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Early Growth Response Protein-1 Expression by Insulin-Like Growth Factor-1 Requires ROS-Dependent Activation of ERK1/2 and PKB Pathways in Vascular Smooth Muscle Cells

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ABSTRACT

Early growth response protein-1 (Egr-1) is a transcription factor that plays an important role in the regulation of several genes implicated in the pathogenesis of cardiovascular disease (CVD) such as atherosclerosis. Insulin-like growth factor-1 (IGF-1), a potent mitogen, is believed to contribute to the development of CVD through the hyperactivation of mitogenic and growth promoting pathways, including the MAPK and PKB pathways, as well as regulation of multiple transcription factors. Reactive oxygen species (ROS) have been shown to mediate the effects of IGF-1 and are believed to contribute to the pathogenesis of vascular abnormalities. We have previously shown that IGF-1 induces the expression of Egr-1 in vascular smooth muscle cells (VSMC); however, the signaling pathways involved in this process remain unexplored. Therefore, we have investigated the involvement of MAPK, PKB, and ROS in IGF-1-induced Egr-1 expression in VSMC. Treatment of VSMC with IGF-1 enhanced Egr-1 protein levels in a time and dose-dependent fashion and PD98059 and SP600125, two selective inhibitors of ERK1/2 and JNK, respectively, significantly decreased IGF-1-induced increase in Egr-1 expression in these cells. In addition, blockade of PI3-K/PKB pathways by Wortmannin/SC-66 respectively, also attenuated IGF-1-induced Egr-1 protein as well as mRNA expression. Diphenyleneiodonium (DPI), an NAD(P)H oxidase inhibitor, blocked the Egr-1 expression in response to IGF-1. In summary, these data demonstrate that ROS-dependent activation of ERK1/2/JNK, PI3-K/PKB signaling events play a critical role in IGF-1 induced expression of Egr-1 in VSMC. J. Cell. Biochem. 117: 152–162, 2016.

KEY WORDS: Egr-1; IGF-1; IGF-1R; ERK1/2; JNK; PKB; PI3-K; VSMC

Aberrant proliferation, migration, and growth of vascular smooth muscle cells (VSMC) are believed to be key events in vascular remodelling and contribute to the pathogenesis of vascular abnormalities such as atherosclerosis, hypertension, and intimal thickening [Jackson and Schwartz, 1992; Vardatsikos and Srivastava, 2011]. The involvement of growth factors and vasoactive peptides such as endothelin-1 (ET-1) and angiotensin-II (Ang-II) in these processes has been well documented in the past decades. We have previously shown an involvement of insulin-like growth factor-1 receptor (IGF-1R) transactivation in triggering ET-1 and Ang-II-induced mitogenic and proliferative responses in VSMC [Bouallegue et al., 2009; Bouallegue et al., 2010]. Insulin-like growth factor-1 (IGF-1), a potent mitogen and vasoactive factor, is known to induce structural alterations in the vessel wall by its ability to trigger proliferation, migration, hypertrophy, and apoptosis of VSMC [Jackson and Schwartz, 1992; Bayes-Genis et al., 2000; Hsieh et al., 2003; Higashi et al., 2012]. These cellular responses are induced through the activation of IGF-1R [Delafontaine et al., 2004] that is highly expressed in VSMC [Delafontaine et al., 2004; Vardatsikos et al., 2009]. The signaling pathways induced by IGF-1R activation include mitogen-activated protein kinases (MAPK) and phosphatidylinositol 3-kinase/protein kinase B (PI3-K/PKB) [Bayes-Genis et al., 2000; Vardatsikos et al., 2009; Jia et al., 2010; Jia et al., 2011] that lead to the regulation of the expression and the activity of multiple transcriptional factors and genes involved in vascular remodelling [Wu et al., 2010]. The contribution of IGF-1R signaling in vascular pathologies has been suggested and is supported by studies showing a significantly higher level of IGF-

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1R concomitantly with increased signaling in VSMC isolated from the aorta of hypertensive animal models [Gomez Sandoval and Anand-Srivastava, 2011; Liu et al., 2011; Youreva et al., 2013). In addition, dominant-negative or antisense oligonucleotides (ASO) of IGF-1R were also reported to attenuate neointimal thickening in injured carotid arteries [Lim et al., 2004].

