ORIGINAL RESEARCH ARTICLE

STIM-1 and ORAI-1 channel mediate Angiotensin-II-induced expression of Egr-1 in vascular smooth muscle cells†

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Abstract

An upregulation of Egr-1 expression has been reported in models of atherosclerosis and intimal hyperplasia and, various vasoactive peptides and growth promoting stimuli have been shown to induce the expression of Egr-1 in vascular smooth muscle cells (VSMC). Angiotensin-II (Ang-II) is a key vasoactive peptide that has been implicated in the pathogenesis of vascular diseases. Ang-II elevates intracellular Ca^{2+} through activation of the store-operated calcium entry (SOCE) involving an inositol-3-phosphate receptor (IP3R)-coupled depletion of endoplasmic reticular Ca^{2+} and a subsequent activation of the stromal interaction molecule 1 (STIM-1) /Orai-1 complex. However, the involvement of IP3R/STIM-1/Orai-1-Ca^{2+}-dependent signaling in Egr-1 expression in VSMC remains unexplored. Therefore, in the present studies, we have examined the role of Ca^{2+} signaling in Ang-II-induced Egr-1 expression in VSMC and investigated the contribution of STIM-1 or Orai-1 in mediating this response. 2-aminoethoxydiphenyl borate (2-APB), a dual non-competitive antagonist of IP3R and inhibitor of SOCE, decreased Ang-II-induced Ca^{2+} release and attenuated Ang-II-induced enhanced expression of Egr-1 protein and mRNA levels. Egr-1 upregulation was also suppressed following blockade of calmodulin and CaMKII. Furthermore, RNA interference-mediated depletion of STIM-1 or Orai-1 attenuated Ang-II-induced Egr-1 expression as well as Ang-II-induced phosphorylation of ERK1/2 and CREB. In addition, siRNA-induced silencing of CREB resulted in a reduction in the expression of Egr-1 stimulated by Ang-II. In summary, our data demonstrate that Ang-II-induced Egr-1 expression is mediated by STIM-1/Orai-1/Ca^{2+}-dependent signaling pathways in A-10 VSMC.

Introduction

Exaggerated vascular smooth muscle cell (VSMC) proliferative responses have been widely described as underlying mechanisms of aberrant neointima formation in vascular diseases(Giachini et al., 2011), Miao et al. (2000). A hallmark of vascular pathologies is the presence of elevated levels of vasoactive peptides, such as angiotensin-II (Ang-II), which is a major vasoconstrictor with a demonstrated causal role in the pathophysiology of vascular disorders (Montezano et al., 2014). Ang-II exerts its biological responses via the stimulation of its seven transmembrane heterotrimeric G-protein coupled receptors (GPCR) type 1 and type 2 (AT1 and AT2) (Murphy et al., 1991). Hyperactivation of Ang-II-induced signal transduction pathways has been demonstrated to contribute to vascular damage by promoting events, such as extracellular matrix accumulation, inflammation, oxidative stress, and, more importantly, VSMC proliferation, hypertrophy and migration(Nakashima et al., 2006, Touyz, 2005, Touyz and Schiffrin, 2000). An increase in the intracellular level of Ca^{2+} ($[Ca^{2+}]i$) is among the early events that occur following Ang-II stimulation of VSMC. This results in part from receptor-mediated activation of phospholipase C (PLC) and formation of inositol-3-phosphate (IP3). IP3 binds to and activates IP3 receptors (IP3R), which releases Ca^{2+} from the endoplasmic reticulum (ER) into the cytosol (Touyz and Schiffrin, 2000). Upon this release, subsequent ER Ca^{2+} depletion is sensed as a signal to trigger an influx of extracellular Ca^{2+} via a store-operated Ca^{2+} entry (SOCE) mechanism. The stromal interaction molecule 1 (STIM-1) located inside the ER membrane has been described as an essential member of the SOCE molecular machinery as it senses the Ca^{2+} depletion and, following conformational changes, associates with the transmembrane pore forming molecules, Orai, to mediate SOCE in the cytoplasm (Roos et al., 2005, Takahashi et al., 2007, Yang et al., 2012, Zhang et al., 2005). Among the different types of Orai channels, type 1 (Orai-1) have been shown to be involved in VSMC proliferation and migration (Potier et al., 2009).

Transcriptional and physiological responses triggered by a rise in $[Ca^{2+}]_i$ are in part mediated by specific Ca^{2+} handling proteins, like calmodulin (CaM), which forms a complex with free Ca^{2+} and triggers the activation of downstream kinases, known as Ca^{2+}/calmodulin-dependent protein kinases (CaMK) (Cheyou et al., 2014). CaMK type II (CaMKII) has been implicated in Ang-II-induced VSMC proliferation (Li et al., 2010)

Early growth response protein-1 (Egr-1), a zinc finger transcription factor, has been shown to be upregulated in models of vascular injury (Khachigian, 2006), and heightened levels of Egr-1 are observed in atherosclerotic lesions of animal models of vascular diseases, as well as in response to growth stimuli in VSMC (Cheyou et al., 2014, Cui et al., 2006, Goetze et al., 2001, Liu et al., 2013a, Midgley and Khachigian, 2004, Santiago et al., 1999, Vazquez-Padron et al., 2010) . Vasoactive peptides and growth factors have been shown to rapidly increase Egr-1 expression via mechanisms involving changes in $[Ca^{2+}]_i$ (Thiel et al., 2010), resulting either from a release from the intracellular stores(Jaimovich and Carrasco, 2002, Rossler and Thiel, 2009) or an influx of extracellular Ca^{2+} (Stefano et al., 2006, Mayer and Thiel, 2009, Mayer et al., 2011) However, the involvement of IP_3R/STIM-1/Orai-1-Ca^{2+}-dependent signalling in the upregulation of Egr-1 in VSMC has not been investigated.

In the present studies, by using pharmacological modulators of Ca^{2+} signaling, as well as RNA interference targeting STIM-1 and Orai-1, we investigated the involvement of SOCE-mediated signaling in Ang-II-induced Egr-1 expression in VSMC.

Materials and methods

Reagents : Ang-II (Cat# A9525) and 2-aminoethoxydiphenyl borate (2-APB) (Cat# D9754) were obtained from Sigma-Aldrich (St. Louis, MO, USA). W-7 (Cat# 681629), KN-93 (Cat# 422711),

and KN-92 (Cat.# 422709) were obtained from EMD Millipore (Burlington, ON, Canada). U0126 (Cat# 109511-58-2) was from Calbiochem (San Diego, CA, USA).

Antibodies : Rabbit polyclonal primary antibodies were used to detect STIM-1 (Sigma-Aldrich Cat# SAB3500365, RRID:AB_10646327), Orai-1 (Santa Cruz Biotechnology Cat# sc-68895, RRID:AB_2283283), total ERK1/2 (Santa Cruz Biotechnology Cat# sc-154, RRID:AB_2141292), Thr202/Tyr204 phosphorylated ERK1/2 (Santa Cruz Biotechnology Cat# sc-16982, RRID:AB_2139990), Ser133 phosphorylated CREB (Cell Signaling Technology Cat# 9198, RRID:AB_2561044), Egr-1 (Cell Signaling Technology Cat# 4153, RRID:AB_2097038) and β-tubulin (Cell Signaling Technology Cat# 2146, RRID:AB_2210545). Rabbit monoclonal primary antibody directed against CREB (Cell Signaling Technology Cat# 9197, RRID:AB_331277) was also used to detect the total amount of CREB.

Cell culture: Experiments were conducted in A-10 VSMC line derived from the medial layer of rat thoracic aorta (ATCC Cat# CRL-1476, RRID:CVCL_0130). The cells were maintained in culture with Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37ºC in a humidified atmosphere of 5% CO_2, as described earlier (Bouallegue et al., 2007). Cells between passages 4 and 8 were grown to 80-90% confluence in 60-mm dishes and incubated in serum and antibiotic-free DMEM 5 hours prior to treatments.

