LFS-D-14-00003_R1

Involvement of peptidyl-prolyl isomerase Pin1 in the inhibitory effect of fluvastatin on endothelin-1-induced cardiomyocyte hypertrophy

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A part of this work was presented at the **13th International Conference on Endothelin** (held at University of Tsukuba, Tokyo Campus; Sep 8th-11th, 2013).

Key words

Pin1, endothelin-1, statin, c-Jun, cardiomyocyte hypertrophy

Total words 5740 6 Figures

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Abstract

Aims: Cardiac hypertrophy is elicited by endothelin (ET)-1 as well as other neurohumoral factors, hemodynamic overload, and oxidative stress; HMG-CoA reductase inhibitors (statins) were shown to inhibit cardiac hypertrophy partly via the anti-oxidative stress. One of the common intracellular pathways of them is the phosphorylation cascade of MEK signaling. Pin1 specifically isomerizes the phosphorylated protein with Ser/Thr-Pro bonds and regulates their activity through conformational changes. There is no report whether the Pin1 activation contributes to ET-1-induced cardiomyocyte hypertrophy and whether the Pin1 inactivation contributes to the inhibitory effect of statins. The aim of this study was to reveal these questions. **Main methods:** We assessed neonatal rat cardiomyocyte hypertrophy using ET-1 and fluvastatin by the cell surface area, ANP mRNA expression, JNK and c-Jun phosphorylation, and [^{3}H]-leucine incorporation. **Key findings:** Fluvastatin inhibited ET-1-induced increase in the cell surface area, ANP expression, and [^{3}H]-leucine incorporation; and it suppressed the signaling cascade from JNK to c-Jun. The phosphorylated Pin1 level, an inactive form, was decreased by ET-1; however, it became basal level by fluvastatin. Furthermore, Pin1 overexpression clearly elicited cardiomyocyte hypertrophy, which was inhibited by fluvastatin. **Significance:** This is the first report that ET-1-induced cardiomyocyte hypertrophy is mediated through the Pin1 activation and that the inhibitory effect of fluvastatin on cardiomyocyte hypertrophy would partly be attributed to the suppression of the Pin1 function. This study firstly suggests that Pin1 determines the size of hypertrophied cardiomyocyte by regulating the activity of phosphorylated molecules and that statins exert their pleiotropic effects partly via Pin1 inactivation.

Introduction

Cardiac hypertrophy is elicited by endothelin (ET)-1 (Suzuki et al., 1990; Yorikane et al., 1993; Ito et al., 1997; Koleitis et al., 2013; Miyauchi and Goto, 2013) as well as other neurohumoral factors, hemodynamic overload, and oxidative stress. One of the common intracellular pathways of them is the phosphorylation cascade of MEK signaling. Peptidyl-prolyl cis-trans isomerase 1 (Pin1) is a highly conserved enzyme that isomerizes specific phosphorylated Ser/Thr-Pro bonds in certain proteins, inducing conformational changes (Lu et al., 1996). It has been reported that Pin1 has the binding activity to the pSer/Thr-Pro pocket of target protein and that Pin1 catalyzes such portion. Both of these activities are exerted when the Ser-16 residue of Pin1 is dephosphorylated; on the other hand, these activities are inhibited when the residue is phosphorylated (Lu et al., 2007). The family of proline-directed protein kinases containing a major regulatory phosphorylation motif (pSer/Thr-Pro) includes c-Jun, Akt, cyclin dependent kinases, Raf-1, SMAD2/SMAD3, etc., which play an important role in the regulation of cell proliferation, tumorigenesis, neurological disorders, and autoimmune and inflammatory diseases (Lee et al., 2011).

The endothelin (ET) system consists of two G protein coupled-receptors, ET type A receptor and ET type B receptor, and three endogenous ligands, ET-1, ET-2, and ET-3 (Davenport, 2002; Horinouchi et al., 2013; Koleitis et al., 2013; Miyauchi and Goto, 2013). ET-1, a potent vasoconstrictive peptide produced by endothelial cells (Yanagisawa et al.,1988), is also produced by cardiomyocytes and contributes to the development of cardiac hypertrophy (Ito et al., 1991; Yorikane et al., 1993; Koleitis et al., 2013; Miyauchi and Goto, 2013). We reported that the production of ET-1 is markedly increased in the failing hearts of rats with chronic heart failure (Sakai et al., 1996a; Sakai et al., 1996b) and that the enhancement of myocardial ET-1 contributes to the modulation of the cardiac function (Sakai et al., 1996a) and cardiac hypertrophy at the

molecular level in the failing hearts (Sakai et al. 2000); moreover, chronic administration of the ET_A receptor antagonist BQ-123 inhibits the cardiac remodeling and ameliorates the cardiac function (Sakai et al., 1996b). In addition, the signaling cascades of the mitogen activated protein kinase (MEK) family including extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) are augmented by ET-1 in cardiomyocyte hypertrophy (Yue et al., 2000; Irukayama-Tomobe et al., 2004; Shimojo et al., 2006). Therefore, in the present study, we hypothesized that Pin1 contributes to the development of cardiomyocyte hypertrophy through the activation of phosphorylated kinases of the MEK family by exerting its catalytic activity.

3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) are widely employed classes of cholesterol-lowering drugs that work through the inhibition of HMG-CoA reductase. Much evidence has demonstrated that statins reduce the cardiovascular risk to a greater extent than that expected based on the blood cholesterol-lowering effect alone; those additional activities of statins are known as pleiotropic effects including the suppression of inflammation and improvement of endothelial dysfunction (Wierzbicki et al., 2003). We have reported that pitavastatin ameliorates the severity of experimental autoimmune myocarditis through the inhibition of T-cell mediated autoimmunity (Tajiri et al., 2013). It was reported that statins interfere with the protein (iso)prenylation processes (Wierzbicki et al., 2003), which may be involved in the regulation of several cellular mechanisms such as signal transduction and cell proliferation and differentiation, etc. Previous studies have shown that statins inhibit cardiomyocyte hypertrophy provoked by angiotensin II by blocking the Rho kinase-mediated cyclin D1 activation (Morikawa-Futamatsu et al., 2006) and that an antioxidant mechanism involving Rac1 inhibition (Takemoto et al., 2001). Such reports suggest that another unresolved mechanism for the pleiotropic effects of statins remains.

