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Nectandrin B activates eNOS phosphorylation in endothelial cells: Role of the AMP-activated protein kinase/estrogen receptor α/ phosphatidylinositol 3-kinase/Akt pathway

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Running title: Effect of nectandrin B on eNOS phosphorylation

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Abbreviations: ACC, acetyl-CoA carboxylase; ACh, acetylcholine; AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; AMPK, adenosine 5'-monophosphate-activated protein kinase; CA-AMPK, constitutive active AMPK; CaMK II, calmodulin-dependent protein kinase II; DAF-2, DA (4,5-diaminofluorescein diacetate); DN-AMPK, dominant negative mutant of AMPK; DPN, Diarylpropionitrile; eNOS, endothelial nitric oxide synthase; ER, estrogen receptor; GR, glucocorticoid receptor; HUVECs, human umbilical vein endothelial cells; MPP, Methyl-piperidino-pyrazole; L-NAME, NG-nitro-L-arginine methyl ester; NO, nitric oxide; NOS, nitric oxide synthase; PI3-kinase, phosphatidylinositol 3-kinase; PPT, 4,4',4''-(4-Propyl-[1*H*]-pyrazole-1,3,5-triyl)*tris*phenol; THC, tetrahydrochrysene. (THC)

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ABSTRACT

We previously revealed that nectandrin B isolated from *Myristica fragrans* (nutmeg, Myristicaceae) functions as a potent AMP-activated protein kinase (AMPK) activator and showed its anti-obesity effect. In this study, we investigated whether nectandrin B affects phosphorylation of endothelial nitric oxide synthase (eNOS) in human endothelial cells. Nectandrin B increased the phosphorylation of eNOS and nitric oxide (NO) production in a concentration dependent manner and maximal effect was found at 10 μg/ml. Nectandrin B activates AMP-activated protein kinase (AMPK) presumably via CaM kinase II activation and nectandrin B-stimulated eNOS phosphorylation was reversed by AMPK inhibition. Both the enzyme activity of phosphatidylinositol 3-kinase (PI3K) and the estrogen receptor (ER)-dependent reporter gene transcription were enhanced by nectandrin B. ERα inhibition by specific antagonist or siRNA suppressed nectandrin B-mediated eNOS phosphorylation. Moreover, AMPK inhibition significantly reversed the activation of ER-dependent transcription and PI3K activation in response to nectandrin B. Nectandrin B evoked endothelium-dependent relaxation in rat aortic rings and this was blocked by inhibition of AMPK, ER or PI3-kinase. These results suggest that potent AMPK activator, nectandrin B enhances NO production via eNOS phosphorylation in endothelial cells and ERα-dependent PI3-kinase activity is required.

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Introduction

Endothelial function is frequently impaired during atherosclerosis, hyperlipidemia and diabetes, and this has been well-correlated with a high risk of cardiovascular events (Widlansky et al., 2003; Gokce et al., 2003). In particular, endothelial dysfunction is a key event in both clinical and experimental type II diabetes (Morcos et al., 2001; Schalkwijk and Stehouwer, 2005). Impaired endothelium-dependent vasodilatation and increased adhesion of monocytes and platelets are frequently found in diabetes (Fatehi-Hassanabad et al., 2010).

The endothelial nitric-oxide synthase (eNOS) has important basal regulatory functions in the vasculature. Constitutively expressed eNOS in endothelial cells oxidizes L-arginine to generate L-citrulline and nitric oxide (NO) in response to diverse stimuli such as shear stress (Moncada and Higgs, 1993; Li et al., 2000). Because endothelial NO production evokes a decrease in vascular tone and inhibits oxidation of low density lipoprotein (Howes et al., 1997), safe compounds that activate eNOS may be beneficial for patients with chronic cardiovascular diseases.

A series of recent studies have revealed that activation of AMP-activated protein kinase (AMPK) has beneficial effects on endothelial dysfunction. AMPK activation inhibits oxidized low density lipoprotein-triggered endoplasmic reticulum

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stress in endothelial cells (Dong et al., 2010) and metformin, a clinical AMPK activator, normalizes endothelial function by suppressing vasoconstrictor prostanoids in arteries from a type II diabetes rat model (Matsumoto et al., 2008). Moreover, AMPK activity is involved in eNOS activation. An experimental AMPK activator, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), increased eNOS activity via Ser 1177 phosphorylation; infusion of AICAR markedly increased muscle microvascular blood volume (Bradley et al., 2010). Conversely, silencing AMPKa1 in human umbilical vein endothelial cells (HUVECs) reduced eNOS content (Colombo and Moncada, 2009).

Plant-derived compounds are becoming of increasing interest as potential anti-atherosclerosis therapeutics. It has been reported that some natural compounds in fruits, vegetables, oil seeds, and herbs have lipid lowering effects and reduce atherosclerotic lesions (Katsuda et al., 2009; Afrose et al., 2009; Magnone et al., 2009). *Myristica fragrans* (nutmeg) has been used as a food and cosmetic as well as a traditional oriental medicine against dysentery, diarrhea and pain (Grover et al., 2002). Furthermore, its seed extracts possess anti-hyperlipidemic and anti-atherosclerotic activities *in vivo* (Sharma et al., 1995). However, the mechanisms underlying these activities have not been clarified. To address this gap in our knowledge, we isolated seven 2,5-bis-aryl-3,4-dimethyltetrahydrofuran lignans from total extracts of *Myristica fragrans* and

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nectandrin B had a strong AMPK stimulation effect at a concentration of 5 μM in differentiated C2C12 cells (Nguyen et al., 2010).

In the present study, we found that nectandrin B potently activates AMPK in both ECV 304 (a human endothelial cell line) and HUVECs in primary cultures. We determined whether nectandrin B affects phosphorylation and expression of eNOS and tried to identify cellular signaling pathways for the phosphorylation and expression of eNOS in response to nectandrin B.

Materials and methods

Nectandrin B isolation. The dried semens of *Myristica fragrans* (nutmeg) were purchased at a folk medicine market in Gwangju city, Republic of Korea. The sample was identified by Professor YH Moon at Chosun University, and its specimen (No. 0010) was deposited at the Department of Pharmacy, Chosun University. The EtOH extract of *M. fragrans* was subjected to an HP-20 column (10 × 60 cm), eluted with a gradient of EtOH in H_2O (60, 80, 90, and 100 %, each 3 L), and finally washed by acetone (2 L) to give five fractions. Bioassay of the five fractions on the AMPK activity revealed that the 80% ethanol-eluted fraction was most active as AMPK activator. This fraction was further chromatographed over silica gel (6 × 60 cm; 63–200

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μm particle size) using a gradient of *n*-hexane/acetone (from 6:1 to 0:1), to yield five fractions (F.1 – F.5) according to their profiles. Nectandrin B was purified from a part of fraction 2 by chromatography on a reversed phase ODS-A column (5.0 × 60 cm, 150 μm particle size) eluted with MeOH/H_2O (1.5:1, to 2:1, each 3 L) and purity was confirmed by high performance liquid chromatography.

Materials. Antibodies against eNOS, phospho-eNOS, phospho-Akt, Akt, phospho-MAP kinase, p38, phospho-extracellular signal regulated kinase (ERK), ERK, phospho-c-Jun N-terminal kinase (JNK), JNK, ERα, phospho-AMP-activated protein kinase (AMPK), AMPK, phospho-acetyl CoA carboxylase (ACC), ACC, phospho-calmodulin-dependent protein kinase II (CaMK II), horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG antibodies were purchased from Cell Signaling Technology (Beverly, MA). LY294002, PD98059, SB203580, SP600125, ICI-182780, A3281, compound C and N^G -nitro-L-arginine methyl ester (L-NAME)(NOS inhibitor) were purchased from Calbiochem (La Jolla, CA). 4,4',4"-(4-Propyl-[1*H*]-pyrazole-1,3,5-triyl)*tris*phenol (PPT), Diarylpropionitrile (DPN), Methyl-piperidino-pyrazole (MPP) and tetrahydrochrysene (THC) were obtained from Tocris Biosciences (Ellisville, MI, USA). ERβ antibody was obtained from abcam (Cambridge, MA, USA). siRNAs targeting for ERα and ERβ were purchased from Santa Cruz Biotechnology (Santa Cruz,

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CA). Actin antibody and other reagents used for molecular studies were obtained from Sigma (St. Louis, MO). Dominant negative mutant (DN-AMPK) or constitutive active forms (CA-AMPK) of AMPK overexpression plasmids were kindly donated by Dr. Ha JH of Kyunghee University (Seoul, Korea).

Cell culture. ECV 304 cells were obtained from the American type culture collection (Bethesda, MD). The cells were maintained at 37 ℃ in an incubator with a humidified atmosphere of 5% CO_2 and cultured in DMEM containing 10% fetal bovine serum. Primary cultured HUVEC cells were purchased from Innopharmascreen (Asan, Chungnam, Korea) and cultured in M199 medium containing 10 units/ml heparin, 20% fetal bovine serum and 20 ng/ml fibroblast growth factor. Nectandrin B was dissolved in dimethylsulfoxide (DMSO) and the stock solutions were added directly to the culture media. Control cells were treated with DMSO only. The final concentration of solvent was always <0.1%.

Cytotoxicity of nectandrin B in ECV 304 cells. Viable adherent cells were stained with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] (2 mg/ml) for 4 h. Media were then removed and the formazan crystals produced were dissolved by adding 200 μl of dimethylsulfoxide. Absorbance was assayed at 570 nm using a microplate reader (LB941, Berthold Technologies) and cell viabilities were

expressed as ratios versus untreated control cells.

Reporter gene analysis. A dual-luciferase reporter assay system (Promega, Madison, WI) was used to determine gene promoter activity. Briefly, cells were plated in 12-well plates and transiently transfected with the reporter and phRL-SV plasmids (hRenilla luciferase expression for normalization; Promega) using Hillymax reagent (Dojindo Molecular Technologies). The cells were then incubated in culture medium without serum for 18 h. Firefly and hRenilla luciferase activities in the cell lysates were measured using a luminometer (LB941, Berthold Technologies). The relative luciferase activity was calculated by normalizing the promoter-driven firefly luciferase activity to the hRenilla luciferase activity.

Western blot analysis. After treatment, cells were collected and washed with cold phosphate-buffered saline (PBS). The harvested cells were then lysed on ice for 30 min in 100 μl lysis buffer [120 mM NaCl, 40 mM Tris (pH 8), 0.1% NP40 (Nonidet P-40)] and centrifuged at 12,000 rpm for 30 min. Supernatants were collected from the lysates and protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, IL). Aliquots of the lysates (30 μg of protein) were boiled for 5 min and electrophoresed on 10% SDS-polyacrylamide gels. Proteins in the gels were transferred onto nitrocellulose membranes, which were then incubated with primary

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antibodies or mouse monoclonal β-actin antibodies. The membranes were further incubated with secondary anti-mouse or anti-rabbit antibodies. Finally, protein bands were detected using an enhanced chemiluminescence western blotting detection kit (Pierce Biotechnology, Rockford, IL).