Cell lysis and immunoblotting: Confluent serum-starved A-10 cells were incubated in the absence or presence of various reagents for 30 minutes followed by incubation with 100 nM Ang-II for indicated times. The cells were washed three times with ice-cold PBS and lysed in 100 µL radio-immunoprecipitation (RIPA) buffer. Equal amounts of proteins measured by Bradford assay were subjected to 10% SDS-polyacrylamide gel electrophoresis, transferred to Immobilion-P polyvinylidinedifluoride membranes (Millipore, USA) and incubated with respective primary antibodies, Egr-1 (1:1000), STIM-1 (1:1000), Orai-1 (1:1000), phospho-ERK1/2 (1:2000), total

ERK1/2 (1:4000), phospho-CREB (1:1000), total CREB (1:4000) or β-tubulin (1:5000). The antigen-antibody complex was detected using a horseradish peroxidase-conjugated secondary anti-rabbit antibody (Cell Signaling Technology Cat# 7074, RRID:AB_2099233) and protein bands were visualized with the enhanced chemiluminescence detection kit (Perkin Elmer, Cat# NEL104, Montreal, QC, Canada).

Preparation of cDNA : After incubations, total RNA was isolated using Trizol reagent (Life Technologies, Burlington, ON, Canada). RNA concentration was quantified with the Eppendorf BioPhotometer D30 (Eppendorf, Mississauga, ON, Canada). Absorbances were measured at wavelengths of 260 nm and 280 nm. The purity of RNA preparation was confirmed when the ratio A260/A280 was comprised in the range of 1.8-2.0. cDNA was synthesized from 1 µg of total pure RNA using High Capacity RNA-to-cDNA Kit (Life Technologies, Cat# 4387406, Grand Island, NY, USA) as per manufacturer's instructions.

Real-time quantitative polymerase chain reaction (qRT-PCR) : qRT-PCR was performed with SYBG (Life Technologies, Grand Island, NY, USA) using 1µL of cDNA in a 20 µL reaction. Amplification was performed using 7500 fast RT-PCR system (Applied Biosystems, Grand Island, NY). Sequences used to design Egr-1 primers were as follow: forward 5'-CTGCTTCATCGTCTTCCTCTG-3' and reverse 5'-GTCAGTGTTGGGAGTAGGAAAG-3'. Egr-1 mRNA expression was measured and normalized with β-actin (Primers: forward 5'-TCTTCCAGCCTTCCTTCCT-3' and reverse 5'-CAGCACTGTGTTGGCATAGA-3') mRNA levels.

Immunofluorescence: Serum-starved A-10 cells grown and treated on glass coverslips were washed with ice-cold PBS and fixed with paraformaldehyde 4% for 30 min at 4°C. Permeabilization was achieved by 10 minutes incubation with 0.1% Triton X-100, 0.1% serum citrate pH 4.0 at room temperature (RT). Cells were then blocked with goat serum diluted in PBS (15 µL/mL PBS) for one hour and incubated overnight at 4°C with Egr-1 antibody diluted in the blocking solution (1:100).

Coverslips were further incubated for two hours at RT with goat anti-rabbit IgG conjugated with Alexa Fluor 488 (1:150) (Thermo Fisher Scientific Cat# A-11034, RRID:AB_2576217). Nuclei were then labelled by staining the coverslips with DAPI (2 µL/1.5 mL H_2O) before being mounted with a buffer made of 30% Glycerol in PBS. The images were taken using X-Cite Serie 120, TE2000-S fluorescence microscope (Youreva and Srivastava, 2016) .

Fura-2 [Ca2+]i imaging : $[Ca^{2+}]_i$ was monitored in A-10 cells after Ang-II stimulation. Briefly, cells were loaded with 10 µM Fura-2-AM (1 hour, at 37 °C in dark), washed in DMEM containing 0.001% cremophor and 2.5 mM of probenecid, followed by washing in DMEM containing 2.5 mM probenecid to achieve de-esterification. Petri dishes containing fura-2-AM-treated A-10 cells were placed on the stage of an inverted microscope (Nikon TE300, Mississauga, ON, Canada). The cells were exposed to alternate (100 ms) excitatory wavelengths at 340 nm and 380 nm with a high-pressure mercury lamp (100 W) via interference filters (Chroma Technology, Brattleboro, VT, USA) mounted on a filter wheel (Sutter Lambda 10-C, Sutter Instrument, Novato, CA, USA) with a dichroic mirror. A cool-coupled device camera recorded fluorescent images from three to ten seconds intervals. Measurements are presented as the F_{340}/F_{380} fluorescence ratio.

siRNA transfection protocol : Transfection was performed using lipofectamine RNAi max (Life Technologies, Cat# 13778-075, Burlington, ON, Canada). A-10 VSMC at 70% confluence were transfected with 10 nM rat siRNA constructs according to the manufacturer's protocol (Origene, Rockville, MD, USA). Briefly, distinct mixtures obtained by addition of lipofectamine to tubes containing siRNA against CREB (Origene, Cat# SR500635, Locus ID 298400), STIM-1 (Origene, Cat# SR512570, locus ID 361618), Orai-1 (Origene, Cat# SR508429, locus ID 84876) or non-targeting scrambled siRNA (Origene, Cat# SR30004) were used to transfect cells for 6 hours. The

medium was replaced afterward with normal supplemented culture medium and the cells were incubated for 48 additional hours at 37^{o}C before stimulation with Ang-II.

Data analysis: Images obtained from immunofluorescence assays were analyzed with the program ImageJ (http://rsb.info.nih.gov/ij/index.html RRID:SCR_003070). The intensity of the bands was quantified by densitometric analysis of immunoblots using Quantity One 1-D Analysis Software (http://www.bio-rad.com/en-us/product/quantity-one-1-d-analysis-software RRID:SCR_014280). Graphs and statistical analysis by one-way standard analysis of variance (ANOVA) were made with Graphpad Prism 5.0 software package (http://www.graphpad.com/ RRID:SCR_002798). Statistical significance of the differences between samples was assessed by a Tukey multiple comparison *post hoc* test. The differences between means were considered significant with $p<0.05$. All quantitative data are expressed as mean ± SEM from at least five independent experiments.

Results

Ang-II induces an increase in Egr-1 protein levels in a time- and dose-dependent fashion in A-10 VSMC.

Ang-II is a key vasoactive peptide with a well-established role in the pathogenesis of vascular diseases and upregulation of Egr-1 has been implicated in neointimal thickening and atherosclerosis. However, the effect of Ang-II on Egr-1 expression in VSMC is not fully characterized. Therefore, we sought to determine the effect of Ang-II on Egr-1 expression in VSMC. As shown in figure 1, Ang-II dose-dependently enhanced the expression of Egr-1 (Fig.1A). The increase in Egr-1 expression could be detected at 10 nM; however, at higher doses, the expression level was further enhanced. Time-course studies using 100 nM Ang-II demonstrated that Egr-1 protein expression was detectable after 30 min of treatment, reached a peak value at 60 min and rapidly declined to basal levels within a 2-hr period (Fig.1B), whereas Egr-1 mRNA was significantly upregulated after 30 min of treatment (Fig.1C).

Consistent with the immunoblotting data, immunofluorescence analysis revealed that the nuclear accumulation of Egr-1 protein also peaked at 60 min following stimulation of VSMC with Ang-II (Fig.1D). Further experiments using actinomycin D, an inhibitor of mRNA transcription, revealed that increased expression of Egr-1 in response to Ang-II required RNA transcription (data not shown). These data demonstrate that Ang-II induces Egr-1 expression, and this action involves an enhanced transcription of Egr-1 mRNA.