However, there is no report whether the Pin1 activation is involved in cardiomyocyte hypertrophy provoked by ET-1; therefore, we hypothesized that the activation of Pin1 activity would contribute to the ET-1-induced cardiomyocyte hypertrophy. In addition, there is no report whether the inhibitory effect of statins on the ET-1-induced cardiomyocyte hypertrophy is mediated through the modulation of the Pin1 activity. The aim of this study was to reveal these questions.

Materials and Methods

Adenovirus vectors Human Pin1 cDNA cloned from 293A cells by PCR using the following primers, (Forward) 5'-CACCATGGCGGACGAGGAGAAGCT-3' and (Reverse) 5'-CTCAGTGCGGAGGATGATGTGGATG-3', was ligated to the pENTR-TOPO plasmid as an entry clone; cDNA was transferred to the pAd/CMV/V5-DEST Gateway vector by an LR recombination (Life Technologies, Carlsbad, CA). The cDNA for human Pin1 was designed to add the V5 tag at C-termianl of the entire Pin1 fusion protein. The adenovirus vector plasmid was digested by Pac I, transfected to 293A cells and amplified, and finally purified by a Vivaspin column system (Sartorius Stadium Biotechnology, Goettingen, Germany). As a control, an adenovirus vector of LacZ was used. Adenovirus for an enhanced green fluorescent protein (EGFP) was used to visualize the morphology of the cardiomyocytes and to take photographs.

Cardiomyocyte culture Neonatal rat cardiomyocytes were isolated from 2- to 3-day-old Sprague-Dawley rats, as described previously (Shimojo et al., 2007; Sakai et al., 2012), and were incubated on fibronectin-coated dishes in DMEM-Ham's F-12 medium (Wako) supplemented with 0.1% fatty acid-free bovine serum albumin (BSA) (Sigma, St. Louis, MO) in 95% air-5% CO_2. The cells were cultured for 2 days after the differential adhesion and then used for further experiments. The animal experiment for

cardiomyocyte isolation was carried out in a humane manner after we received approval from the Institutional Animal Experiment Committee of University of Tsukuba and were in accordance with the Regulation for Animal Experiments in our university.

Study Protocol Cardiomyocytes were pretreated with a vehicle or fluvastatin (0.1-10 μM) (Wako Pure Chemical Industries, Osaka, Japan) and 12 hours later, human/rat ET-1 (10 nM) (Peptide Institute Inc., Osaka, Japan) was applied and incubated for 48 hours; the cell surface area was evaluated as describing below; and the sampling for the [^{3}H]-leucine uptake, real-time PCR, and Pin1 expression was done in an individual experiment. A Western blot was performed for ERK, JNK, and c-Jun, and the samples were collected 30 minutes after the ET-1 stimulation. In the transient transfection experiments, cardiomyocytes were transfected with an adenovirus for Pin1 or LacZ (control) at a titer with 20 multiplicity of an infection (MOI); in the experiment of taking photographs of cardiomyocytes, the adenovirus for EGFP (10 MOI) was transfected additionally; 12 hours later after adenoviral transfection, cardiomyocytes were treated with a vehicle or fluvastatin and harvested for 48 hours. There are some reasons why we selected fluvastatin in this experiments; (1) as fluvastatin is known as one of lipophilic statins contrasted to a hydrophilic statin, pravastatin, and is suspected to be easily incorporated into the muscle cells, it suggested us that using fluvastatin leads the easier evaluation for determining the inhibitory effect of statin on cardiomyocyte hypertrophy; (2) other researchers previously reported that fluvastatin has the inhibitory effect on cardimyocyte hypertrophy (Morikawa-Futamatsu et al., 2006); and (3) other lipophilic statins, simvastatin and atorvastatin were reported to prevent cardiac hypertrophy (Liu et al., 2008; Indolfi et al., 2002; Planavila et al., 2005).

Cardiomyocyte size measurement Cardiomyocytes were observed by microscope (DM1L, Leica Japan, Tokyo, Japan) and captured by a charge-coupled device camera (Olympus, Tokyo, Japan). The surface area of the attached cardiomyocytes on the dish

(the area of cardiomyocytes in 2D image) for 10 cells/field with 5 fields in each group was evaluated by the software NIH ImageJ ver 1.47 (National Institute of Health, Bethesda, MD) and repeated by 3 times.

Protein synthetic rate The rates of protein synthesis in cultured neonatal rat cardiomyocytes were assessed by measuring the [^{3}H]-leucine uptake into acid-insoluble cellular materials as described previously (Sano et al., 2002; Shimojo et al., 2007). The cells were plated on 24-well dishes at a density of 1x10^5 cells/well and pretreated with fluvastatin or vehicle 12 hours before the ET-1 stimulation. Twenty-four hours after the ET-1 treatment, 0.1 mCi/ml [^{3}H]-leucine (GE Healthcare, Piscataway, NJ) was added and the cells were incubated for 24 hours. The cells were finally fixed by 5% trichloroacetic acid and detached by 0.25% trypsin, and the cell residues were solubilized in 0.5M NaOH. Aliquots were counted with a scintillation counter (LS-6500 scintillation counter; Beckman Coulter, Fullerton, CA). In the transient transfection assay, cardiomyocytes were transfected with an adenovirus for Pin1 or LacZ; 12 hours later, fluvastatin or vehicle was added and cardiomyocytes were incubated for 48 hours; [^{3}H]-leucine was added and incubated for last 24 hours.

Gene expression analysis The total RNA from 1x10^6 cardiomyocytes was isolated using RNeasy (Qiagen, Valencia, CA) and was reverse transcribed to cDNA by a synthesis kit (Qiagen). The mRNA expression levels of the target genes were analyzed by real-time quantitative PCR with a TaqMan probe using an ABI Prism 7700 sequence detector (Applied Biosystems, Foster, CA), as described previously (Shimojo et al., 2007; Sakai et al., 2012). The commercially available gene-specific primers and TaqMan probe sets were obtained from AppliedBiosystems. The PCR mixture (25 microL total volume) consisted of forward and reverse primers for each gene at 450 nM each, FAM-labeled primer probes at 200 nM, and a TaqMan Universal PCR Master Mix (AppliedBiosystems). PCR amplification was performed in duplicate as follows: 1 cycle

at 95^{o}C for 10 min and 40 cycles at 94 ^{o}C for 15 s and 60 ^{o}C for 1 min. The quantitative values of target mRNA were normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression. The primers and probes sets were as follows: atrial natriuretic peptide (ANP), Rn00561661_m1; B-type natriuretic peptide (BNP), Rn00580641_m1; c-jun, Rn99999045_s1; and GAPDH, Rn01775763_g1.