Measurement of NO production. Production of NO was assessed using the NO-specific fluorescent dye 4,5-diaminofluorescein diacetate (DAF-2 DA; Cayman Chemical, Ann Arbor, MI) as described previously (Formoso et al.,2006). Briefly, ECV 304 cells were grown to 95% confluence in chamber slides (Lab-Tek, Rochester, NY) and serum-starved overnight. Cells were then loaded with DAF-2 DA (final concentration, 2 μM) for 30 min at 37 °C, rinsed 3 times with DMEM media and kept in the dark. Cells were then treated without or with nectandrin B as indicated in the figure legends. The cells were fixed in 5% paraformaldehyde for 5 min at 4 °C. Fixed cells were visualized using a fluorescence microscope (Axiovert 200M; Carl Zeiss, Germany) and an inverted epifluorescence microscope with an attached charge-coupled device camera using appropriate filters with a peak excitation wavelength of 480 nm and a peak emission wavelength of 510 nm.

The amount of NO in the culture media was also determined using the Griess reagent (Assay Designs, Ann Arbor, MI). Culture media obtained 24 h after treatment

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with various concentrations of nectandrin B were incubated with nitrate reductase for 1 h at 37°C to reduce nitrate to nitrite. An equal volume of Griess reagent was then added and the optical density of the samples measured at a wavelength of 540 nm. Data were expressed as fold change over untreated controls.

Recombinant ERα binding assay. Vehicle or test chemicals were incubated with 1 nM tritiated estradiol (^{3}H-E_2; Perkinelmer, Boston, MA) and 0.6 nM recombinant human ERα (MyBioSource, San Diego, CA) in TE buffer (10 mM Tris, 1 mM EDTA, PH 7.5) at 4 °C overnight. Hydroxylapatite (60% in TE buffer) was added, mixed well, and incubated for 15 min at room temperature. The resulting slurry was washed three times by centrifugation with TE buffer. Bound ligand was extracted by incubation of the slurry with absolute ethanol at 30 °C for 10 min. Tritium (^{3}H) decay (counts per minute) was measured by liquid scintillation in β-counter (Wallac, Gaithersburg, MD).

Organ chamber study. Male Sprague–Dawley rats (270–330 g) were sacrificed and thoracic aortas were carefully removed and placed in a modified Krebs–Ringer-bicarbonate solution containing (in mM) NaCl, 118.3; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25.0; Ca^{2+}EDTA, 0.016 and glucose, 11.1 (control solution). The aortas were cleaned of loose connective tissue and then cut into rings (2 mm wide), The aortic rings were suspended horizontally between two stainless steel

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stirrups in the organ chambers filled with 5 ml of control solution (37 °C, pH 7.4) and bubbled with 95% O_2 and 5% CO_2. The change in tension was measured isometrically with Grass FT03 force transducers (Grass Instrument Co., Quincy, MA), and data were acquired and analyzed with a PowerLab 8/30 Data Acquisition System and LabChart pro software (AD Instruments, Colorado Springs, CO). The rings were stretched progressively to the optimal tension (2 g) before the addition of 90 mM KCl. Once the plateau of the contraction elicited by KCl was obtained, the aortic rings were rinsed three times with warm control solution and 1 μM acetylcholine-mediated relaxation was tested in the precontracted rings by 1 μM phenylephrine to check endothelium-dependent relaxation responsiveness. In some experiments, rings were incubated for 30 min with LY294002 (10 μM), ICI-182,780 (100 nM) or compound C (20 μM). After 30-min incubation, 10 μg/ml nectandrin B was added and the relaxation response was monitored in the precontracted aortic rings after addition of 1 μM phenylephrine.

Statistical analysis. Unpaired Student's t-test was used to determine the significance of differences between treatment groups. Statistical significance was accepted for p values of <0.05.

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Results

Cytotoxicity of nectandrin B in ECV 304 cells. Initially, we determined the cytotoxicity of nectandrin B to ECV 304 cells by MTT assay. Fig. 1B shows that nectandrin B at the tested concentrations did not cause cytotoxicity except at concentrations above 30 μg/ml. Thus, we treated cells with nectandrin B in the concentration range 1-10 μg/ml during subsequent experiments.

Nectandrin B increases phosphorylation of eNOS and production of NO in endothelial cells. NO production from eNOS activation plays a protective physiological role in the vasculature (Li and Förstermann, 2000). We did western blot analyses to detect changes in eNOS phosphorylation. When cells were treated with 1-10 μg/ml nectandrin B for 1 h and 10 μg/ml nectandrin B at the indicated time points (5 min to 60 min), the levels of phosphorylated eNOS were increased in a concentration- and time-dependent manner (Fig. 2A and 2B). However, nectandrin B incubation for 3 to 24 h did not affect the expression level of eNOS in ECV 304 cells (Fig. 2C). To examine whether eNOS phosphorylation by nectandrin B stimulates NO production, ECV 304 cells were loaded with DAF-2 DA, a dye that upon binding to an oxidized species of NO results in fluorescence. As shown in Fig. 2D and 2E, green fluorescence (indicative

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of NO production) was increased by nectandrin B in a concentration- and time-dependent manner. We further determined nitrite levels in culture medium. Nitrite production was significantly enhanced by 3 or 10 μg/ml nectandrin B (Fig. 3A). To confirm these results, HUVECs were treated with 1-10 μg/ml nectandrin B and eNOS phosphorylation and NO production were detected. As expected, phosphorylated eNOS level and DAF-2 DA fluorescence were enhanced by nectandrin B treatment in HUVECs (Fig. 3B and 3C).

Nectandrin B increases eNOS phosphorylation via AMP-activated protein kinase through calmodulin-dependent protein kinase II. It is known phosphorylation of Ser-1177 in eNOS, which plays an important role in the regulation of eNOS activity, is induced by AMPK and results in NO production in endothelial cells (Chen et al., 1999; Morrow et al., 2003). In our previous study, we reported that nectandrin B potently activates AMPK in differentiated skeletal muscle cells (Nguyen et al., 2010). Hence, we examined the effect of nectandrin B on AMPK activity in human endothelial cells. Representative AMPK activation markers, phosphorylation of AMPK and ACC, were increased by nectandrin B in concentration- and time-dependent manners in ECV 304 cells (Fig. 4A and 4B). The same results were also found in HUVECs (Fig. 4C).

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LKB1 (also called STK11) is a mammalian kinase that activates AMPK by phosphorylating Thr172 on its catalytic (α) subunit (Carling et al., 2008). Recent studies have reported evidence that Ca^{2+}/calmodulin-dependent protein kinase II (CaMKs) can act upstream of AMPK, at least in some cell types (Hurley et al., 2005; Woods et al., 2005). ECV 304 cells were treated with nectandrin B for the indicated times and we determined the active phosphorylated form of CaMK II and LKB1. Fig. 4B shows that the levels of phospho-CaMK II were increased by 10 μg/ml nectandrin B, but this treatment did not affect LKB1 phosphorylation (data not shown), which suggest that nectandrin B-induced AMPK activation may be mediated through CaMK II activation.

We then tested whether AMPK is necessary for eNOS phosphorylation induced by nectandrin B. AMPK activation by 1 mM AICAR treatment increased NO production in ECV 304 cells (Fig. 5A). ECV 304 cells were pretreated with compound C, an AMPK inhibitor and we examined nectandrin B-dependent eNOS phosphorylation. Nectandrin B-induced phosphorylation of ACC was impaired in compound C-pretreated ECV 304 cells, and eNOS phosphorylation was attenuated (Fig. 5B), which suggests that AMPK is required for eNOS activation. To confirm these results, we used overexpression vector for DN-AMPK or CA-AMPK of AMPK. Transfection of DN-AMPK prior to nectandrin B treatment reduced nectandrin B-stimulated ACC and eNOS

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phosphorylation (Fig. 5C). Conversely, CA-AMPK transfection markedly increased phosphorylation levels of ACC and eNOS (Fig. 5D). To investigate whether the CaMK II pathway is involved in the process by which nectandrin B causes AMPK and eNOS phosphorylation, ECV 304 cells were pretreated with a CaMK II inhibitor, A3281 (10 μM), and exposed to nectandrin B. As shown in Fig. 5E, A3281 blocked nectandrin B-induced phosphorylation of eNOS, ACC and AMPK. These results suggest that nectandrin B-mediated eNOS phosphorylation is dependent on AMPK signaling through CaMK II.

Role of the PI3-kinase/Akt pathway in eNOS phosphorylation by nectandrin B. Diverse kinases such as PI3K/Akt, p38 kinase, ERK and JNK have been shown to be involved in cellular signaling for vascular relaxation and NO production (Merla et al., 2007; Grossini et al., 2008). To further elucidate the upstream signaling pathways involved in nectandrin B-mediated eNOS phosphorylation and subsequent NO production, we examined the activity of PI3K, ERK, p38 kinase and JNK in nectandrin B-treated ECV 304 cells. Western blot analyses using phospho-specific antibodies showed that nectandrin B incubation caused a sustained phosphorylation of Akt or JNK, but not ERK and p38 (Fig. 6A). To address the role of PI3K or JNK

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activation in eNOS phosphorylation by nectandrin B, the effects of specific kinase inhibitors were investigated. LY294002, a PI3K inhibitor significantly reduced nectandrin B-induced eNOS phosphorylation (Fig. 6B). However, an inhibitor of the JNK pathway (SP600125: JNK inhibitor) had no effect (Fig. 6C).

Involvement of the ERα-dependent PI3K/Akt pathway in phosphorylation of eNOS by nectandrin B. A recent study suggests that AMPK activation restores estrogen responsiveness in human endothelial cells (Chakrabarti and Davidge, 2009). It has also been shown that a lignan, nordihydroguaiaretic acid, has estrogenic activity (Fujimoto et al., 2004). Until now, no evidence has been reported of a correlation between estrogen receptor (ER) and eNOS phosphorylation induced by nectandrin B. When we determined ER-dependent transcription by using an ERE reporter, nectandrin B significantly increased the reporter activities of ERE in a concentration-dependent manner, but the increase was marginal compared to the effects of a full ER agonist, 17-β-estradiol (Fig. 7A). It has been shown that ERα directly interacts with PI3K and modulates its activity in human vascular endothelial cells (Simoncini et al., 2000). To further test the possible role of ER activation in PI3K-dependent eNOS phosphorylation in nectandrin B-exposed endothelial cells, we examined whether ICI-182780, an ER

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specific inhibitor, affects the eNOS phosphorylation that occurs in response to nectandrin B. ICI-182780 potently suppressed eNOS phosphorylation as well as Akt phosphorylation (Fig. 7B). These results indicate that nectandrin B-stimulated eNOS phosphorylation is linked with ER-dependent PI3-kinase/Akt pathways. We then confirmed the effects of an ER antagonist and a PI3-kinase inhibitor on NO production induced by nectandrin B. As shown in Fig. 7C, nectandrin B-mediated NO production (DAF-2 fluorescence) was suppressed by ICI-182780 or LY294002 pretreatment. These data suggest that ER activation is critical for PI3-kinase/Akt-mediated eNOS phosphorylation by nectandrin B.