IP3 receptor blockade attenuates Ang-II-induced Egr-1 expression and alters [Ca2+]i responses in A10 VSMC

IP_3 generation is among the earliest events subsequent to Ang-II receptor activation. Ang-II raises cytosolic Ca^{2+} concentration through release mechanisms involving PLC-dependent generation of IP_3 that activates IP_3R-coupled release of ER Ca^{2+} (Liu et al., 2009, Duff et al., 1995). IP_3R-dependent events have been widely shown to mediate receptor-mediated signaling to Ca^{2+} entry in multiple cell types. Therefore, by using 2-APB, an allosteric non-competitive inhibitor of IP_3R that antagonizes SOCE (Ma et al., 2000, Peppiatt et al., 2003, van Rossum et al., 2000), we wished to determine whether IP_3R-dependent Ca^{2+} signaling plays a role in Egr-1 expression. As shown in Figure 2, 2-APB dose-dependently inhibited Ang-II-induced Egr-1 protein expression (Fig.2A) and significantly blunted Ang-II-induced upregulation of Egr-1 mRNA (Fig.2B). Consistent with the immunoblotting results, immunofluorescence localization of Egr-1 in cells also revealed that 2-APB-mediated blockade of IP_3R decreased the nuclear accumulation of Egr-1 in VSMC (Fig.2C) suggesting a role of IP_3R-mediated calcium signaling events in Ang-II-induced Egr-1 expression. It should be noted that 2-APB treatment alone resulted in the reduction of Egr-1 levels in unstimulated cells although in these cells, only trace amounts of Egr-1 were detectable.

We further investigated whether the attenuation of Egr-1 expression by IP_3R blockade could be attributed to the alterations in Ang-II-induced $[Ca^{2+}]i$ mobilization in VSMC. As shown in figures 3A and 3B, stimulation of Fura-2-AM-loaded A-10 VSMC with Ang-II resulted in a rapid rise in $[Ca^{2+}]i$. Pre-incubation of the cells with 2-APB abolished the $[Ca^{2+}]i$ response (Fig.3A and Fig.3B) confirming the ability of 2-APB to modify Ang-II-induced $[Ca^{2+}]i$ dynamics.

siRNA-mediated silencing of STIM-1 or Orai-1 inhibits Ang-II-induced Egr-1 expression

IP_3R-mediated depletion of ER Ca^{2+} induces SOCE through a process where STIM-1/Orai-1 cooperation plays a pivotal role (Roos et al., 2005, Zhang et al., 2005, Zou et al., 2011). Our data using 2-APB suggested an involvement of IP_3R/SOCE in Ang-II-induced Ca^{2+} release and Egr-1 expression, but the contribution of STIM-1 and Orai-1 in Ang-II-induced expression of Egr-1 remains unexplored. Therefore, by using RNA interference technique, we assessed whether these key components of SOCE participate in this process. As shown in Figure 4, treatment of VSMC with 10 nM of siRNA targeting STIM-1 (siSTIM-1) (Fig.4A) or Orai-1 (siORAI-1) (Fig.4B) significantly reduced the expression of these molecules, whereas scrambled siRNA (siSCR) exerted no effect on STIM-1 (Fig.4A) or Orai-1 (Fig.4B) expression. Furthermore, siRNA-induced silencing of either STIM-1 or Orai-1 significantly reduced Ang-II-induced expression of Egr-1 in VSMC, suggesting their involvement in this process.

Calmodulin and CaMKII inhibitors attenuate Ang-II-induced Egr-1 expression in VSMC

Since the downstream effects of Ca^{2+} are in part mediated by Ca^{2+} binding proteins, such as calmodulin (CaM) and CaMKinases (CaMKII), and because we have shown earlier that CaM/CaMKII plays a role in mediating endothelin-1 (ET-1)-induced signaling responses and Egr-1 expression in VSMC (Bouallegue et al., 2013), we investigated if these effectors of Ca^{2+} signaling also mediate Ang-II-induced expression of Egr-1. As shown in Figure 5, pre-treatment of the cells with 10 µM of W-7 (Fig. 5A) or KN-93 (Fig.5B), respective pharmacological inhibitors of calmodulin and CaMKII activity, significantly reduced Ang-II-induced expression of Egr-1. In contrast, KN-92, the inactive analog of KN-93, produced no effect on Ang-II-induced response. Collectively, these data support the involvement of Ca^{2+}/CaM/CaMKII-dependent signaling in Ang-II-induced Egr-1 expression.

siRNA-mediated silencing of STIM-1 or Orai-1 attenuates Ang-II-mediated activation of ERK1/2 and CREB

We have shown earlier that CaM/CaMKII pathway mediates MEK/ERK1/2 activation in response to ET-1 in VSMC (Bouallegue et al., 2013). Additionally, we and others have observed that the MEK/ERK1/2 pathway plays a key role in Egr-1 expression induced by several stimuli (Hasan and Schafer, 2008, Liu et al., 2013a, Youreva and Srivastava, 2016). Therefore, we investigated if the attenuation of Ang-II-induced Egr-1 expression due to STIM-1 and Orai-1 silencing in VSMC was associated with a change in ERK1/2 phosphorylation. As shown in Figure 6, siRNA-induced silencing of either STIM-1 (Fig.6A) or Orai-1 (Fig.6B) resulted in a significant reduction in Ang-II-induced phosphorylation of ERK1/2 (Fig.6C and Fig.6D). This suggests that ERK1/2 is a downstream effector of STIM-1/Orai-1-mediated signaling in response to Ang-II in VSMC.

Two cyclic-AMP response elements (CRE) are found in Egr-1 promoter (Cheyou et al., 2014, Cui et al., 2006) and the transcription factor CRE-binding protein (CREB) has previously been shown to regulate Egr-1 induction in response to GPCR agonist stimulation in VSMC (Cui et al., 2006). Additionally, a role of Ang-II-induced activation of CREB in mediating VSMC hypertrophy, proliferation and neointimal formation after vascular injury has been reported (Molnar et al., 2014, Funakoshi et al., 2002). Thus, we sought to investigate if attenuation of Ang-II-induced Egr-1 expression and ERK1/2 phosphorylation observed following STIM-1 and Orai-1 silencing were accompanied by a change in CREB activation as evidenced by an altered phosphorylation of CREB on Ser133 (Funakoshi et al., 2002, Liu et al., 2013b, Shaywitz and Greenberg, 1999). As shown in figure 6, siRNA-induced reduction in either STIM-1 (Fig.6A and Fig.6E) or Orai-1 (Fig. 6B and Fig.6F) expression also suppressed the phosphorylation of CREB in response to Ang-II, suggesting that STIM-1/Orai-1-mediated SOCE is required to trigger Ang-II-induced CREB phosphorylation in VSMC.

Activation of ERK1/2 and CREB is required to regulate Ang-II-induced Egr-1 expression

ERK1/2 has been shown to mediate the phosphorylation of CREB in response to Ang-II, (Cui et al., 2016, Funakoshi et al., 2002, Molnar et al., 2014) and CREB was demonstrated to mediate LPA-induced increase in Egr-1 expression (Cui et al., 2006). Therefore, to clarify the sequence of the molecular events regulating Ang-II-induced Egr-1 expression, it was of interest to investigate the effect of ERK1/2 blockade on Ang-II-mediated CREB phosphorylation and to assess the consequences of CREB depletion on Ang-II-induced Egr-1 expression. Therefore, cells were pretreated with the MEK/ERK1/2 inhibitor U0126 prior to stimulation with Ang-II. As shown in fig.7A, Ang-II induced a potent increase in CREB phosphorylation on Ser133 (Fig.7A) which was almost completely attenuated by U0126. Similarly, Ang-II-induced Egr-1 expression was also suppressed by U0126 (Fig.7B). To further substantiate the involvement of CREB in Ang-II-induced Egr-1 expression we examined the effect of siRNA- induced depletion of CREB on Egr-1 expression. As shown in figure 7 C, CREB depletion resulted in almost total inhibition of Ang-II-induced Egr-1 expression in VSMC. Altogether, these data reveal for the first time that ERK1/2/CREB pathway is a downstream effector of SOCE-mediated signaling leading to Egr-1 induction in VSMC stimulated with Ang-II.

Discussion

In this study, we have demonstrated a key role of Ca^{2+}-dependent signaling pathways in Ang-II-induced expression of Egr-1 in VSMC. Our data reveal that pharmacological blockade of IP_3R by 2-APB reduced Ang-II-induced increase in $[Ca^{2+}]_i$ levels and Egr-1 expression in VSMC. In addition, our data showing that siRNA-induced silencing of either STIM-1 or Orai-1, key components of SOCE complex, resulted in a significant reduction in Ang-II-induced Egr-1 expression, indicate that a functional SOCE plays a critical role in triggering the signaling cascade leading to Egr-1 expression. Furthermore, by using pharmacological blockers of calmodulin and CaMKII, we have provided evidence that SOCE triggered by STIM-1/Orai-1 activation signals Egr-1 expression via

CaM/CaMKII-dependent downstream pathways in VSMC. Moreover, we demonstrated that STIM-1/Orai-1 plays a key role in triggering Ang-II-induced activation of MEK/ERK1/2- and CREB-dependent signaling in VSMC.