Western blot Cardiomyocytes in a concentration of 2x10^{6} from each group were lysed on ice with a buffer (10 mM Tris HCl, 150 mM NaCl, 1 mM ethylenediamine- N, N, N', N'-tetraacetic acid [EDTA], 0.1% sodium dodecyl sulfate [SDS]), as described previously (Shimojo et al. 2007; Sakai et al. 2012). The protein concentration of the supernatant was determined with a bicinchoninic acid protein assay (Pierce, Rockford, IL). Samples were run on an SDS-polyacrylamide gel electrophoresis (PAGE), using 10% or gradient 10-17.5% polyacrylamide gels, and electrotransferred to a PVDF membrane. To reduce any nonspecific binding, the membrane was blocked for 1 h at room temperature with 5% BSA or skim milk in tris-buffered saline (TBS) containing 0.1% Tween 20 (TBST). Thereafter, the membrane was incubated overnight at 4^{o}C with primary antibodies. We incubated the membrane with a horseradish peroxidase-conjugated goat anti-rabbit antibody or horse anti-mouse antibody (Cell Signaling Technology, Boston, MA) at 1:10000 in a TBST at room temperature for 60 min. The blots were visualized with an enhanced chemiluminescence detection system (GE Healthcare), exposed to X-ray film or captured by CCD camera system (AE-6981, Atto Corporation, Tokyo, Japan), and analyzed by CS Analyzer (Atto Corporation). The following was used as the first antibodies: rabbit anti-Pin1, rabbit anti-phospho-Pin1, rabbit anti-p44/42 MAP Kinase, rabbit anti-phospho-p44/42 MAP Kinase, rabbit anti-SAPK/JNK, rabbit anti-phospho-SAPK/JNK, rabbit anti-c-Jun, rabbit anti-phospho-c-Jun (these antibodies were purchased from Cell Signaling Technology), mouse anti-β-actin (Sigma-Aldrich, St. Louis MO), and mouse anti-V5 (Life Technology).

Statistical Analysis The values are shown as means ± SE. Data were compared by a one-way ANOVA with a Turkey-Kramer's HSD using the JMP ver 8.0 statistical software (SAS Institute, Cary, NC). Differences were considered significant at a $P<0.05$.

Results

Cardiomyocyte hypertrophy and fluvastatin: The fluvastatin treatment markedly attenuated the increase in cell surface area stimulated by ET-1 in a dose-dependent manner (Figure 1A); the treatment with only fluvastatin alone slightly and significantly decreased the cardiomyocyte surface area more than the dosage of 1 μM (Figure 1A). The fluvastatin treatment markedly inhibited the ET-1-induced increase of the protein synthesis evaluated by [^{3}H]-leucine incorporation in a dose-dependent manner (Figure 1B); the treatment with fluvastatin alone significantly suppressed the incorporation only at the dosage of 10 μM (Actual values were followings: ET-1 [E] 0 [nM], Fluvastatin [F] 0 [μM], 5125 ± 294 [cpm, mean ± SE]; E0, F0.1, 4498 ± 405; E0, F1, 3791 ± 125; E0, F10, 2415 ± 149; E10, F0, 7348 ± 406; E10, F0.1, 7144 ± 677; E10, F1, 5159 ± 29; E10, F10, 3586 ± 89) (Figure 1B). These data indicated that fluvastatin has an inhibitory effect on cardiomyocyte hypertrophy provoked by ET-1. Moreover, there was a possibility that fluvastatin has a cytotoxic effect at a dosage of 10 μM, because of the marked suppression in protein synthesis at this dosage and of the report that 3 μM fluvastatin induced apoptosis in cardiomyocyte (Ogata et al., 2002); therefore, we used fluvastatin at a dosage of 1 μM when we analyzed the effect of fluvastatin on cardiomyocyte. The mRNA expression of the cardiomyocyte hypertrophy markers ANP and BNP was significantly increased by ET-1, and fluvastatin significantly decreased both expressions

(Figures 2A, 2B), suggesting that the inhibitory effect of fluvastatin on hypertrophy was also confirmed by the alteration of the hypertrophy markers.

Intracellular signaling of cardiomyocyte hypertrophy and fluvastatin: Fluvastatin markedly decreased both phospho-JNK and phospho-c-Jun level activated by ET-1 (Figure 3B), suggesting the inhibition of the JNK pathway by fluvastatin; however, fluvastatin did not alter the phospho-ERK level augmented by ET-1 (Figure 3A). These data suggest that the inhibitory effect of fluvastatin on cardiomyocyte hypertrophy is mediated mainly via the JNK pathway rather than ERK. One of the downstream targets of JNK is c-Jun, which consists the transcription factor activator protein-1 (AP-1) as the homodimer by itself or heterodimer with c-Fos. The increased expression of c-jun mRNA induced by ET-1 was significantly suppressed by fluvastatin (Figure 4A); phospho-c-Jun was augmented by ET-1, and fluvastatin markedly attenuated the phosphorylation (Figure 4B). These data suggest that the inhibitory effect of fluvastatin on cardiomyocyte hypertrophy is predominantly associated with the JNK to c-Jun signaling, although ET-1 mobilizes both the ERK and JNK pathways.