It has been shown that both ERα and ERβ are involved in eNOS phosphorylation in endothelial cells (Mineo and Shaul, 2006). Immunoblot analyses showed that both the receptor types were expressed in ECV 304 cells (Fig. 8A, left). Either DPN (ERα selective agonist) or PPT (ERβ selective agonist) increased eNOS phosphorylation in ECV 304 cells, demonstrating both the ER subtypes are coupled with eNOS phosphorylation process in this cell type (Fig. 8A, right). We further determined the effects of specific antagonists targeting ERα and ERβ on eNOS phosphorylation by nectandrin B. Methyl-piperidino-pyrazole (MPP, a selective ERα antagonist) potently suppressed nectandrin B-mediated eNOS phosphorylation, while

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tetrahydrochrysene (THC, a selective ERβ antagonist) did not affect (Fig. 8B). Moreover, ERα siRNA potently suppressed nectandrin B-mediated eNOS phosphorylation, but ERβ siRNA marginally affected eNOS phosphorylation in response to nectandrin B (Fig. 8C). In order to assess whether nectandrin B directly bind to ERα, we further performed ERα ligand binding assay using human recombinant ERα. 3 and 10 μg/ml nectandrin B significantly inhibited tritiated estradiol binding to human ERα, but the inhibition intensity is lower than 10 nM 17-β-estradiol (Fig. 8D). These data suggest that nectandrin B acts as a relatively selective agonist on ERα.

ER-dependent eNOS phosphorylation is coupled with diverse signaling molecules. It has been shown that Src kinase mediates PI3K/Akt-dependent rapid eNOS activation in endothelial cells (Haynes et al., 2003). We found that Src specific inhibor, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2) suppressed eNOS phosphorylation by nectandrin B (Fig. 8E). Coimmunoprecipitation studies of plasma membranes from COS-7 cells transfected with ERα and Gα proteins demonstrated estrogen-stimulated selective interaction between ERα and Gαi. Moreover, Gαi inhibitor pertussis toxin blocked estrogen-dependent eNOS activation (Mineo and Shaul, 2006). However, pertussis toxin did not abrogate nectandrin B-stimulated eNOS phosphorylation in ECV 304 cells (Fig. 8E). Hence, Src tyrosine

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kinase, but not G proteins, may be involved in the ERα and subsequent eNOS activation process by nectandrin B.

Both PI3K/Akt and AMPK pathways are required for eNOS phosphorylation by nectandrin B. Hence, we studied possible cross-talk between the two kinase pathways. PI3K inhibition by LY294002 pretreatment failed to inhibit the phosphorylation of ACC in response to nectandrin B (Fig. 9A). However, AMPK inhibition by compound C impaired nectandrin B-induced Akt phosphorylation (Fig. 9B). In addition, compound C attenuated ER-dependent reporter activity induced by nectandrin B (Fig. 9C). These data support the conclusion that AMPK/ERα/PI3-kinase/Akt regulates eNOS phosphorylation in response to nectandrin B.

Nectandrin B-mediated endothelium-dependent relaxation is reversed by inhibition of ER, PI3-kinase or AMPK. We did organ chamber studies using endothelium-intact aortic rings. Nectandrin B (10 μg/ml) relaxed phenylephrine-precontracted aortic rings and LY294002 (PI3K inhibitor) and ICI-182,780 (ER antagonist) completely blocked nectandrin B-mediated vasorelaxation (Fig. 10A). Although, compound C (AMPK inhibitor) significantly reversed nectandrin B's relaxation effect, the inhibition intensity was weaker than that of LY294002 or ICI-

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182,780 (Fig. 10A). Immunoblot analysis using the homogenates of aortic rings showed that nectandrin B increased the level of phosphorylated eNOS at 15-30 min after 10 μg/ml nectandrin B exposure to aortic rings (Fig. 10B), which suggest that eNOS phosphorylation and subsequent NO production by nectandrin B is directly coupled with vascular relaxation.

Discussion

The species *M. fragrans* has been used traditionally for spices and various medicinal purposes – as a stomachic, carminative, tonic, aphrodisiac, and nervous system stimulant (Nguyen et al., 2010). Several studies have shown that lignan compounds from *M. fragrans* have beneficial anti-inflammatory, anti-diabetes and anti-oxidant effects (Han et al., 2008; Anggakusuma et al., 2009; Ma et al., 2009; Kwon et al., 2008). AMPK is activated either by an increase in the AMP/ATP ratio during metabolic stress or by activation of upstream kinases such as LKB1 and CaMK (Hardie, 2007). Because AMPK activation suppresses ATP-consuming anabolic pathways and conversely activates ATP-generating catabolic pathways, a selective AMPK activator could function as an anti-diabetes and anti-obesity agent (Misra, 2008). In this sense, nectandrin B is an attractive natural compound, since the lignan is a potent AMPK

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activator at relatively low concentrations compared to other phytochemicals.

Here, we demonstrated that nectandrin B stimulates eNOS phosphorylation in human endothelial cells. Although it has been reported that eNOS levels are reduced in AMPK silencing cells (Colombo and Moncada, 2009), protein expression of eNOS was not affected by nectandrin B. The PI3K/Akt pathway was reported to be essential for Ser 1177 or Ser1179 eNOS phosphorylation (Dimmeler et al., 1999; Fulton et al., 1999) and therefore functions as an essential kinase in regulating eNOS activity and NO production in various circumstances (Thomas et al., 2002; Cai et al., 2003). The current experiments show that nectandrin B strongly activates PI3K, and that eNOS phosphorylation and NO production in response to nectandrin B are attenuated by PI3K inhibition. A recent study showed that ERα directly interacts with PI3K and modulates its activity in human vascular endothelial cells (Simoncini et al., 2000). Furthermore, PI3-kinase is included in ER-dependent signaling (Campbell et al., 2001). In our study, we found that nectandrin B has weak agonistic activities on the ERα, whereas nectandrin B-induced eNOS and Akt phosphorylations are suppressed by ER antagonists. Furthermore, nectandrin B-stimulated eNOS phosphorylation was inhibited by ERα antagonist or ERα siRNA, but not by ERβ blocking. These data suggest that ERα activation is critical for PI3K/Akt-mediated eNOS phosphorylation by nectandrin

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B.

In addition to the PI3K/Akt pathway, MAP kinase pathways have been reported to be involved in eNOS regulation. Both the activities of ERK and p38 kinase were related with eNOS activation by several vasodilators (Grossini et al., 2009; Kan et al., 2008) and the JNK pathway is a downstream target kinase after eNOS activation (Go et al., 2001). Here, we found that nectandrin B activates JNK but neither ERK nor p38, and were confirmed that these pathways are not involved in nectandrin B-induced eNOS phosphorylation.

Although it is obvious that PI3K/Akt is a key kinase in regulating eNOS phosphorylation (Shiojima et al., 2002), regulation of eNOS phosphorylation can be under the control of other kinases as well, including AMPK (Chen et al., 1999; Morrow et al., 2003), protein kinase A (Namkoong et al., 2009) and protein kinase C (Michell et al., 2001). AMPK is a serine/threonine protein kinase that is a critical mediator of energy metabolism (Hardie and Hawley, 2001; Carling, 2004). We showed that overexpression of the constitutively active form of AMPK alone was enough to increase eNOS phosphorylation in endothelial cells, indicating that AMPK also functions as an eNOS activator in our system. Moreover, nectandrin B potently activated AMPK, and this activation is closely associated with eNOS phosphorylation, which was shown

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using chemical inhibitors or a dominant negative mutant of AMPK. Previous studies demonstrated that CaMKs and LKB1 act upstream of the AMPK pathway (Woods et al., 2005) and regulate eNOS phosphorylation (Mount et al., 2008; Bair et al., 2009). In our study, CaMK II phosphorylation was increased by nectandrin B, and, furthermore, CaMK II inhibitors significantly inhibit nectandrin B-induced phosphorylation of eNOS as well as AMPK. These results indicate that nectandrin B stimulates eNOS phosphorylation via the AMPK pathway, presumably through CaMK II activation. Levine et al. showed that AMPK lies upstream of Akt in the pathway leading from receptor activation to eNOS stimulation (Dimmeler et al., 1999). Because inhibition of either PI3-kinase or AMPK simultaneously blocks nectandrin B-stimulated eNOS phosphorylation, we further tested whether cross-talk between PI3-kinase/Akt and AMPK is associated with eNOS phosphorylation by nectandrin B. We found that AKT-phosphorylation induced by nectandrin B was attenuated by compound C, but that LY294002 did not affect nectandrin B-mediated AMPK phosphorylation. In addition, compound C decreased the ER reporter activity stimulated by nectandrin B. Thus, we can conclude that nectandrin B first activates AMPK, and that this may be responsible for further serial activation of the ERα/PI3K/Akt pathway and subsequent eNOS phosphorylation.

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In an organ chamber study, we found that 10 μg/ml nectandrin B potently relaxed rat aortic rings, and inhibitors against ER or PI3K completely suppressed nectandrin B-stimulated vascular relaxation. Although compound C also results in significant inhibition, the inhibition intensity was marginal compared to LY294002 and ICI-182,780. The discrepancy between the effectiveness of compound C in endothelial cell culture and that in organ chamber system may be due to the incomplete tissue uptake of compound C in the rat aortic rings.

In a previous study, 200 mg/kg tetrahydrofuran mixture of *Myristica fragrans*, which contains seven lignan compounds including nectandrin B, was daily administered in C57/BL6 mice for 6 weeks. The mixture showed anti-obesty effect against high fat-diet feeding, but it did not cause any toxic response in mice (Nguyen et al., 2010). We also found that oral administration of 20 mg/kg nectandrin B in C57/BL6 mice for 6 weeks did not make any significant change in the organs of mice (data not shown). Considering relatively long treatment schedules in these studies, we believe 1-10 μg/ml nectandrin B seems to be a clinically relevant concentration range *in vivo*. In summary, the present study shows that nectandrin B activates eNOS via eNOS phosphorylation, and that the ER/PI3K/Akt pathway under the control of AMPK plays a critical role in nectandrin B-mediated eNOS phosphorylation. Nectandrin B would be applicable to

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prevent cardiovascular diseases and our observations have important implications for the elucidation of the pharmacological mechanism of nectandrin B.