Upregulated levels of IP_3 and IP_3R have been observed in VSMC and mesenteric arteries of hypertensive rats as compared to normotensive rats and correlated with heightened vascular reactivity in hypertension (Abou-Saleh et al., 2013). Studies have also reported an attenuation of VSMC proliferation following blockade of IP_3R (Wilkerson et al., 2006) suggesting the participation of IP_3R-mediated events in hypertension and vascular resistance. Our data obtained using 2-APB suggests that Egr-1 induction may be among the molecular events that underlie the importance of IP_3R in increased agonist-mediated vascular resistance in hypertension. 2-APB has been largely considered as a direct SOCE inhibitor (Peppiatt et al., 2003) and pharmacological inhibition of SOCE using SKF 96465 was recently shown to exert positive effects on blood pressure reduction, Ang-II-induced $[Ca^{2+}]_i$ release and LPA-induced VSMC proliferation (Xu et al., 2015). Thus, our data demonstrating that 2-APB decreases Ang-II-induced Egr-1 expression suggest that Egr-1 downregulation may be one of the mechanism by which SOCE blockers exert their vasculoprotective effects.

Although earlier studies have reported that Ca^{2+} plays an important role in Egr-1 expression, the data presented here are the first to report an involvement of STIM-1/Orai-1 in enhancing the transcription of Egr-1 in response to Ang-II. A critical role of STIM-1/Orai-1-mediated SOCE in Ang-II-induced proliferation of VSMC has been reported (Guo et al., 2012). These authors also reported that siRNA-induced silencing of either STIM-1 or Orai-1 reduced Ang-II-induced neointimal growth and intimal thickening in balloon-injured carotid arteries (Guo et al., 2012). Interestingly, Egr-1 expression is enhanced in balloon-injured carotid arteries (Ohtani et al., 2004); thus, it is possible that the reduction of intimal thickening by silencing STIM-1/Orai-1 noted by Guo *et al*. may be occurring via a decrease in Egr-1 expression (Guo et al., 2012).

An exaggerated expression of STIM-1 and Orai-1 has been observed in the aorta isolated from stroke-prone spontaneously hypertensive rats (Giachini et al., 2009) and Ang-II has been reported to induce the expression of both STIM-1 and Orai-1 in carotid artery neointimal VSMC (Guo et al., 2012). Importantly, the knockdown of STIM-1 or Orai-1 reduced neointimal formation, and was also associated with a decreased VSMC proliferation and migration (Bisaillon et al., 2010). Considering that a similar reduction of Egr-1 either by antisense oligonucleotides or DNAzymes results in attenuation of neointimal growth (Bhindi et al., 2006, Chen et al., 2009, Ohtani et al., 2004), it may be suggested that modulation of Egr-1 expression by STIM-1/Orai-1-induced SOCE plays a key role in vascular damage. Our studies showing that pharmacological blockade of CaM/CaMKII pathways by using W-7 and KN-93 resulted in a significant reduction in Ang-II-induced Egr-1 implicated CaM/CaMKII as a downstream effector of SOCE-induced Ca^{2+} signals in VSMC. CaMKII has been demonstrated to participate in the proliferation of VSMC in response to GPCR ligands and contributes to neointimal growth in animal models of vascular injury (Li et al., 2010, Giachini et al., 2011). Notably, knock down of Orai-1, while reducing neointima formation, was also found to decrease the enhanced levels of CaMKII (Zhang et al., 2011) reinforcing the involvement of CaMKII as an effector of SOCE pathway.

Earlier work has demonstrated that MAP kinase signaling plays a key role in inducing Egr-1 expression in a wide variety of cell types in response to a large number of stimuli (Liu et al., 2013a, Stefano et al., 2007, Youreva and Srivastava, 2016). However, our data demonstrating that siRNA-induced silencing of either STIM-1 or Orai-1 resulted in the suppression of ERK1/2 as well as CREB phosphorylation indicated that Ang-II-induced SOCE is crucial to induce ERK and CREB phosphorylation in VSMC. A role of Ca^{2+} and CaM pathway in signaling ERK1/2 phosphorylation has been reported earlier, however, the data presented here provide the first evidence supporting a key role of SOCE in Ang-II-induced signaling pathways and reinforce the involvement of ERK1/2 signaling in Egr-1 expression in VSMC (Figure 8). Moreover, ERK1/2 has been demonstrated to control CREB

activation via the mitogen and stress-activated kinase that directly phosphorylates the serine 133 motif on CREB. Our data showing that siRNA-induced silencing of CREB attenuated Ang-II –stimulated Egr-1 expression strengthens the notion that ERK-dependent CREB activation plays a key role in provoking Ang-II-induced signaling events leading to Egr-1 expression. In summary, we have demonstrated that STIM-1/Orai-1- induced SOCE, through ERK1/2/CREB–dependent signaling pathways participates in the expression of Egr-1 in response to Ang-II in VSMC.

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Disclosures

The authors of this manuscript do not have any conflict of interest to disclose.

Figure legends

Figure 1: Ang-II induces Egr-1 synthesis and accumulation in A-10 VSMC

Quiescent A-10 cells were stimulated with increasing concentrations of Ang-II for one hour (**A**) or with 100 nM Ang-II for the indicated time periods (**B)**. Cell lysates were immunoblotted with Egr-1 antibody (top panels in **A** and **B**) or β-tubulin (middle panels). Bar diagrams in each section represent average data quantified by densitometric scanning of immunoblots. The control is defined as 1 and each value aside is expressed as fold increase compared to the control values. *p<0.05, **p<0.01, ***p<0.001 versus control values. **C**) Quiescent A-10 cells were incubated with 100 nM Ang-II for the indicated time periods. Analysis of relative Egr-1 mRNA levels was performed by qRT-PCR. Relative level of Egr-1 mRNA is measured as fold variation compared to the control and normalized with β-actin level taken as a standard. ** p<0.01 versus control values. **D**) Cells were treated with 100 nM Ang-II for the indicated time periods, fixed and stained with anti-Egr-1 antibody (green signal). Nuclei were stained with DAPI (blue signal). Merged pictures show the DAPI-stained image superimposed on the Egr-1-stained image.

Figure 2: Attenuation of Ang-II-induced Egr-1 upregulation by 2-APB in A-10 VSMC

A) Quiescent A-10 cells were pre-treated with increasing concentrations of 2-APB for 30 minutes, followed by stimulation with 100 nM Ang-II for one hour. Cell lysates were probed with Egr-1 antibody (top panel) and β-tubulin (middle panel). Bar diagrams represent the densitometric quantifications of Egr-1. Values are expressed as fold increase compared to the control value (CTL) defined as 1. *p<0.05 versus control values; $^{\#}$p<0.05 versus VSMC treated with Ang-II alone. **B**) Quiescent A-10 cells were treated without (CTL) or with 2-APB (50 μM) for 30 minutes followed by stimulation with Ang-II for one hour. Analysis of relative Egr-1 mRNA levels was performed by qRT-PCR. Relative level of Egr-1 mRNA is measured as fold variation compared to the control and normalized with β-actin level taken as a standard. ***p<0.001 versus control values. $^{\#}$p<0.001 versus VSMC treated with Ang-II alone. **C**) Cells were treated without (CTL) or with 2-APB (50 μM) for 30 minutes followed by stimulation with Ang-II for one hour. Cells were fixed and stained with anti-Egr-1 antibody (green signal). Nuclei were stained with DAPI (blue signal). Merged pictures show the DAPI-stained image superimposed on the Egr-1-stained image.