Involvement of Pin1 in cardiomyocyte hypertrophy. The phospho-Pin1 level was significantly lower in the cardiomyocytes stimulated by ET-1 than the control (without ET-1); the reduced phospho-Pin1 level was reversed to the control level by fluvastatin treatment (Figure 5). The data suggest that a functional alteration of Pin1 occurred in the hypertrophied cardiomyocyte induced by ET-1, and that fluvastatin inhibited the ET-1-induced cardiomyocyte hypertrophy partly via the normalization of Pin1 phosphorylation. To study the role of Pin1 in the cardiomyocyte hypertrophy and how fluvastatin is involved in the Pin1 regulation, transient transfection of Pin1 was done using an adenovirus vector (Figures 6A-6E). Morphologically, Pin1 overexpression induced cardiomyocyte hypertrophy (Figures 6A, 6B, 6D) and fluvastatin inhibited the Pin1-induced hypertrophy (Figures 6B, 6C, 6D). These data suggested that a gain of

the Pin1 function induces cardiomyocyte hypertrophy and that Pin1-induced hypertrophy is suppressed by fluvastatin. In Western blot analysis, the V5-tagged Pin1 (Pin1-V5) transgene product was verified by the anti-V5 antibody; the expression level of endogenous Pin1 did not differ among 3 groups (LacZ, Pin1 without fluvastatin, and Pin1 with fluvastatin); exogenously transfected Pin1-V5 was detected at the upper part compared to endogenous Pin1 because of the increase in molecular weight; phosphorylated Pin1 level was markedly increased in Pin1-V5 transfected groups compared to LacZ group in this experimental condition and phosphorylated Pin1 level was tended to be increased in fluvastatin-treated Pin1 group compared to vehicle-treated Pin1 group (Figure 6E).

Discussion

This is the first report that ET-1-induced cardiomyocyte hypertrophy is mediated through the Pin1 activation and that the inhibitory effect of fluvastatin on cardiomyocyte hypertrophy would partly be attributed to the suppression of the Pin1 function. These findings suggest that the ET-1-induced cardiomyocyte hypertrophy is mediated through the activation of Pin1, which may be a key molecule determining the size of hypertrophied cardiomyocytes; furthermore, this is the first study to demonstrate that the inhibitory effect of fluvastatin on ET-1-induced hypertrophy would partly be attributed to the suppression of the Pin1 function by affecting the signaling from JNK to c-Jun.

Pin1 involvement in the cardiomyocyte hypertrophy

Pin1 is a small protein with an N-terminal WW domain and a C-terminal PPIase domain (Lu et al., 2007). The WW domain binds phosphorylated Ser/Thr-Pro (pSer/Thr-Pro) motifs; the PPIase domain catalyzes cis/trans-isomerization of the

proline-containing peptides; thus, Pin1 changes the activity of interacting proteins (Lu et al., 2007). It has been reported that Pin1 is overexpressed in breast cancer and promotes Ras signaling by increasing the transcriptional activity of c-Jun (Wulf et al., 2001). In the present study, ET-1 activated the ERK, JNK, and c-Jun, a downstream target of JNK, simultaneously, and ET-1 attenuated the phosphorylated Pin1, an inactive form of Pin1, suggesting that ET-1 increases the catalytic activity of Pin1. Human c-Jun has a phosphorylated Ser63/Pro73 sequence and is known to be one of the Pin1 target proteins (Wulf et al., 2001). Therefore, Pin1 may participate in ET-1-induced cardiomyocyte hypertrophy partly via the activation of c-Jun, a component of the transcription factor activator protein (AP)-1.

Recently, it has been reported that human JNK1, an upstream precursor of c-Jun, also has four pSer/Thr-Pro motifs and Thr-183 on JNK1 is critical for JNK1 activation and Pin1 binding (Park et al., 2012); therefore, Pin1 might participate in ET-1-induced hypertrophy via the activation of JNK as well as c-Jun. Furthermore, it has also been reported that ERK and c-Fos, a downstream target of ERK, have pSer/Thr-Pro motifs and that Pin1 can bind to ERK and c-Fos (Monje et al., 2005; Toko et al., 2013); it suggests that Pin1 is involved in cardiomyocyte hypertrophy by cooperating with c-Jun and c-Fos to regulate the AP-1-dependent gene transcription upon activation of the MEK family members. In addition, the C-terminal domain of the largest subunit in RNA polymerase II, the global regulator machinery of transcription, is phosphorylated at Ser-2 and Ser-5 residues prior to the Pro at hypertrophy (Sano et al., 2004; Sakai et al., 2012); Pin1 would contribute to the activation of RNA polymerase II and be involved in the hypertrophic responses. Therefore, Pin1 may play an important role in the development of cardiomyocyte hypertrophy not only via the activation of c-Jun but also via several kinds of functional molecules.

Inhibitory effect of fluvastatin on ET-1-induced cardiomyocyte hypertrophy

Some researchers have reported the inhibitory effects of statins on cardiac hypertrophy both in vivo and in vitro; simvastatin prevented cardiac hypertrophy induced by a pressure overload through p21ras inactivation (Indolfi et al., 2002) and angiotensin II-induced hypertrophy through an antioxidant mechanism involving Rac1 inhibition (Takemoto et al., 2001); and fluvastatin inhibited cardiac hypertrophy via the suppression of cyclin D1 linked to Rho kinase (Morikawa-Futamatsu et al., 2006). The finding of the present study that fluvastatin inhibited the cardiomyocyte hypertrophy induced by ET-1 is also expected from the previous research mentioned above, and not a surprising idea because cardiomyocyte stimuli including angiotensin II, ET-1, and a pressure overload finally activate similar signaling pathways, i.e., the activation of the MEK family and transcription factor AP-1 (Heineke and Molkentine. 2006).

The new finding of the present study was that the inhibitory effect of fluvastatin on hypertrophy could partly be mediated via the suppression of the Pin1 function; one of the Pin1 targets inactivated by fluvastatin was suspected to be the JNK to c-Jun pathway rather than ERK, because fluvastatin predominantly decreased the expression of phospho-JNK and phospho-c-Jun, but not phospho-ERK, under ET-1 stimulation. Cyclin D1, whose function is suppressed by statins, is also known to be a target of Pin1 (Liou et al., 2002); therefore, there is a possibility that the involvement of cyclin D1 inhibition by fluvastatin on the anti-hypertrophy effect is partly mediated via the suppression of Pin1.