Authorship Contribution

Participated in research design: Kang, and Lee

Conducted experiments: Hien, Hung, and Oh, S.J.

Contributed new reagents or analytic tools: Oh, W.K.

Performed data analysis: Hien, and Lee

Wrote or contributed to the writing of the manuscript: Hien, and Kang

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FOOTNOTES

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Figure legends

Fig. 1. (A) Structure of nectandrin B. (B) Cytotoxicity of nectandrin B in ECV 304 cells. Cells were seed in a 48-well plate and various concentrations of nectandrin B were incubated for 24 h. Cell viability were estimated by MTT assay. Each bar represents the mean ± SD calculated from eight different samples (significant as compared to control, *$p<0.05$).

Fig. 2. Effect of nectandrin B on eNOS phosphorylation and expression. (A) Concentration-dependent eNOS phosphorylation by nectandrin B. ECV 304 cells were treated with nectandrin B (1 - 10 μg/ml) for 1 h and the total cell lysates were subjected to immunoblottings with antibody against Ser-1177 phosphorylated eNOS or total eNOS. Relative changes in the eNOS phosphorylation were assessed by scanning densitometry. Data represent the means±SD of 3 separate experiments (significant as compared to control, *$p<0.05$; control level = 1). (B) Time course of eNOS phosphorylation by nectandrin B. ECV 304 cells were treated with nectandrin B (10 μg/ml) for the indicated time (5 min to 60 min). Under identical condition, phosphorylated eNOS and total eNOS levels were detected by Western blot analyses. Relative changes in the eNOS phosphorylation were assessed by scanning densitometry. Data represent the means±SD of 3 separate experiments (significant as compared to control, *$p<0.05$; control level = 1). (C) No change of eNOS expression by nectandrin B. ECV 304 cells were treated with nectandrin B (10 μg/ml) for 3 to 24 h and the total cell lysates were subjected to immunoblottings with eNOS and β-actin antibodies. Relative change in the eNOS protein expression was assessed by scanning densitometry. Data

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represent the means±SD of 3 separate experiments (control level = 1). Concentration- (D) and time-dependent (E) NO production by nectandrin B. ECV 304 cells were serum-starved overnight and loaded with DAF2-DA as described under "Materials and Methods". Cells were then stimulated with nectandrin B (0.5 - 10 μg/ml) for 60 min or nectandrin B (10 μg/ml) for the indicated time. After nectandrin B treatments, cells were fixed in 5% paraformaldehyde and visualized with an epifluorescent microscope. Emission of green fluorescence is indicative of NO production.

Fig. 3. Effect of nectandrin B on eNOS phosphorylation and NO production in HUVEC cells. (A) Nitrite amounts in culture medium. ECV 304 cells were serum-starved overnight, incubated with nectandrin B (1 - 10 μg/ml) for additional 24 h and culture media were collected for nitrite determination. Data represent the means±SD of 3 separate experiments (significant as compared to control, *$p<0.05$; control level = 1). (B) Concentration-dependent eNOS phosphorylation in HUVEC cells. HUVEC cells were treated with nectandrin B (1 - 10 μg/ml) for 1 h and the total cell lysates were subjected to immunoblottings with antibodies against Ser-1177 phosphorylated eNOS, total eNOS and β-actin. (C) NO production by nectandrin B in HUVEC cells. HUVEC cells were serum-starved overnight and loaded with DAF2-DA as described under "Materials and Methods."

Fig. 4. AMPK activation by nectandrin B. (A) Concentration-dependent phosphorylation of AMPK or ACC by nectandrin B in ECV 304 cells. The cells were incubated with nectandrin B (1 - 10 μg/ml) for 1 h. 1 mM AICAR was used as a representative AMPK activator. (B) Time course phosphorylation of AMPK, ACC or

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CaMK II by nectandrin B in ECV 304 cells. 10 μg/ml nectandrin B was treated for the indicated time. (C) Concentration-dependent phosphorylation of AMPK or ACC by nectandrin B in HUVEC cells. The cells were incubated with nectandrin B (1 - 10 μg/ml) for 1 h.

Fig. 5. Involvement of AMPK in nectandrin B-induced eNOS phosphorylation. (A) NO production by AICAR. 1 mM AICAR was treated for 1 h in ECV 304 cells. (B) Effect of compound C on nectandrin B-induced eNOS phosphorylation. ECV cells were pretreated with 10 μM compound C (AMPK inhibitor) for 30 min and then incubated with 10 μg/ml nectandrin B for additional 60 min. Phosphorylated eNOS and ACC were detected by Western blot analyses. Relative changes in the eNOS phosphorylation were assessed by scanning densitometry. Data represent the means±SD of 3 separate experiments (significant as compared to control, *$p<0.05$; control level = 1; significant as compared to nectandrin B-treated group, $^{\#}p<0.05$). (C) Effect of DN-AMPK transfection on nectandrin B-induced eNOS phosphorylation. ECV 304 cells were transfected with DN-AMPK and pcDNA control for 24 h, the cells were then treated with 10 μg/ml nectandrin B for 60 min. Phosphorylated eNOS and ACC were detected by Western blot analyses. Relative changes in the eNOS phosphorylation were assessed by scanning densitometry. Data represent the means±SD of 3 separate experiments (significant as compared to control, *$p<0.05$; control level = 1; significant as compared to nectandrin B-treated group, $^{\#}p<0.05$). (D) Effect of CA-AMPK on the phosphorylation of eNOS and ACC. ECV 304 cells were transfected with CA-AMPK and pcDNA control for 24 h, the cells were then treated with or without 10 μg/ml nectandrin B for 60 min. Phosphorylated eNOS and ACC were detected by Western blot

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analyses. Relative changes in the eNOS phosphorylation were assessed by scanning densitometry. Data represent the means±SD of 3 separate experiments (significant as compared to control, *$p<0.05$; control level = 1). (E) Role of CaMK II in nectandrin B-mediated AMPK activation and eNOS phosphorylation. ECV 304 cells were pretretaed with 10 μM A3281 (Calmodulin inhibitor) for 30 min and then incubated with 10 μg/ml nectandrin B for additional 60 min. Phosphorylation intensity of eNOS, AMPK or ACC were detected by Western blot analyses. Relative changes in the eNOS phosphorylation were assessed by scanning densitometry. Data represent the means±SD of 3 separate experiments (significant as compared to control, *$p<0.05$; control level = 1; significant as compared to nectandrin B-treated group, $^{\#}p<0.05$).

Fig. 6. Role of PI3K/Akt pathway in nectandrin B-stimulated eNOS phosphorylation. (A) Effect of nectandrin B on the activities of PI3-kinase, ERK, JNK and p38 kinase. ECV 304 Cells were treated with 10 μg/ml nectandrin B for the indicated times and then immunoblotted with phosphorylation-specific antibodies that recognize phospho-Akt (p-Akt), phospho-ERK (p-ERK), phospho-p38 kinase (p-p38) and phospho-JNK (p-JNK). Parallel immunoblots were analyzed for total kinase levels with anti-Akt, ERK, p38, and JNK antibodies. (B) Effect of PI3-kinase inhibitor on nectandrin B-stimulated eNOS phosphorylation. ECV 304 cells were preincubated with 10 μM LY 294002 and then cells were incubated with 10 μg/ml nectandrin B for 60 min. Cell lysates were subjected to Western blotting analyses with antibodies against phosphortlated eNOS, eNOS, phosphorylated Akt or Akt. (C) Effects of JNK inhibitor on nectandrin B-stimulated eNOS phosphorylation. ECV 304 cells were preincubated with 10 μM SP600125 (SP) and then cells were incubated with 10 μg/ml nectandrin B for 60 min.

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Fig. 7. Role of estrogen receptor signaling in Akt-dependent eNOS phosphorylation. (A) Nectandrin B-induced ERE reporter activation. ECV 304 cells were transiently transfected with ERE-Luc plasmid. Following transfection, cells were treated with nectandrin B (1 - 10 μg/ml) or 17-β-estradiol (E2, 100 nM) for 24 h prior to lysis and measurement of ERE reporter activity. Data represent the means±SD of 4 separate samples (significant as compared to control, *$p<0.05$). (B) Effect of ER antagonist on nectandrin B-induced phosphorylation of Akt and eNOS. ECV 304 cells were preincubated with 100 nM ICI-182780 (ER antagonist). The cells were then incubated with 10 μg/ml nectandrin B for 60 min. Cell lysates were subjected to Western blotting analyses using antibodies against phosphorylated eNOS, eNOS, phosphorylated Akt or Akt. (C) Effects of PI3K inhibitor or ER antagonist on nectandrin B-mediated NO production. Under identical condition, the cells were serum-starved overnight and loaded with DAF2-DA as described under "Materials and Methods." Cells were preincubated with inhibitors for 30 min and then incubated with with 10 μg/ml nectandrin B. L-NAME was used as a NOS inhibitor.

Fig. 8. Crucial role of ERα/Src in nectandrin B-mediated eNOS phosphorylation. (A) eNOS activation effects of ERα and ERβ agonists in ECV 304 cells. Left, ERα and ERβ expression was detected by immunoblottings using specific antibodies. Right, DPN (ERα agonist, 100 nM) and PPT (ERβ agonist, 100 nM) were exposed to ECV 304 cells for 60 min and eNOS phosphorylation was determined. (B) Effects of ERα (MPP) and ERβ (THC) antagonists on eNOS phosphorylation induced by nectandrin B. ECV 304 cells were preincubated with 10 μM MPP or 10 μM THC and then cells were

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incubated with 10 μg/ml nectandrin B for 60 min. (C) Effects of siRNAs for ERα (left) and ERβ (right) on eNOS phosphorylation induced by nectandrin B. ECV 304 cells were preincubated with 60 *p*mole control siRNA, ERα siRNA or ERβ siRNA and then cells were incubated with 10 μg/ml nectandrin B for 60 min. (D) ERα binding activity of nectandrin B. 10 nM 17-β-estradiol (E2) or 1-10 μg/ml nectandrin B were incubated with 1 nM tritiated estradiol and 0.6 nM recombinant human ERα protein. Data represent the means±SD of 3 separate samples (significant as compared to control, *$p<0.05$; **$p<0.01$). (E) Effects of Gαi inhibitor (pertussis toxin, PTX, 10 nM) and Src inhibitor (PP2, 10 μM) on eNOS phosphorylation induced by nectandrin B. ECV 304 cells were preincubated with 10 nM PTX or 10 μM PP2 and then cells were incubated with 10 μg/ml nectandrin B for 60 min.