Figure 3: Attenuation of Ang-II-induced $[Ca^{2+}]_i$ mobilization by 2-APB in A-10 VSMC

Quiescent VSMC were labeled with Fura 2-AM prior to treatments and imaging was conducted by alternating excitation wavelengths to excite Ca^{2+}-bound Fura 2 (340 nm) and Ca^{2+}-free Fura 2 (380 nm). The corresponding emissions were recorded and analyzed for the Fura 2 ratio (F_{340}/F_{380}). Graph in **A** represents the average measures of intracellular ratios F_{340}/F_{380} obatined after stimulation with either 100 nM Ang-II alone (Black) or 50 µM 2-APB (Blue), prior to Ang-II stimulation. The bar diagrams in **B** correspond to measurements from at least 10 cells from selected regions. *** p<0.001.

Figure 4: Knockdown of STIM-1 or Orai-1 inhibited Ang-II-induced Egr-1 expression in A-10 VSMC.

Cells were transfected with 10 nM STIM-1 siRNA (siSTIM-1), 10 nM Orai-1 siRNA (siORAI-1) or 10 nM control siRNA (siSCR) prior to stimulation with 100 nM Ang-II for one hour. Cell lysates were immunoblotted with STIM-1 (top panel in **A**), Orai-1 (top panel in **B**), Egr-1 (middle panels in **A** and **B**) or β-tubulin. Bar diagrams in **C**, **D**, **E** and **F** represent average data quantified by densitometric scanning of immunoblots. Values are expressed as fold increase compared to the control value (CTL) defined as 1. *p<0.05, **p<0.01, ***p<0.001 versus CTL. #p<0.05, ###p<0.001 versus siSCR+Ang-II,

Figure 5: Pharmacological blockade of calmodulin and CaMKII inhibited Ang-II-induced Egr-1 expression in A-10 VSMC

Quiescent A-10 cells were pre-treated with or without 10 µM of the calmodulin inhibitor W-7 (**A**) or the CaMKII inhibitor KN-93 (**B**), as well as its inactive analog KN-92 for 30 minutes, followed by stimulation with 100 nM Ang-II for one hour. Cell lysates were probed with Egr-1 antibody (top panels in **A** and **B**) or β-tubulin (middle panels). Bar diagrams in each section represent average data quantified by densitometric scanning of immunoblots. The control is defined as 1 and each value aside is expressed as fold increase compared to the control value defined as 1. *p<0.05, ***p<0.001 versus CTL values. #p<0.05, ###p<0.001 versus VSMC treated with Ang-II alone.

Figure 6: STIM-1 and Orai-1 are required for Ang-II–induced activation of ERK1/2 and CREB in A-10 VSMC

Cells were transfected with 10 nM STIM-1 siRNA (siSTIM-1), 10 nM Orai-1 (siORAI-1) or 10 nM control siRNA (siSCR) prior to stimulation with 100 nM Ang-II for five minutes. **A** and **B** show immunoblotting of cell lysates with antibodies corresponding to ERK1/2 and CREB respectively

phosphorylated on Thr202/Tyr204 and Ser133. Blots were also analyzed for total ERK1/2 and β-tubulin. Bar diagrams in **C**, **D**, **E** and **F** represent average data quantified by densitometric scanning of phospho-ERK1/2 and phospho-CREB immunoblots. Values are expressed as fold increase compared to the control value (CTL) defined as 1. *p<0.05, **p<0.01, ***p<0.001 versus CTL. #p<0.05, ##p<0.01, ###p<0.001, versus siSCR+Ang-II.

Figure 7: Activation of ERK1/2 and CREB is required to regulate Ang-II-induced Egr-1 expression in A-10 VSMC

A) Quiescent A-10 cells were pre-treated with or without U0126 (10 μM) followed by stimulation with 100 nM Ang-II for five minutes. Cell lysates were immunoblotted with an antibody corresponding to CREB phosphorylated on Ser133 (top panel). The blots were also analyzed for total CREB (middle panel). **B)** Quiescent A-10 cells were pre-treated with or without U0126 (10 μM) followed by stimulation with 100 nM Ang-II for one hour. Cell lysates were immunoblotted with Egr-1 (top panel) or β-tubulin (middle panel). Bar diagrams represent average data quantified by densitometric scanning of immunoblots.Values are expressed as fold increase compared to the control value (CTL) defined as 1. **p<0.01, ***p<0.001, versus CTL values. ##p<0.01, ###p<0.001, versus sample with Ang-II alone. **C**) Cells were transfected with 10 nM CREB siRNA (siCREB) prior to stimulation with 100 nM Ang-II for one hour. Cell lysates were immunoblotted with Egr-1, total CREB or β-tubulin. Bar diagrams represent average data quantified by densitometric scanning of Egr-1 immunoblots. Values are expressed as fold increase compared to the control value (CTL) defined as 1. **p<0.01 versus CTL. ##p<0.01 versus siSCR+Ang-II.

Figure 8: Schematic model of the involvement of STIM-1/Orai-1 and Ca^{2+} signaling in Ang-II-induced Egr-1 expression in A-10 VSMC

Ang-II binds to its G-protein coupled receptor, activates the Gq subunit and increases the intracellular levels of inositol-1,4,5-trisphosphate (IP_3) through a phospholipase C-β(PLC-β)-dependent hydrolysis of phosphatidylinositol-,4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and IP3. IP3 binds to its ligand-activated receptor (IP3R) located within the endoplasmic reticulum (ER) membrane and triggers Ca^{2+} efflux inside the cytosol. Resulting ER Ca^{2+} depletion activates the stromal interaction molecule 1 (STIM1) known to mediate Ca^{2+} entry via a change in conformation that results in its accumulation near the plasma membrane, where it can activate Orai-1 calcium channels. Calcium diffusion from the

extracellular compartment through Orai-1 channels results in heightened intracellular Ca^{2+} that binds to calmodulin (CaM) and interacts with CaMKII further leading to ERK1/2 and CREB activation and subsequent induction of Egr-1.

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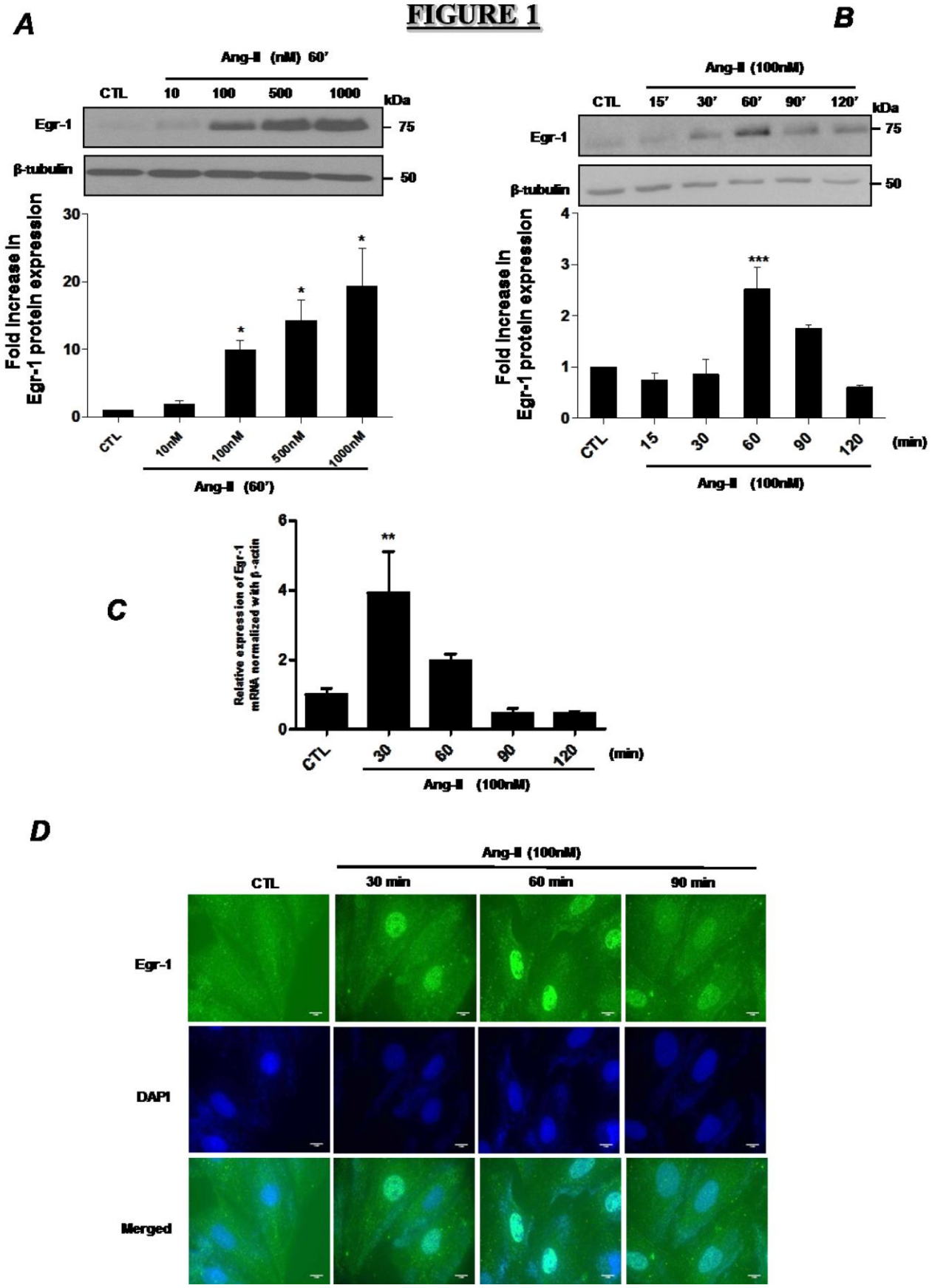
FIGURE 1