Early growth response protein-1 (Egr-1) is a zinc finger transcription factor that is activated in response to a large number of stimuli, including growth factors, vasoactive peptides, shear stress, and hypoxia [Chen et al., 2009; Wu et al., 2010; Pagel and Deindl, 2011; Kapakos et al., 2012]. Egr-1 regulates the transcription of several genes that have been attributed in the pathogenesis of vascular disease. It has been implicated in the development of atherosclerotic lesions and plays critical roles in regulating neointimal thickening in response to vascular injury [Blaschke et al., 2004; Harja et al., 2004; Lowe et al., 2001]. Egr-1 expression is highly upregulated in atherosclerotic vessels as compared to normal vessels [McCaffrey et al., 2000]. In injured arteries, Egr-1 expression has also been shown to be rapidly and transiently increased in both endothelial cells and VSMC [Khachigian et al., 1996]. Moreover, studies have demonstrated that reduction of Egr-1 expression or function by ASO, DNAzymes, or siRNA inhibit proliferation of VSMC and attenuate neointima formation in rat carotid arteries as well as in vein graft, suggesting its critical role in vessel damage [Fahmy and Khachigian, 2007; Chen et al., 2009; Han and Liu, 2010; Wu et al., 2010; Pagel et al., 2012]. We have recently demonstrated that Egr-1 expression is upregulated in response to IGF-1 in VSMC [Youreva et al., 2013]. However, the signaling pathways implicated in IGF-1-induced Egr-1 expression in VSMC remain poorly characterized. In this study, we have investigated the involvement of MAPK and PI3-K/PKB signaling pathway, as well as reactive oxygen species (ROS) generation in transducing the effect of IGF-1 on Egr-1 expression in VSMC.

MATERIALS AND METHODS

ANTIBODIES AND REAGENTS

IGF-1 was obtained from Peprotech Inc and cell culture reagents were from Invitrogen Corp. (Grand Island, New York). Actinomycin D and Cycloheximide were procured by Sigma-Aldrich (Oakville, Ontario). AG1024 (IGF-1R inhibitor), PD98059 (MEK inhibitor), SP600125 (JNK inhibitor), Wortmannin (PI3-K inhibitor), SC-66 (AKT inhibitor XVIII) and DPI (NAD(P)H oxidase inhibitor) were purchased from Calbiochem (San Diego, California). Phospho-Ser^{473}-specific-PKB, anti-PKB, phospho-Thr^{183}/Tyr^{185}-specific-JNK, anti-JNK, Egr-1, β-tubulin and anti-rabbit antibodies were from Cell Signaling (Beverly, Massachusetts). Phospho-Thr^{202}/Tyr^{204}-specific-ERK1/2, anti-ERK1/2 and horseradish peroxidase-conjugated goat anti-mouse immunoglobulin antibodies were from Santa Cruz Biotechnology (Santa Cruz, California). Alexa Fluor 488 goat anti-rabbit IgG was obtained from Life Technologies, Molecular Probes (Grand Island, New York). The enhanced chemiluminescence (ECL) detection system kit was procured from Perkin Elmer (Montreal, Quebec).

CELL CULTURE

A-10 VSMC derived from thoracic aorta of embryonic rat were maintained in culture with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, in a humidified atmosphere of 5% CO_2 exchange at 37°C. They were sub-cultured twice a week by harvesting them with trypsin/ethylenediaminetetraacetic acid (EDTA). The cells were grown to 80–90% confluence in 60-mm culture plates and made quiescent by incubating in FBS-free DMEM 5 h prior to the experimental treatment.