Fig. 9. AMPK is required for the activation of ER/Akt pathway by nectandrin B. (A) Effect of PI3K inhibitor on nectandrin B-stimulated AMPK activation. ECV cells were pretreated with 10 μM LY294002 (LY, PI3K inhibitor) for 30 min and then incubated with 10 μg/ml nectandrin B for additional 60 min. Phosphorylated AMPK was detected by Western blot analysis. (B) Effect of AMPK inhibitor on nectandrin B-stimulated Akt activation. ECV cells were pretreated with 10 μM compound C (AMPK inhibitor) for 30 min and then incubated with 10 μg/ml nectandrin B for additional 60 min. Phosphorylated Akt was detected by Western blot analysis. (C) Effect of compound C on nectandrin B-induced increase in ERE activity. ECV 304 Cells were transiently transfected with ERE-Luc plasmid. Following transfection, cells were incubated with compound C (10 μM) and 10 μg/ml nectandrin B for 24 h prior to lysis and measurement of ERE reporter activity. Data represent the means±SD of 4 separate

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samples (significant as compared to control, *$p<0.05$; significant as compared to nectandrin B-treated group, $^{\#}p<0.05$).

Fig. 10. Role of ER/PI3-kinase and AMPK pathways in nectandrin B-mediated vasorelaxation. (A) Effects of inhibitors targeting ER, PI3-kinase and AMPK on nectandrin B-mediated vasorelaxation. Endothelium-intact aortic rings were anchored to the organ chamber and preincubated with or without LY294002 (LY, 10 μM), ICI-182780 (ICI, 100 nM), or compound C (20 μM) for 30 min and 10 μg/ml nectandrin B-mediated vascular relaxation was monitored in the precontracted aortic rings by 1 μM phenylephrine. Data are expressed as relative relaxation percent to acetylcholine (ACh, 1 μM)-mediated relaxation and represent the means±SD of 4 separate experiments (significant as compared to nectandrin B-treated sample, *$p<0.05$; control level=1). (B) Effect of nectandrin B on eNOS phosphorylation in aortic rings. Rat aortic rings were incubated with 10 μM nectandrin B for the indicated time (5 min to 90 min) and the homogenates of aortic rings were subjected to phosphorylated eNOS immunoblotting.

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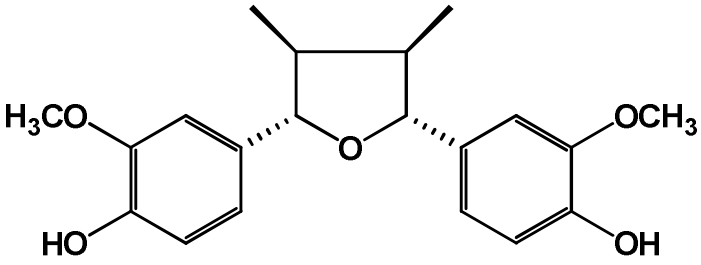
Fig. 1