In the transient transfection assay using Pin1 adenovirus vector, the gain of the Pin1 function by the Pin1 overexpression caused cardiomyocyte hypertrophy, whereas fluvastatin attenuated the Pin1-induced hypertrophy. The ET-1-induced reduction in phospho-Pin1 level was reversed by fluvastatin (Fig 5), whereas the expression level of phospho-Pin1 did not differ significantly between in fluvastatin treated Pin1 group and in

vehicle-treated Pin1 group (Figure 6E). Therefore, we consider a mechanism for an inhibition of Pin1-induced cardiomyocyte hypertrophy by fluvastatin with no alteration of the phospho-Pin1 level as followings. Because it has been reported that death-associated protein kinase 1 (DAPK1) inhibits the catalytic activity of Pin1 (Lee et al., 2011), it would be suspected that the suppressive effect of statins on Pin1-mediated hypertrophic response is partly be attributed to the magnitude of DAPK1 activation. Alternatively, the following discussion may be also possible. The overexpression experiment of Pin1 transgene, which was forcedly expressed by CMV promoter and out of the transcriptional control by primarily or secondarily statin-regulated transcriptional factors, is an artificial condition. Thus, the transgene product may be a large amount compared with the endogenous Pin1 protein; therefore, we could not detect a significant difference of phospho-Pin1 expression level between in vehicle-treated Pin1 group and in fluvastatin-treated Pin1. In addition to above consideration, the precise mechanism for statins in the inactivation of Pin1 function still remains to be resolved.

Conclusion

The present study revealed for the first time that (1) ET-1-induced cardiomyocyte hypertrophy is mediated through the activation of Pin1, which may be a key molecule determining the size of hypertrophied cardiomyocyte; (2) the inhibitory effect of fluvastatin on ET-1-induced hypertrophy would partly be attributed to the suppression of the Pin1 function; (3) the pathway from JNK to c-Jun would be a major target of Pin1. These findings firstly suggest that Pin1 is an important molecule for aggravating cardiac hypertrophy and that the inhibition of the Pin1 function by HMG-CoA reductase inhibitors is a novel mechanism for pleiotropic effects of them.

Acknowledgements

This work was supported by the Ministry of Education, Science, Sports, and Culture of Japan Grants-in-Aid for Scientific Research (25293125, 23592025, 25861714, 25860581, 22790687, 25670757, 23500835, 22390334, 23659279, 24590654) and a grant from the Miyauchi Project of Tsukuba Advanced Research Alliance at the University of Tsukuba. We thank Sayaka Inoue, Ayami Nakamura, Naomi Koharazawa, and Mari Nakagawa for their technical assistance.

Conflict of interest

There are no conflicts of interests.

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Figure Legends

Figure 1. Fluvastatin inhibited ET-1-induced cardiomyocyte hypertrophy. ET-1 (10 nM)-induced cardiomyocyte hypertrophy was inhibited by fluvastatin in a dose dependent manner evaluated by the cell surface area (10 cells/field with 5 fields [n=50] in each combination) **(A)** and by [^{3}H]-leucine uptake (4 wells [n=4] in each combination) **(B)**. Data were analyzed by a one-way ANOVA and expressed as the mean ± SE. *P<0.05, **P<0.01 compared with the control (treatments without ET-1 and fluvastatin). $^{\#}$P<0.05, $^{\#\#}$P<0.01 compared with only ET-1 treatment.

Figure 2. Effects of fluvastatin (Flu, 1 μM) and ET-1 (10 nM) treatments on the expression of the cardiomyocyte hypertrophy markers. ANP mRNA (n=6 for each group) **(A)** and BNP mRNA (n=6 for each group) **(B)** evaluated by RT-PCR. GAPDH mRNA was used as an internal control. Data were analyzed by a one-way ANOVA and expressed as the mean ± SE. *P<0.05 compared with the control (treatments without ET-1 and fluvastatin). $^{\#}$P<0.05 compared with only ET-1 treatment.

Figure 3. Effects of fluvastatin (Flu, 1 μM) and ET-1 (10 nM) treatments on the expression of ERK and JNK evaluated by a Western blot. **A**, Representative blots showed the phospho-ERK and total ERK levels, and the bar graph represented the ratio of the blot intensity of the phospho-ERK to the total ERK (n=4 for each group); **B,** blots showed the phosph-JNK and total JNK, and the bar graph represented that of the phospho-JNK to the total JNK (n=4 for each group). Data were expressed as the mean ± SE. **P<0.01 compared with the control (treatments without ET-1 and fluvastatin). $^{\#\#}$P<0.01 compared with ET-1 treatment.

Figure 4. Effects of fluvastatin (Flu, 1 μM) and ET-1 (10 nM) treatments on the expression of c-Jun. **A,** Expression of c-jun mRNA expression, and the bar graph represented the ratio of the c-jun expression to the GAPDH expression (n=6 for each group); **B**, representative Western blots showing the phospho-c-Jun and total c-Jun, and the bar graph represented the ratio of the blot intensity of the phospho-c-Jun to the total c-Jun (n=4 for each group). Data were expressed as the mean ± SE. **P<0.01 compared with the control (treatments without ET-1 and fluvastatin). $^{\#}$P<0.05 compared with ET-1 treatment.

Figure 5. Effects of fluvastatin (Flu, 1 μM) and ET-1 (10 nM) treatments on the expression of phospho-Pin1 evaluated by a Western blot. Representative blots showing the phospho-Pin1 and total Pin1 levels, and the bar graph represented the ratio in the blot intensity of the phospho-Pin1 to the total Pin1. Relative expression level of the phospho-Pin1 to the total Pin1 (n=4 for each group). Data were expressed as the mean ± SE. **P<0.01 compared with the control (no treatments). $^{\#\#}$P<0.01 compared with ET-1 treatment.

Figure 6. Cardiomyocyte hypertrophy induced by Pin1 overexpression was inhibited by fluvastatin (1 μM). Representative photographs of cardiomyocytes transiently transfected by adenoviruses (20 MOI); **(A)** LacZ (control), **(B)** Pin1, **(C)** Pin1 treated with fluvastatin. Cardiomyocytes were labeled by adenovirus for EGFP (10 MOI), additionally. Bar represents 100 μM. **D,** The bar graph represented the [^{3}H]-leucine uptake (4 wells [n=4] in each combination). **E**, Representative Western blot showing the phospho-Pin1, total Pin1, and V5 protein level overexpressed by the V5-tagged Pin1 adenovirus transfection. Data were expressed as the mean ± SE. *P<0.05, **P<0.01 compared with LacZ transfection without fluvastatin treatment. $^{\#}$P<0.05 compared with

1 Pin1 transfection without fluvastatin treatment.

Figure 1

Figures 1A and 1B Sakai S, et al.