CELL LYSIS AND IMMUNOBLOTTING

Confluent, serum-starved quiescent A-10 VSMC were pre-treated in the presence of various agents for 30 min, followed by stimulation with 6.5 nmol/L IGF-1 for 1 h. Then cells were washed twice with ice-cold PBS and lysed in 200 μl of lysis buffer (25 nmol/L Tris-HCl, pH 7.5. 25 mmol/L NaCl, 1 mmol/L Na orthovanadate, 10 mmol/L Na fluoride, 10 mmol/L Na pyrophosphate, 2 mmol/L benzamidine, 2 mmol/L ethylene glycol tetraacetic acid (EGTA), 2 mmol/L ethylenediaminetetraacetic acid (EDTA), 1 mmol/L phenylmethylsulphonyl fluoride (PMSF), 10 μg/ml aprotinin, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS) and 0.5 μg/ml leupeptin) on ice. The cell lysates were clarified by centrifugation at 12000g for 10 min at 4°C. Protein concentrations were determined by Bradford assay. Equal amounts of protein were subjected to either 7.5% or 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidinedifluoride (PVDF) membranes, purchased from Millipore (Etobicoke, Ontario) and incubated overnight at 4°C with respective primary antibodies Egr-1 (1:1000), phospho-Thr^{202}/Tyr^{204}-specific-ERK1/2 (1:4000), phospho-Ser^{473}-specific-PKB (1:2000) and phospho-Thr^{183}/Tyr^{185}-specific-JNK (1:2000). The antigen-antibody complex was detected by horseradish peroxidase-conjugated secondary antibody (1:4000), and protein bands were visualized by ECL. The same blots were subsequently reprobed with corresponding total antibodies of β-tubulin (1:8000), ERK1/2 (1:4000), PKB (1:4000) and JNK (1:4000) to detect the total amount of these proteins. The intensity of the bands was quantified by densitometric analysis using Quantity One Bio-Rad Corp. imaging and Graphpad Prism 5 (San Diego, California) software programs.

PREPARATION OF THE NUCLEAR FRACTION

Cells incubated in the absence or presence of various agents were washed twice with ice-cold PBS and lysed in 200μl of lysis buffer solution containing 10 mmol/L Hepes, 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L PMSF, 1 mmol/L protease cocktail inhibitor and 1 mmol/L Na orthovanadate. The cells lysates were vortexed for 10 s at highest setting after adding NP40 10% detergent and were centrifuged at 18327g for 4 min at 4°C. The pellet was resuspended in 200μl of buffer containing 10 mmol/L Hepes, 400 mmol/L NaCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L PMSF, 1 mmol/L protease cocktail inhibitor and 1 mmol/L Na orthovanadate. The suspension was sonicated for 6 cycles at 10 s per cycle with 30 s intervals between each cycle and then centrifuged at 18327*g* for 5 min at 4°C. The pellet was discarded and the supernatant, which corresponds to the nuclear