A



Nectandrin B

B

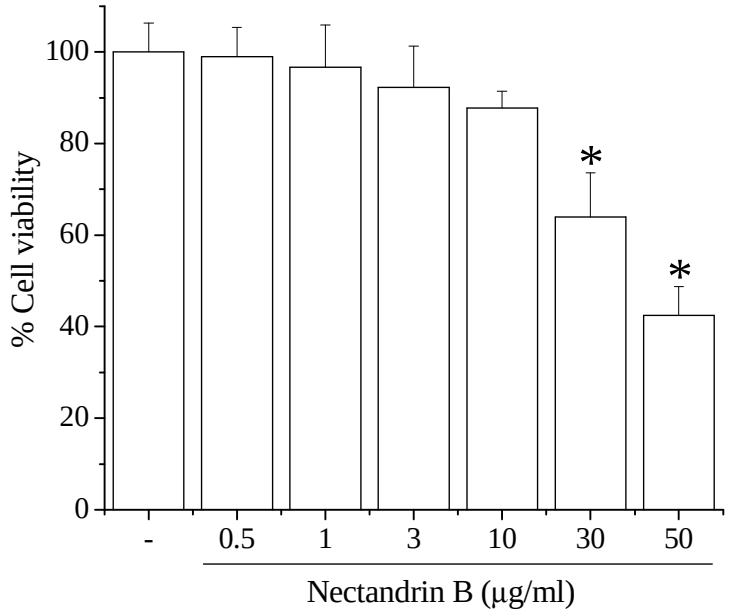


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Fig. 2

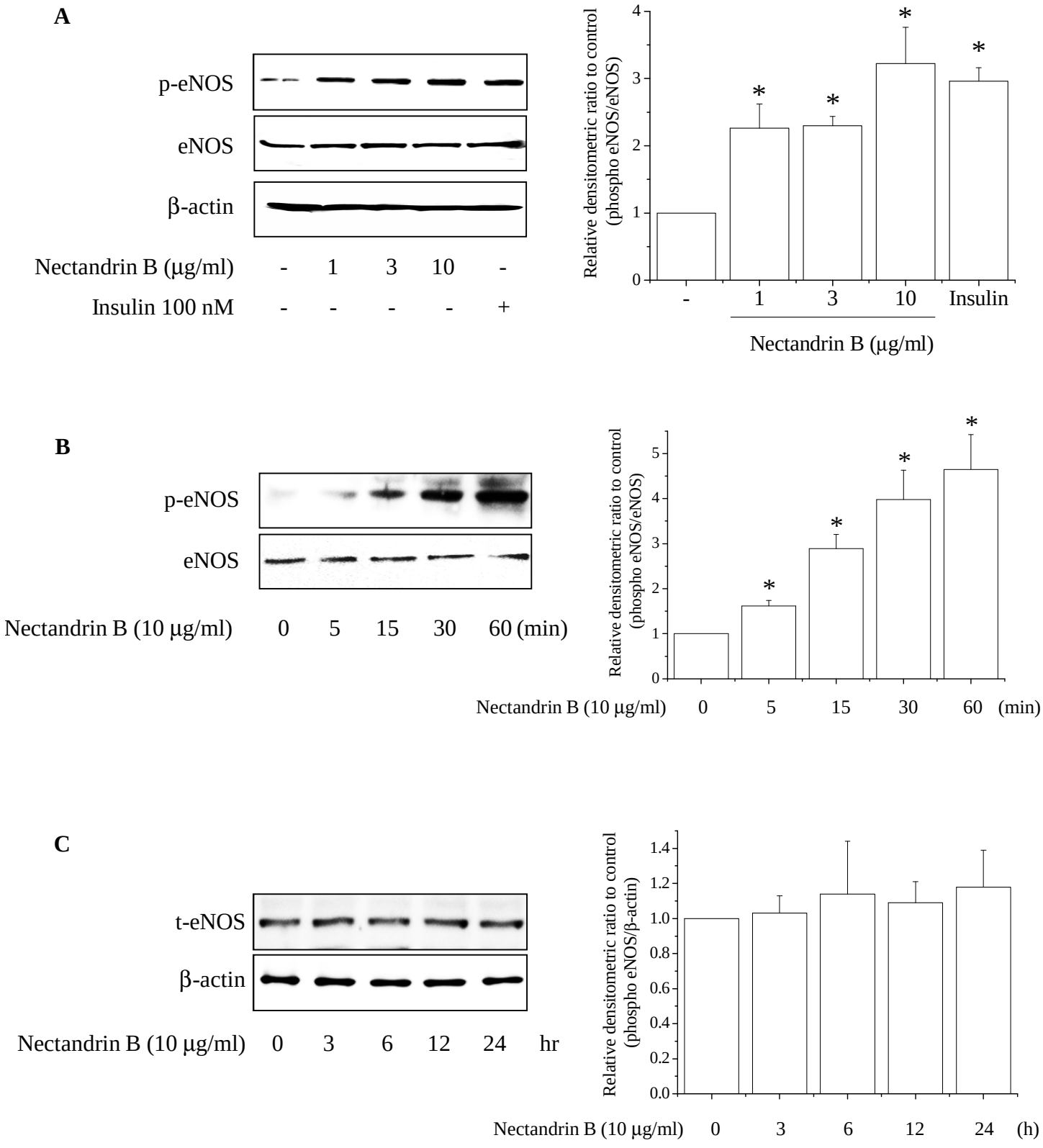

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Fig. 2

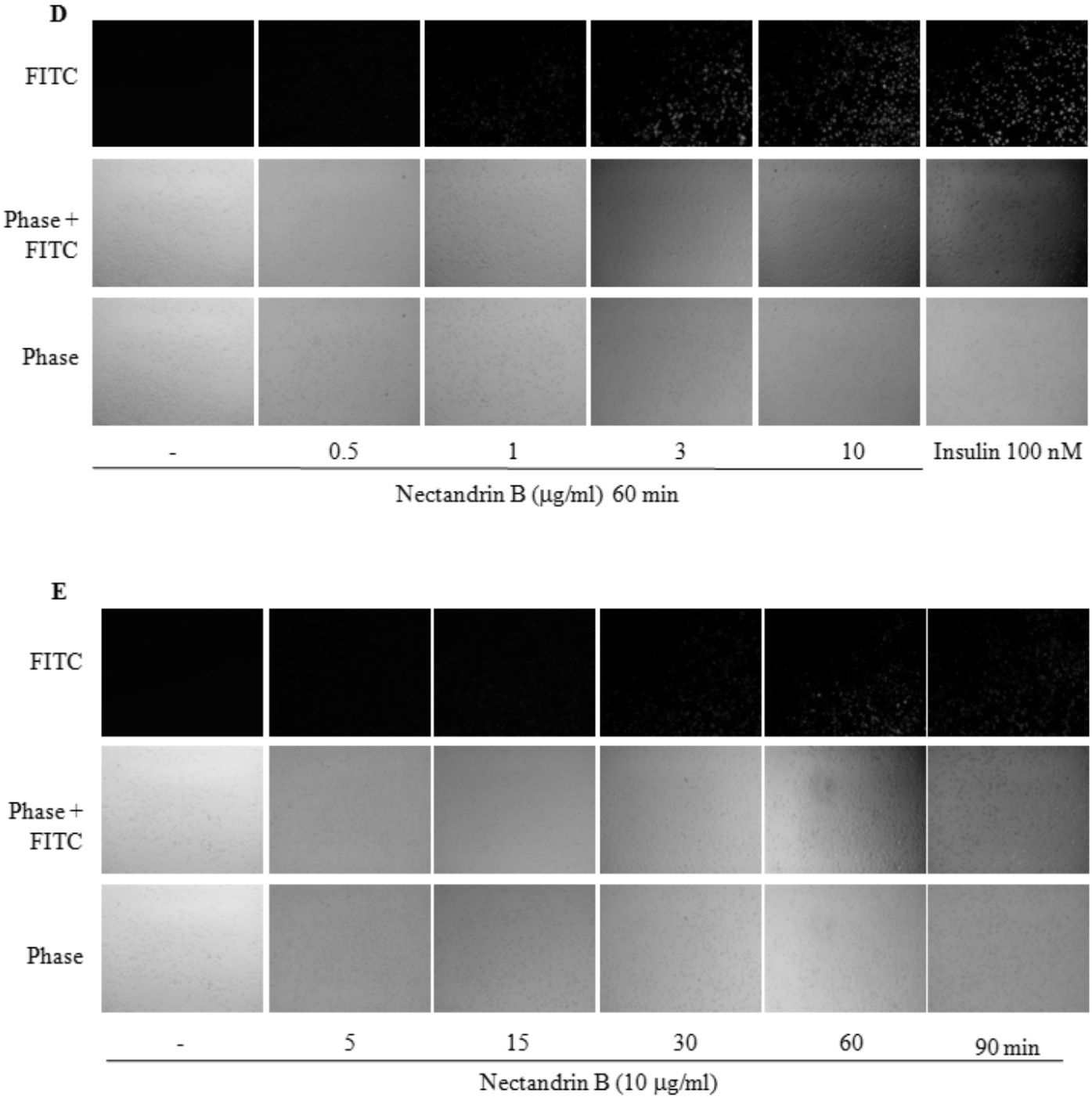

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Fig. 3

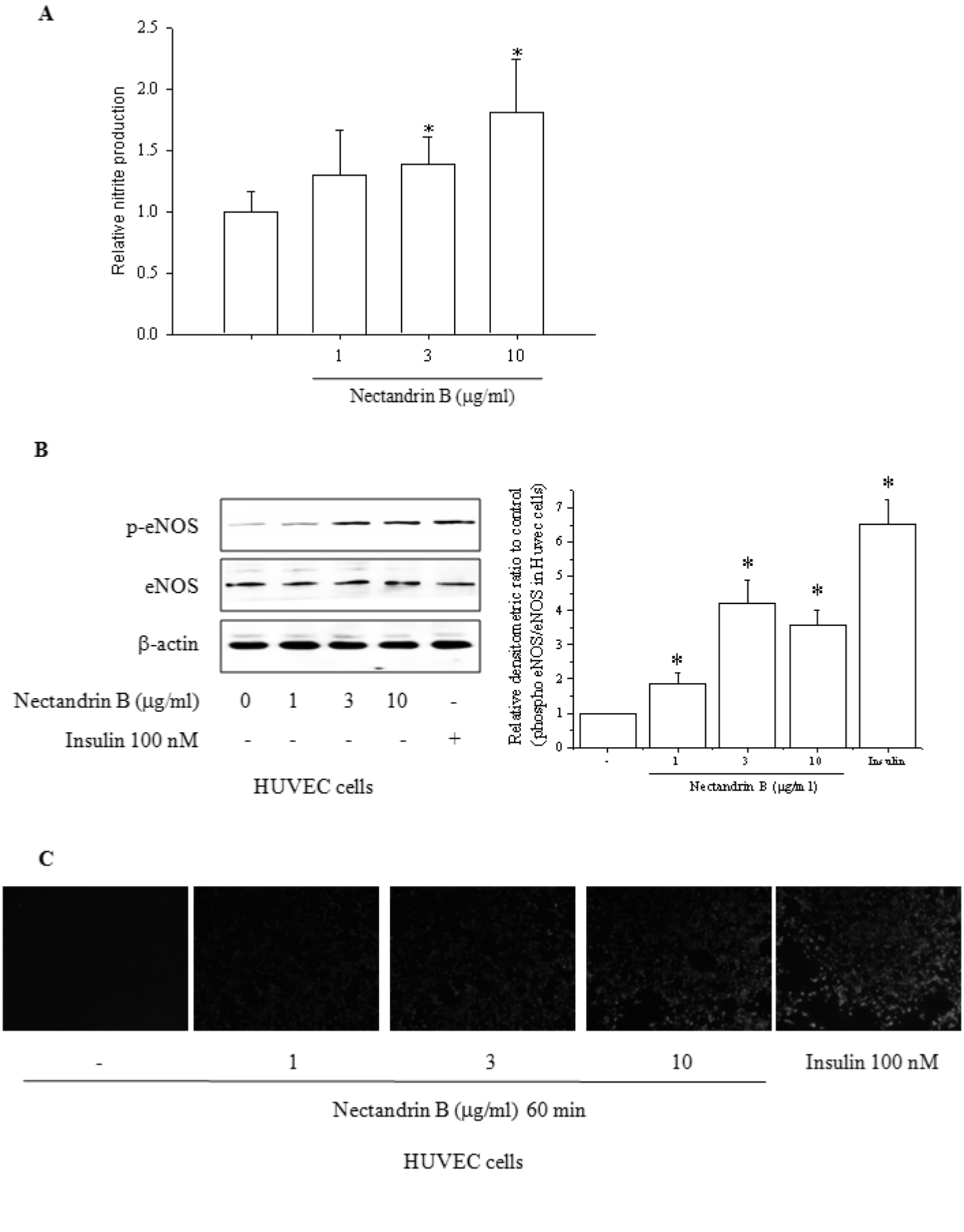