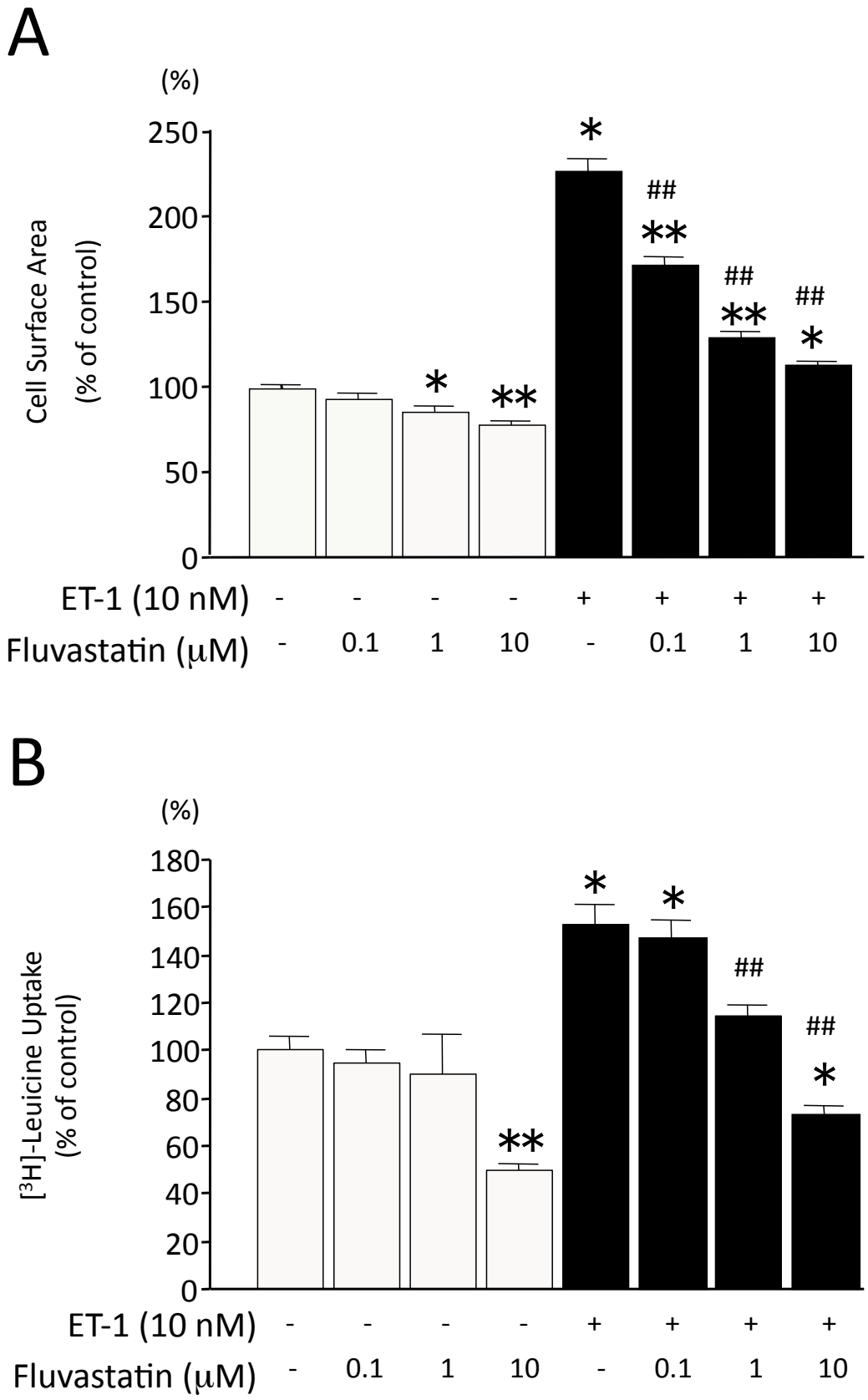

Figure 2

Figures 2A and 2B Sakai S, et al.

