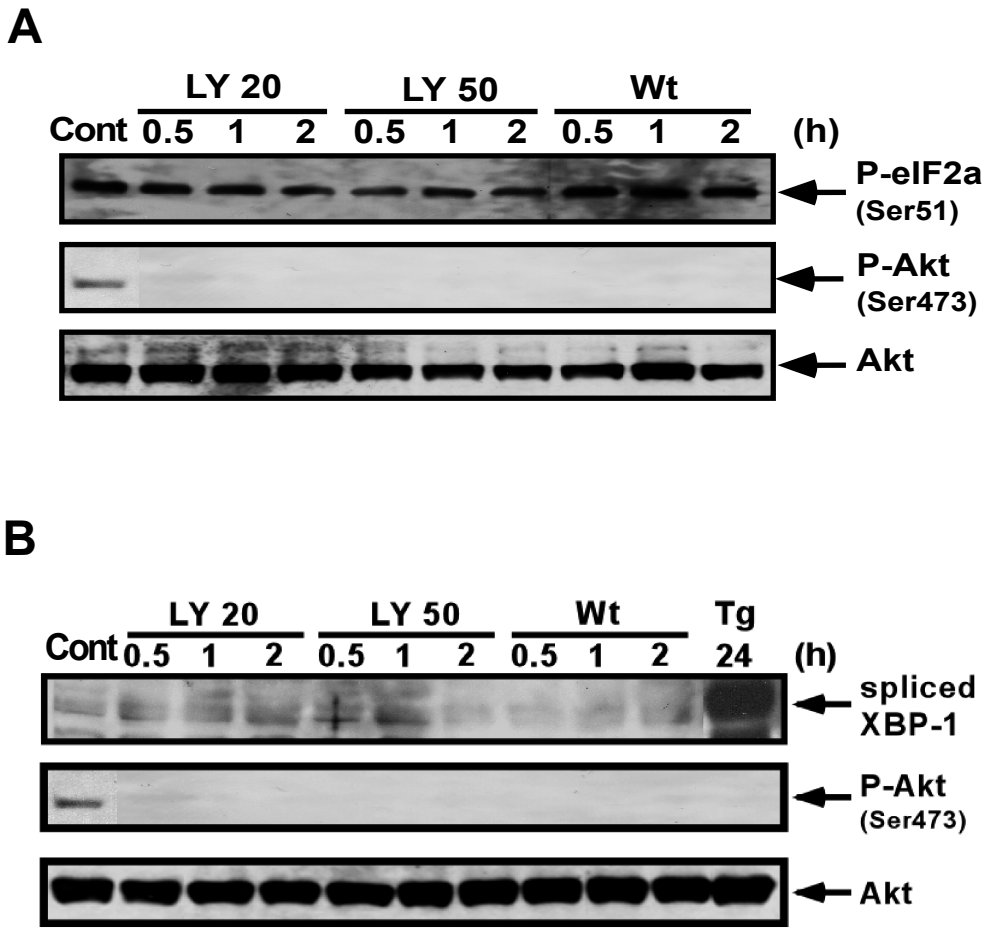


Fig. 2 Hosoi T. et al