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Fig. 4

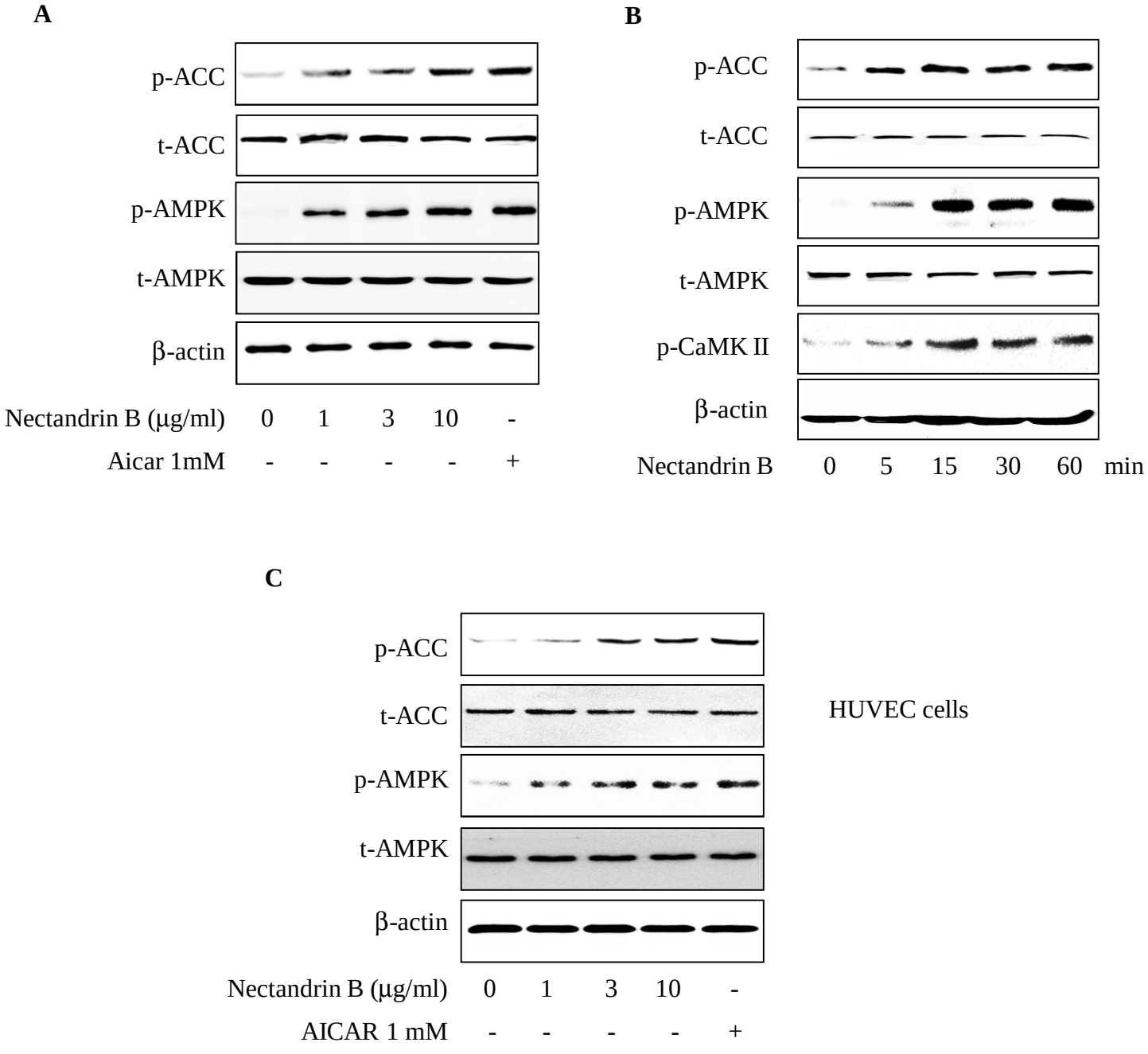

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Fig. 5

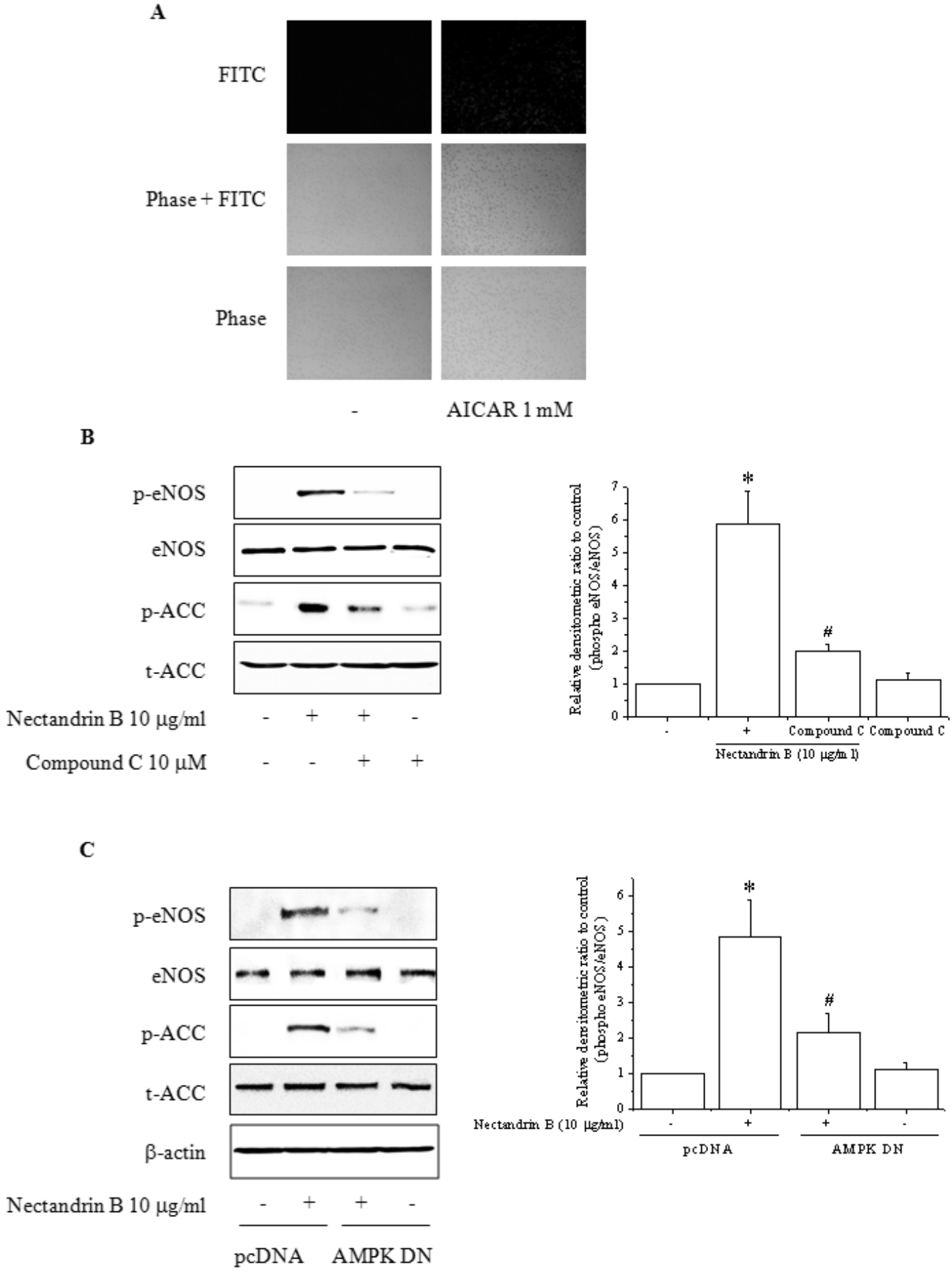

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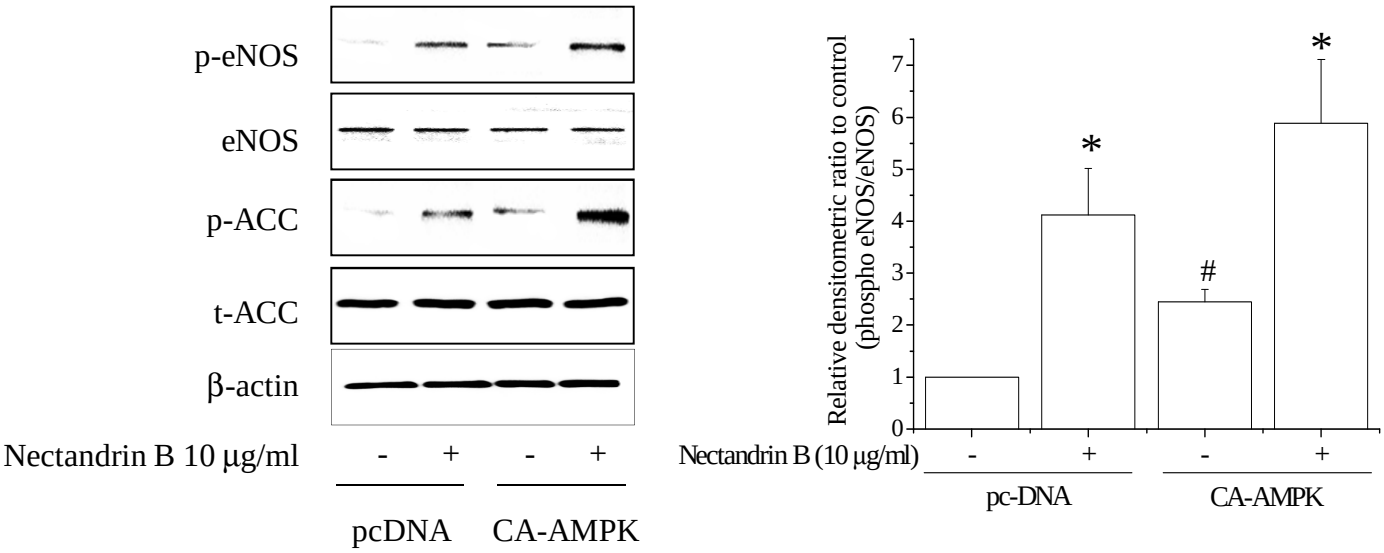
Fig. 5

D



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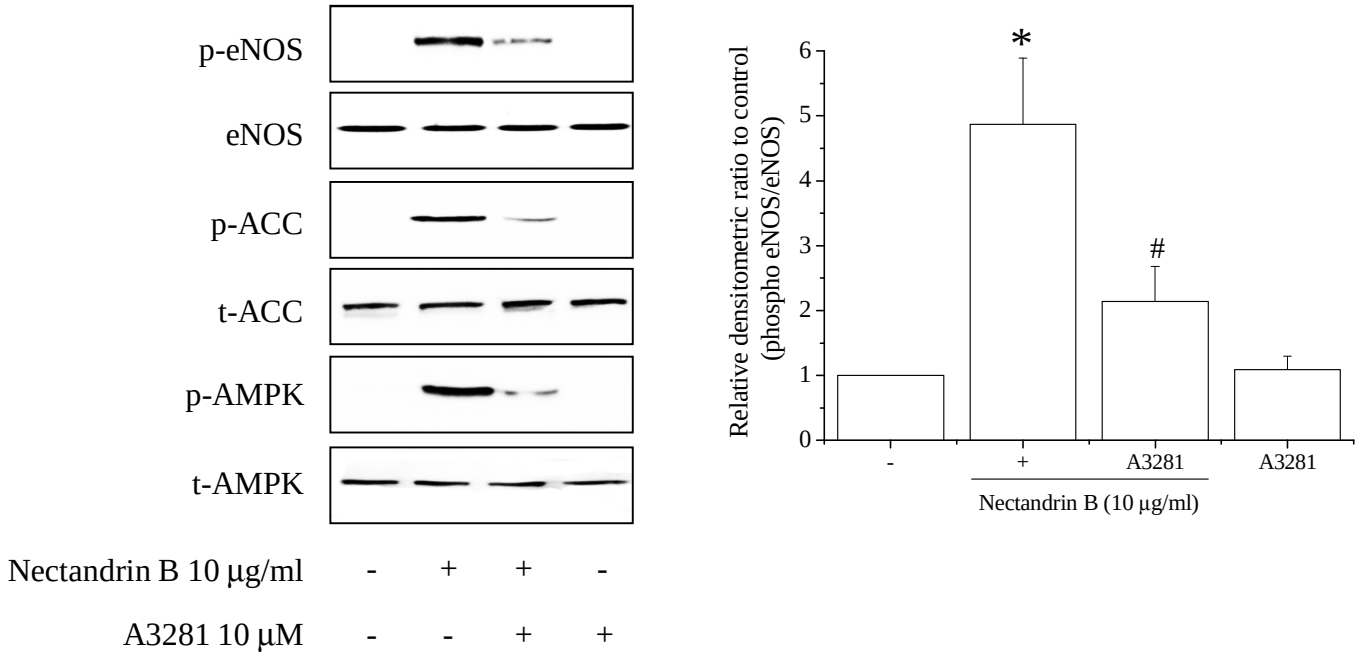