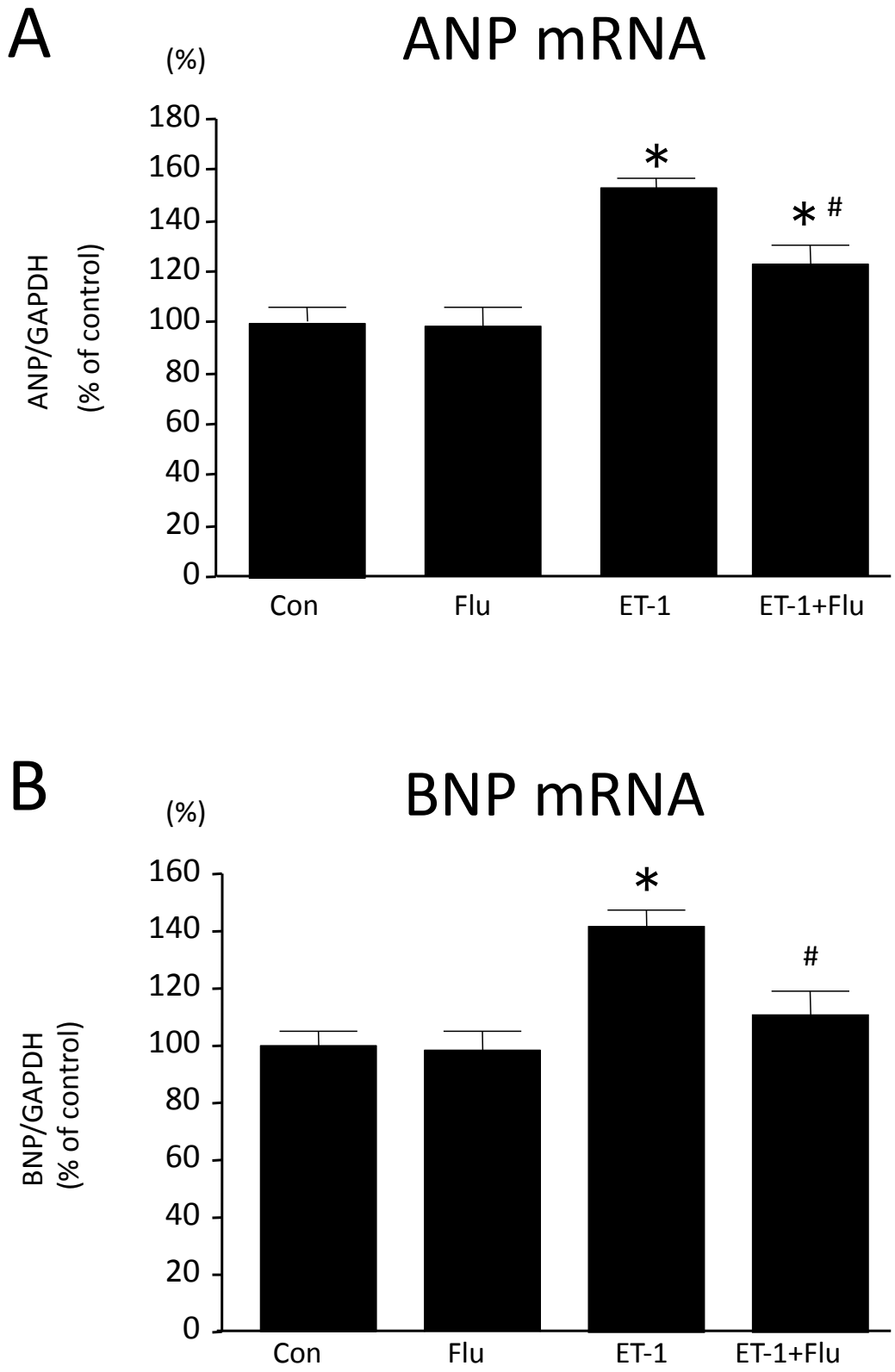

Figure 3

Figures 3A and 3B Sakai S, et al.

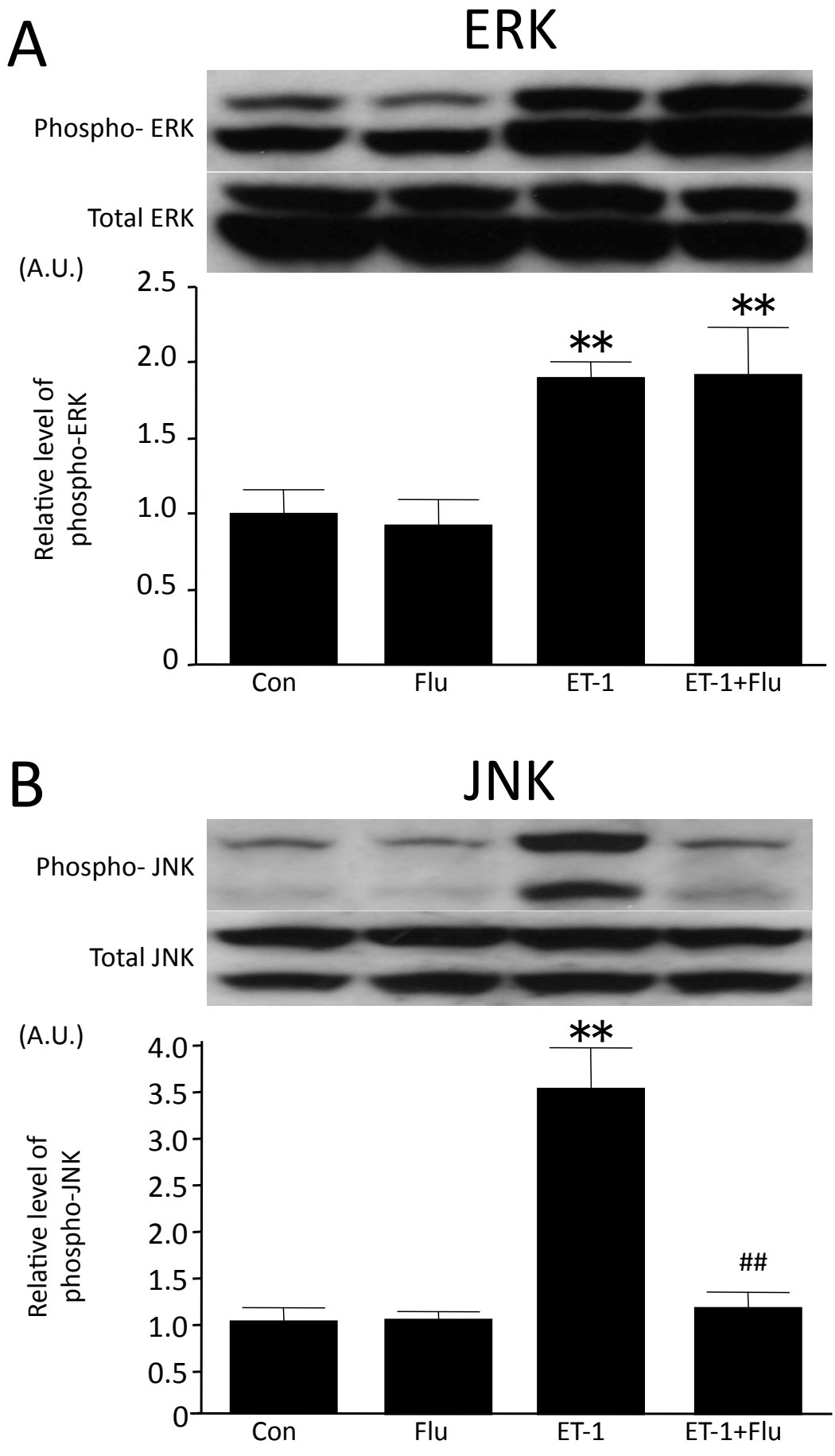

Figure 4

Figures 4A and 4B Sakai S, et al.