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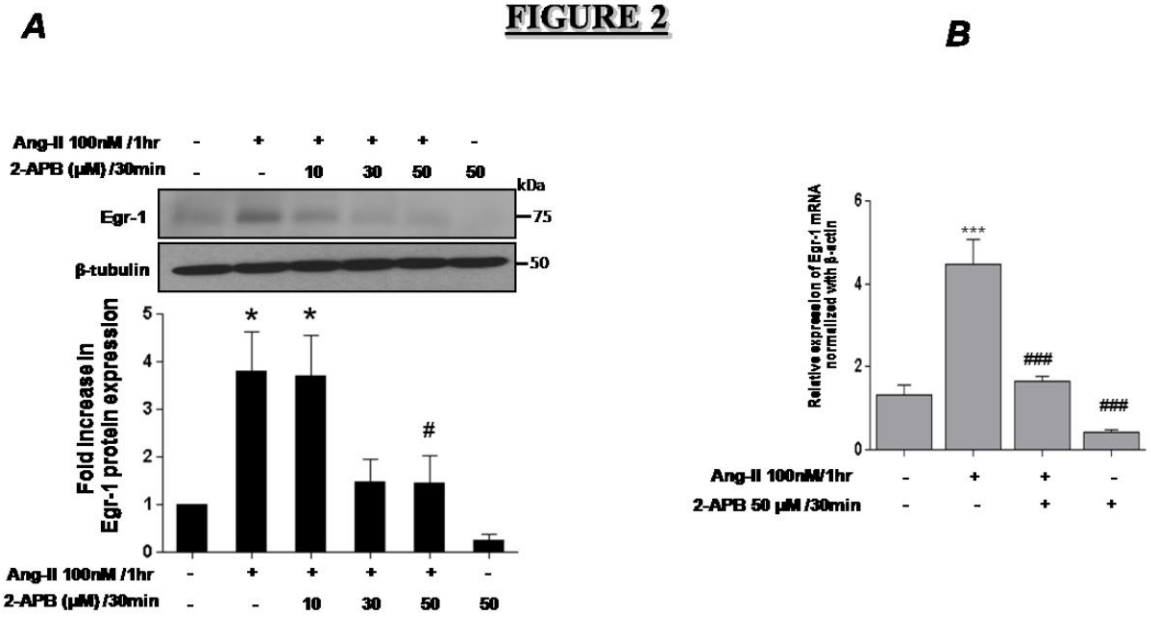

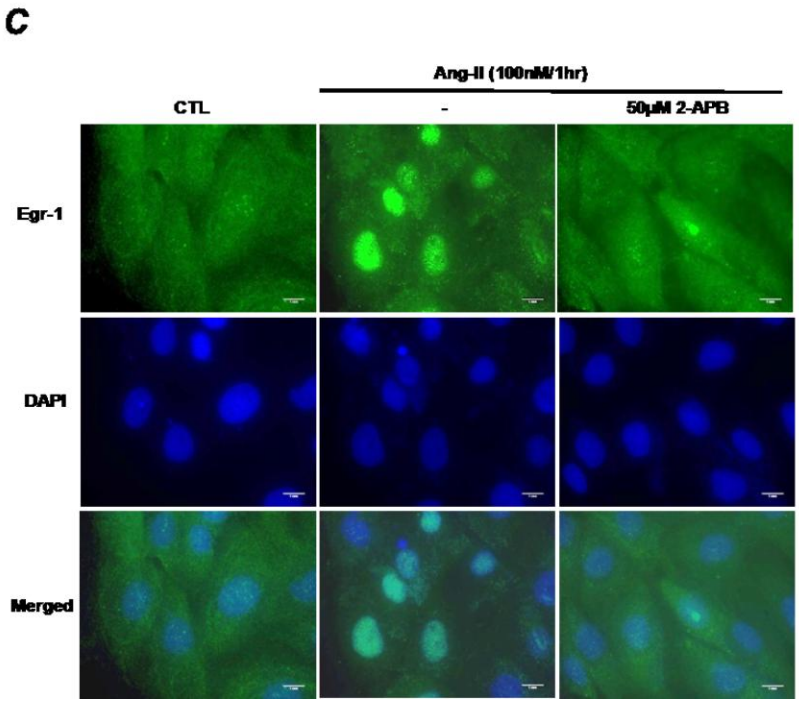

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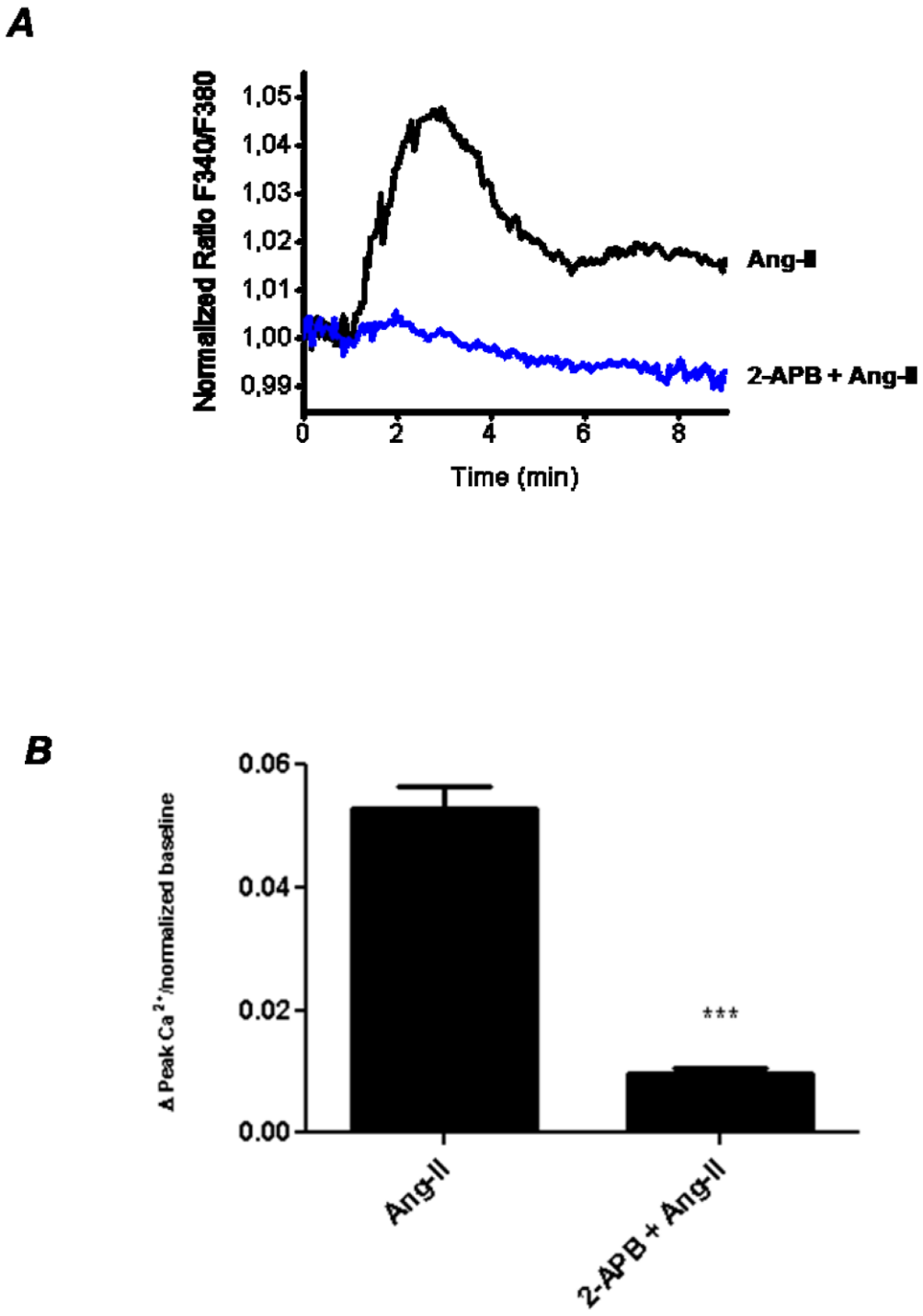