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Fig. 6

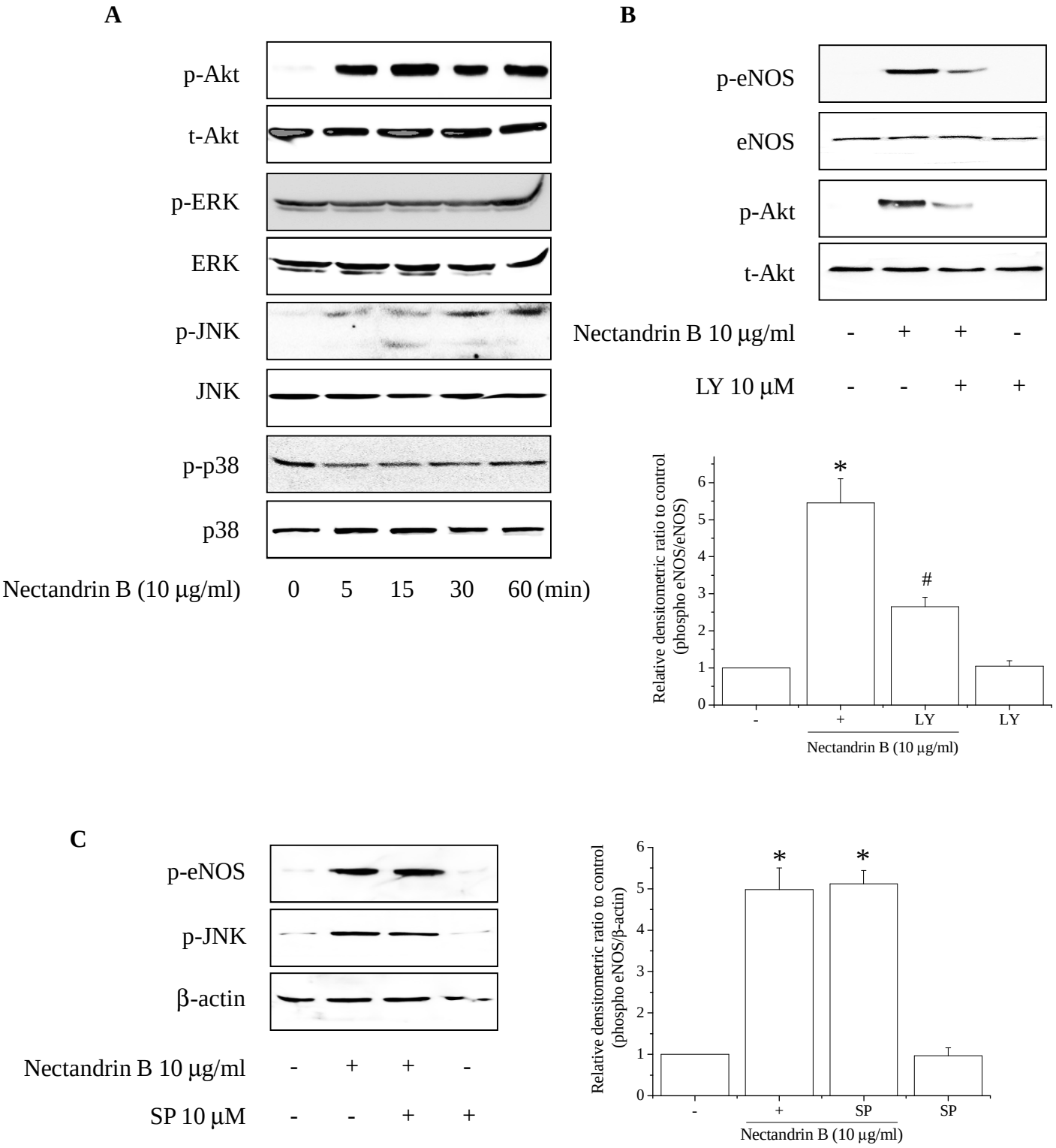

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Fig. 7

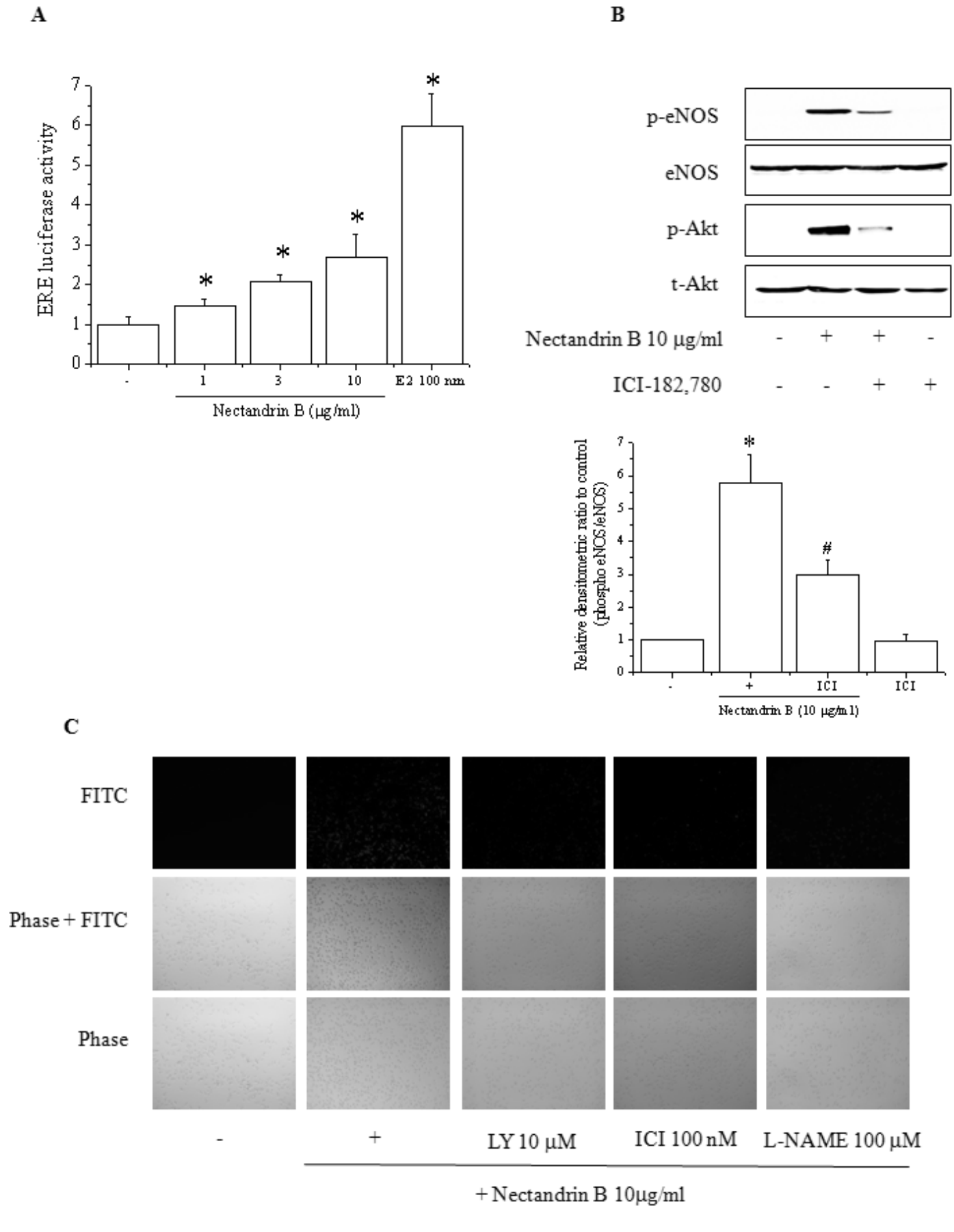

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Fig. 8

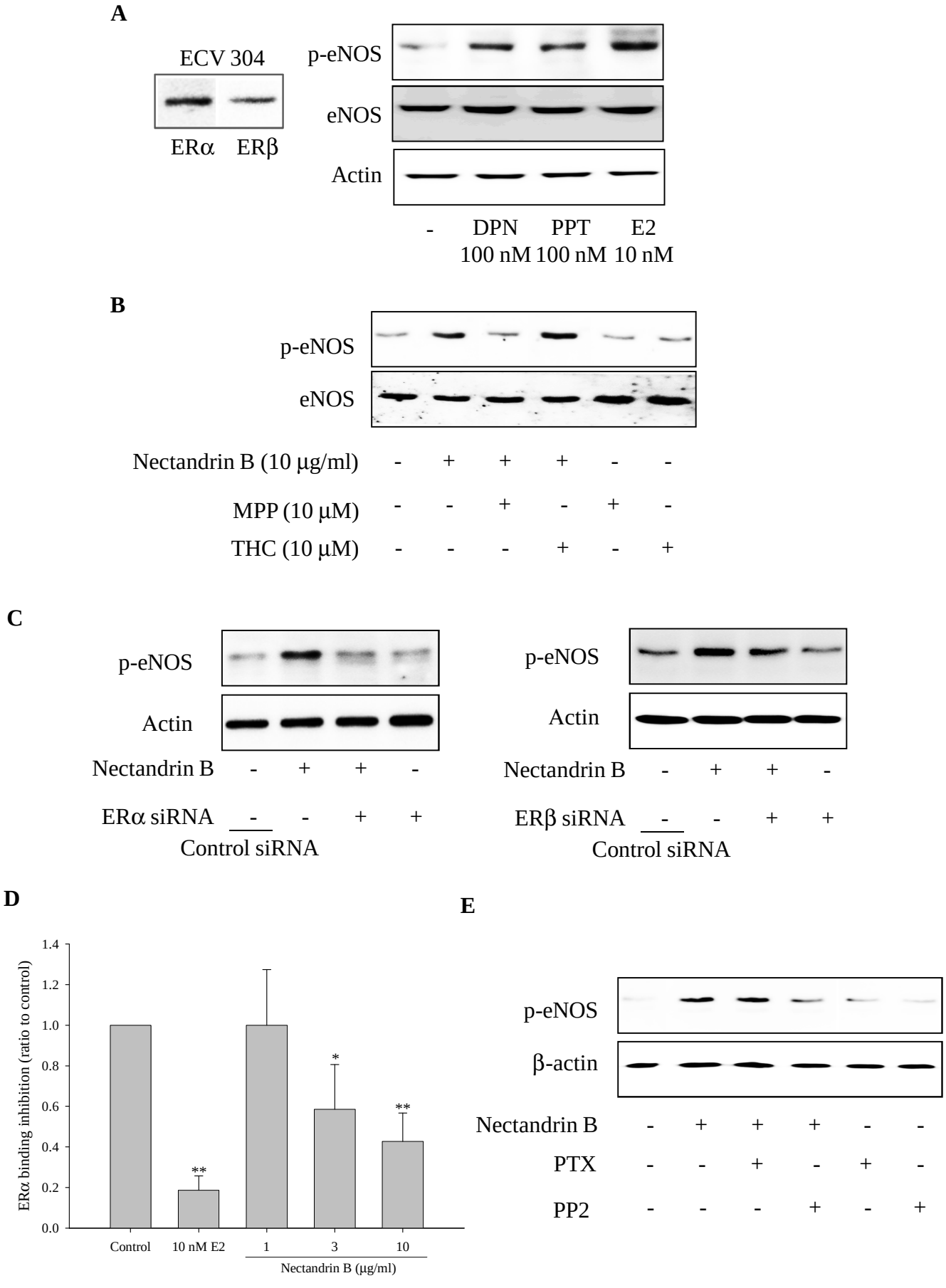

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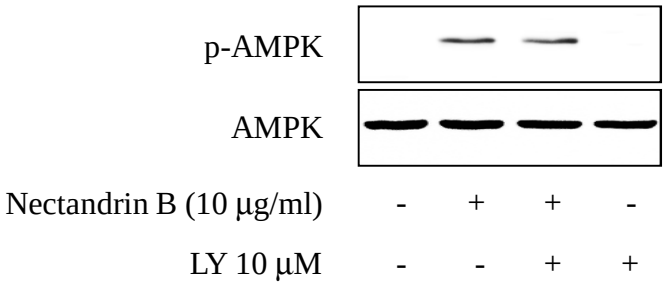
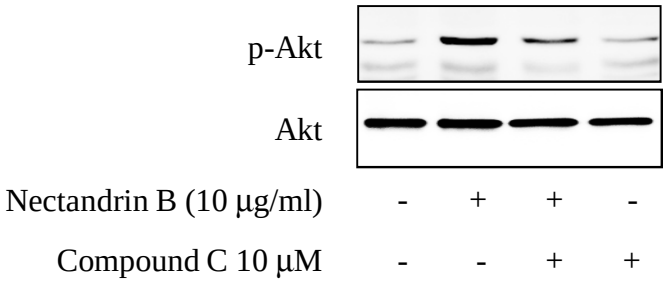
Fig. 9

A



B



C

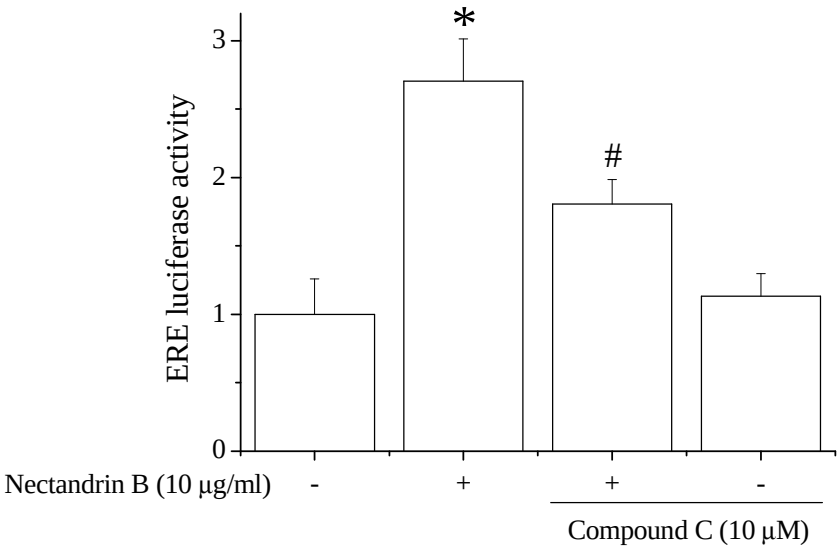


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Fig. 10