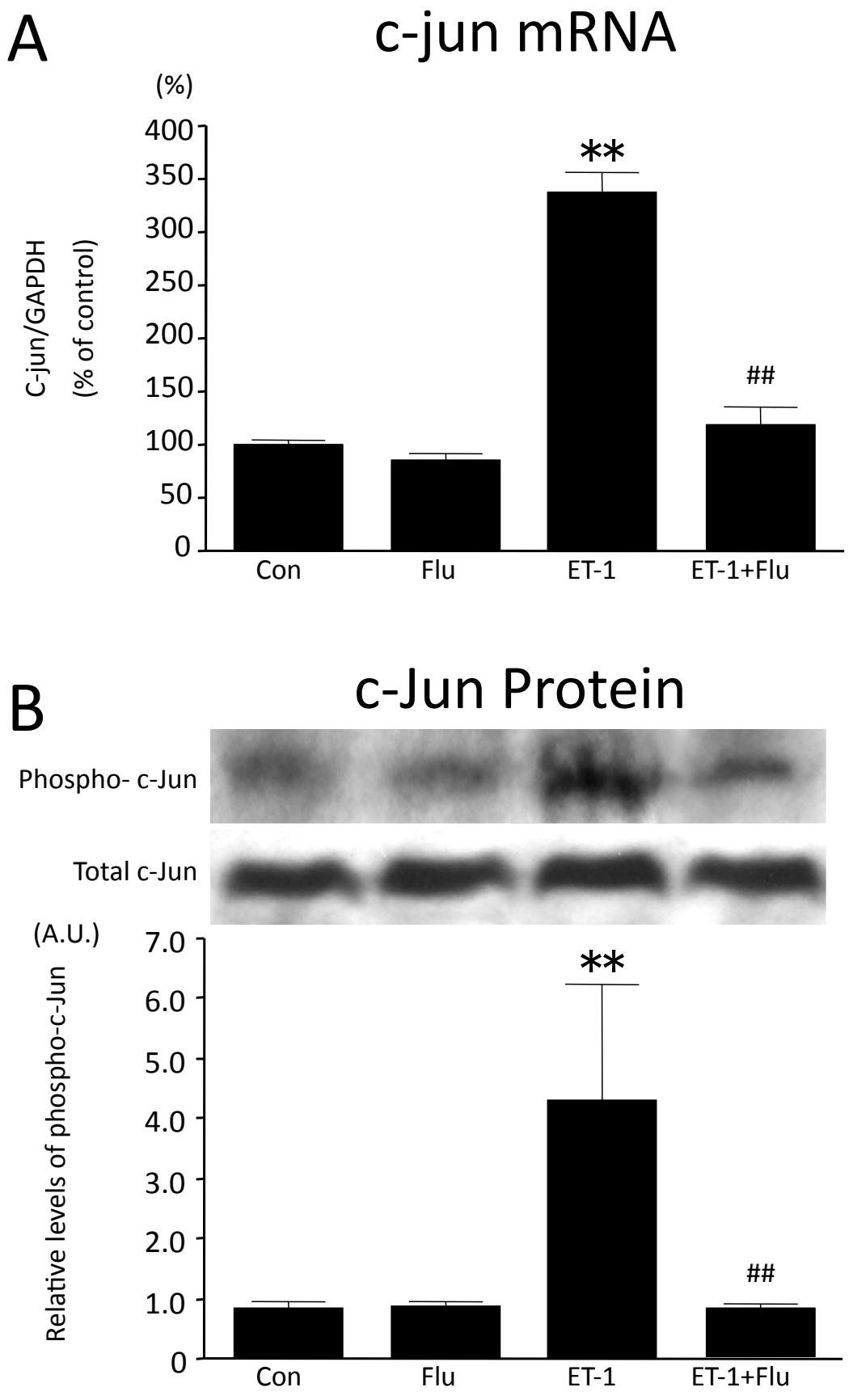

Figure 5 Sakai S, et al.

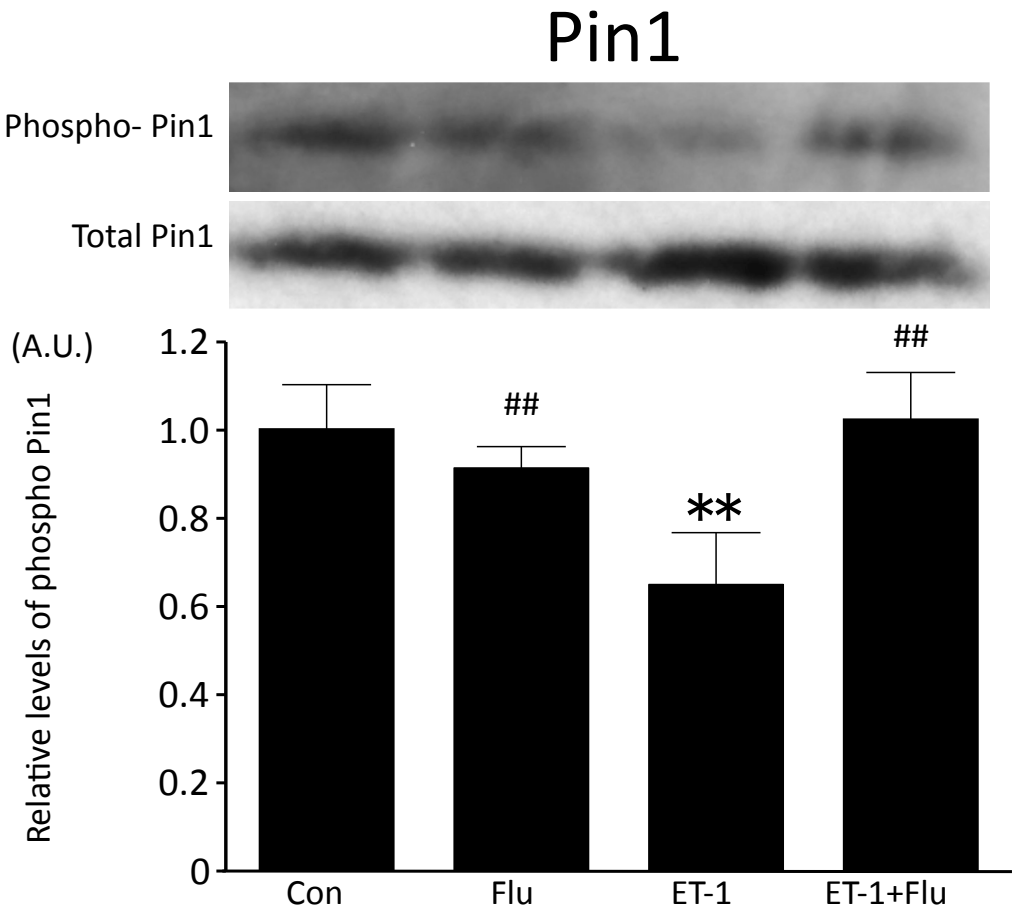

Figure 6

Figures 6A-6E Sakai S, et al