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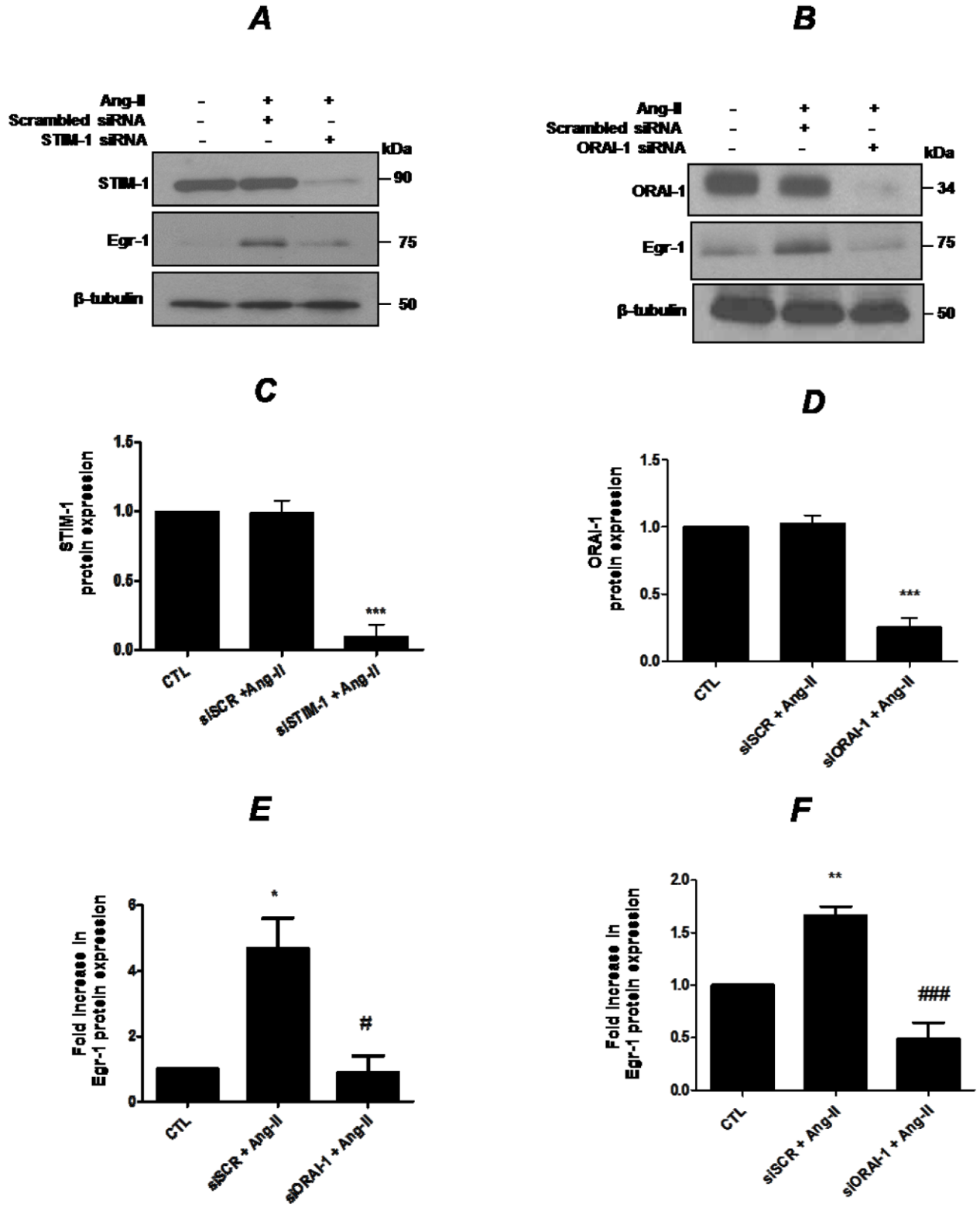

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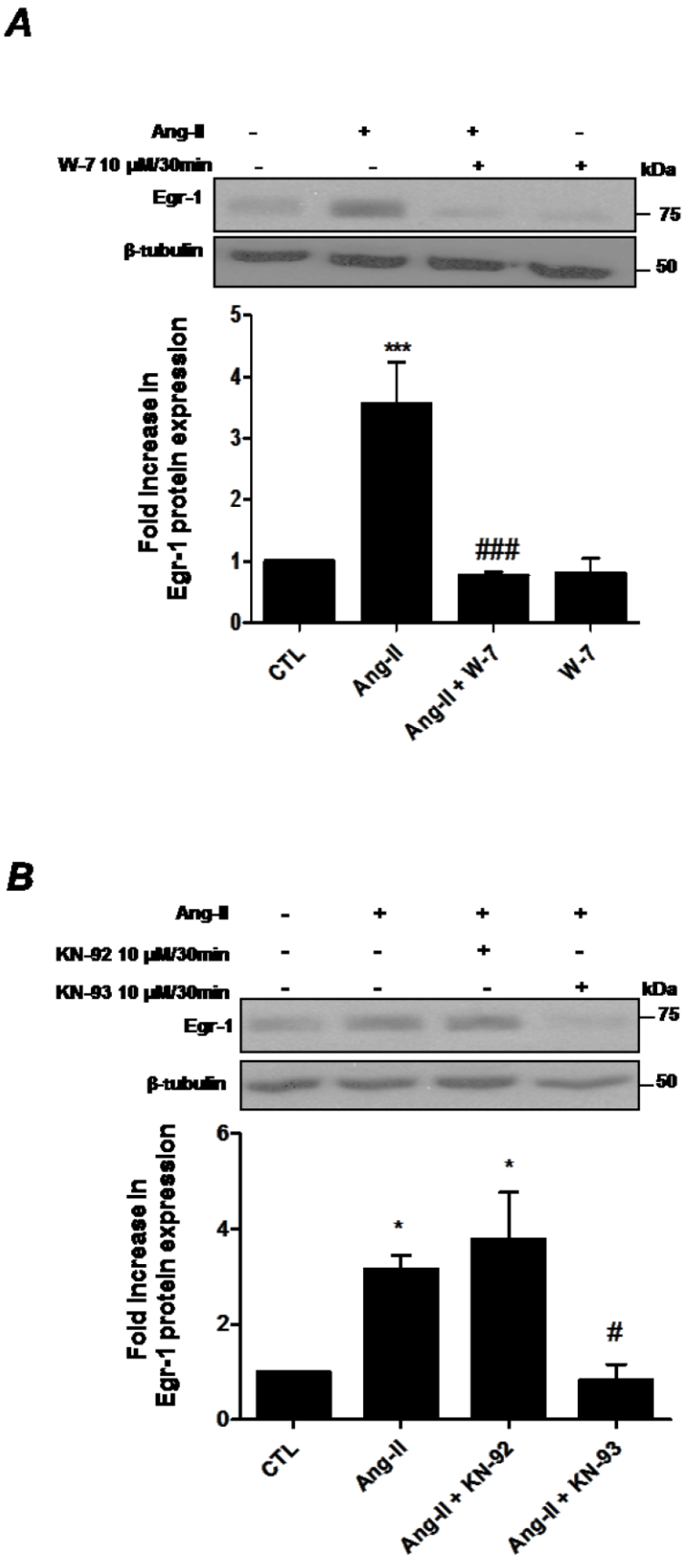

FIGURE 6

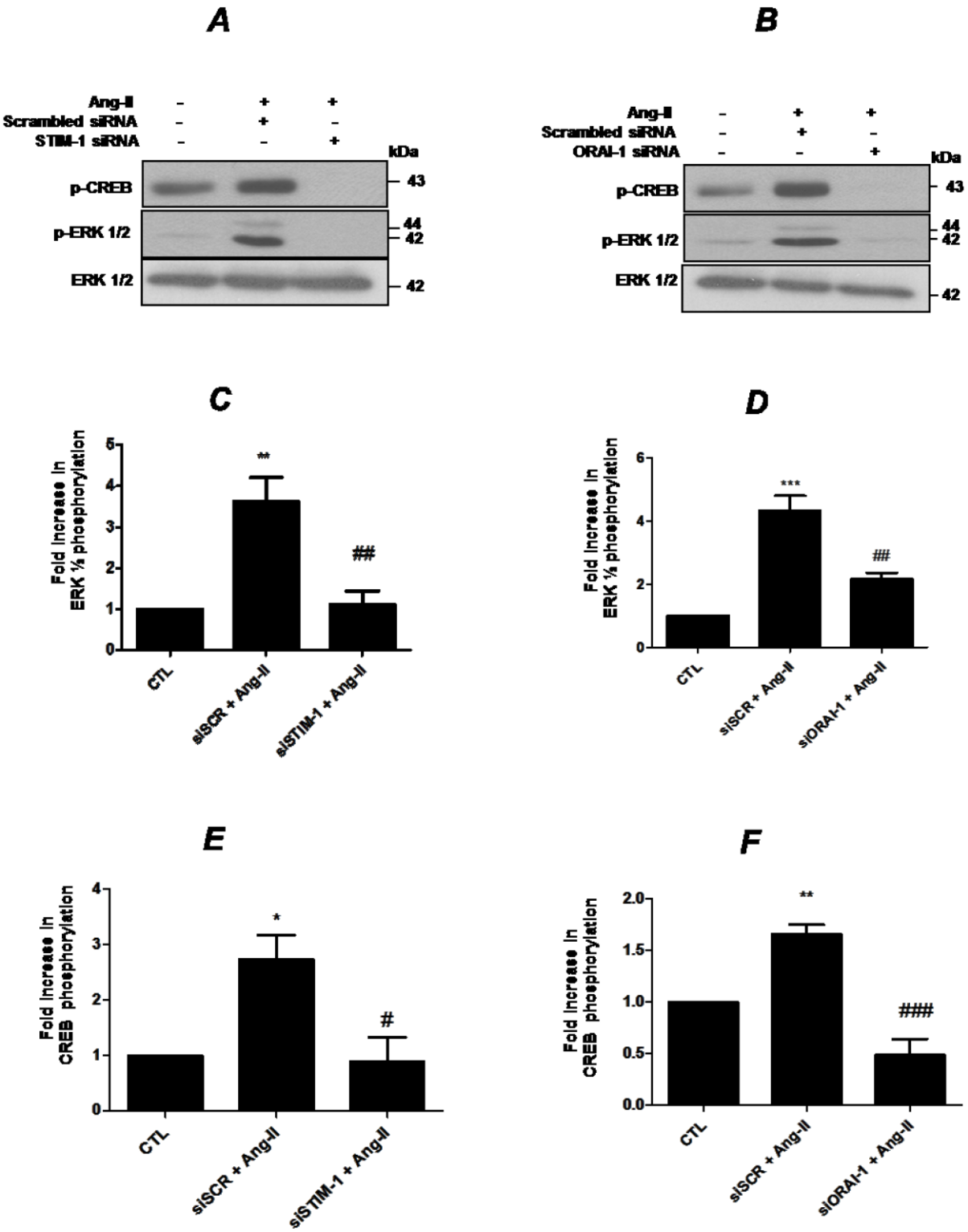

FIGURE 7

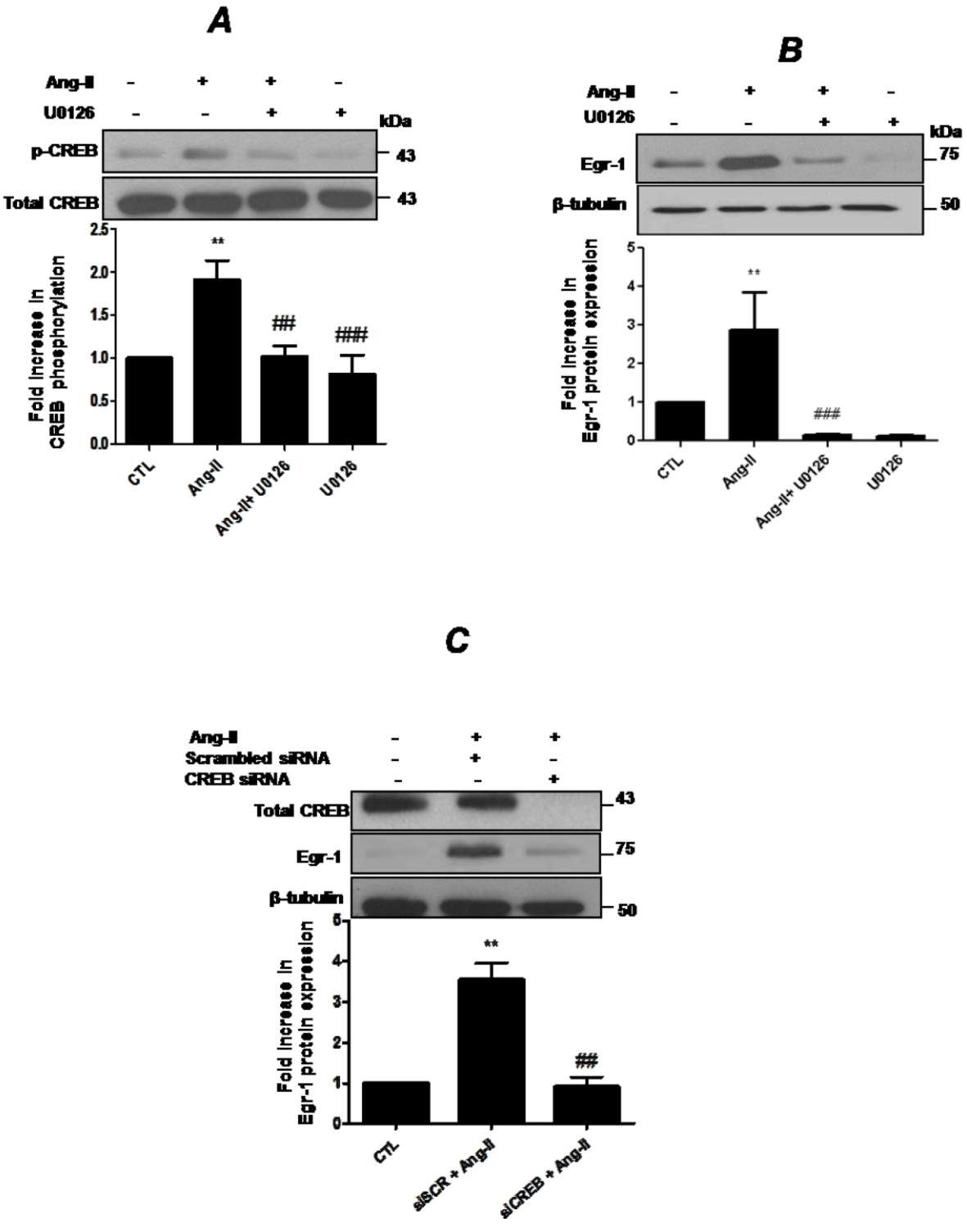

FIGURE 8