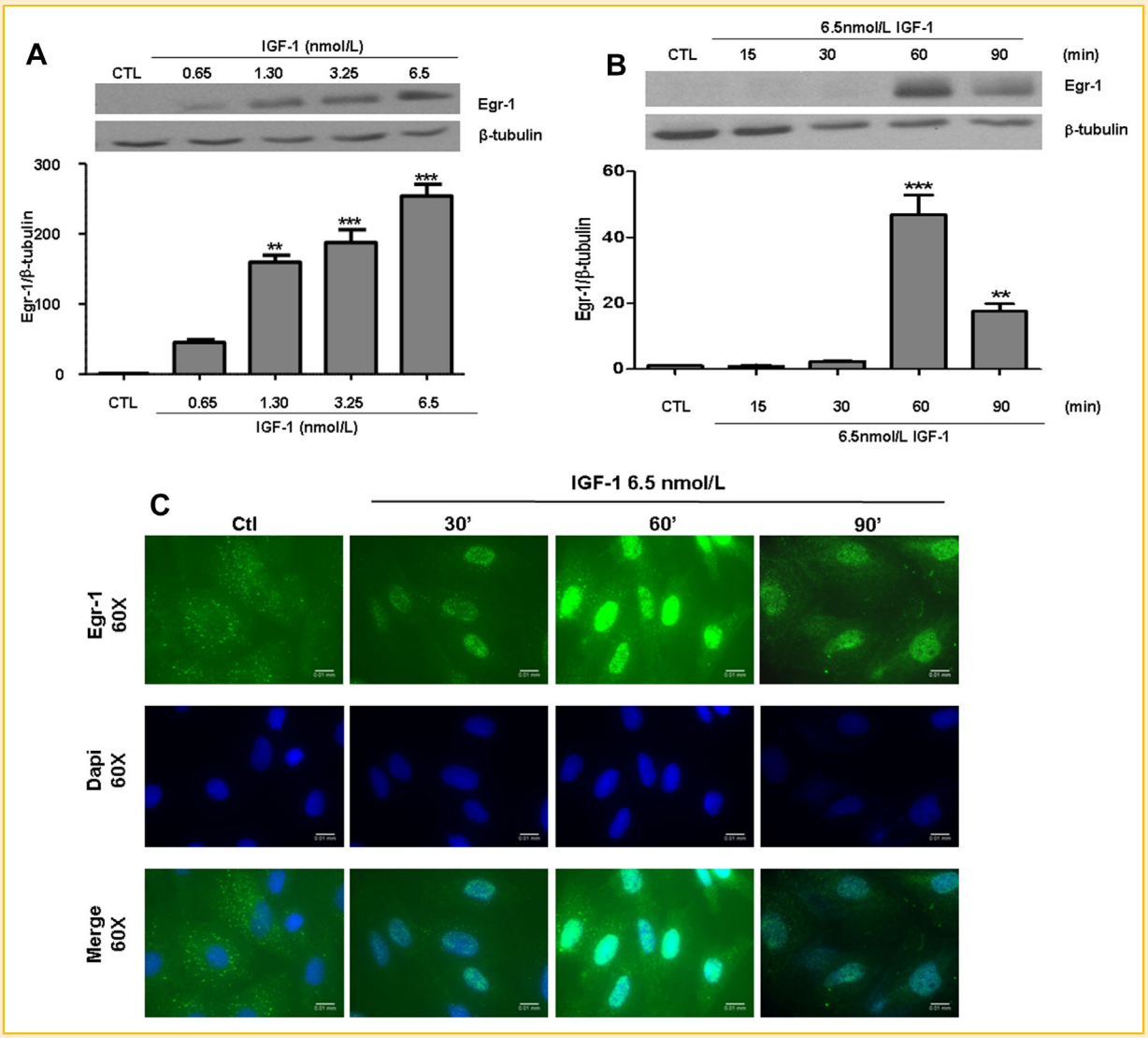

Fig. 1. Insulin-like growth factor-1 (IGF-1) enhances early growth response protein-1 (Egr-1) expression in A-10 vascular smooth muscle cells (VSMC) in a time and dose-dependent fashion. Confluent, serum-starved quiescent A-10 VSMC were treated in the absence (CTL) or presence of 6.5nmol/L IGF-1 for indicated times (B), or with increasing doses of IGF-1 for 60 min (A). Nuclear fractions of the cell lysates were probed with Egr-1 antibody (top panel) and also analyzed for protein loading, using β-tubulin (middle panel). Bar diagrams (bottom panel) represent the densitometric quantifications of Egr-1 relative to β-tubulin. Values are the mean ± SE of 3 independent experiments and are expressed as a ratio where the control values are taken as 1. $^{**}P < 0.005$ and $^{***}P < 0.0005$, compared to CTL. For immunofluorescence microscopy experiments, confluent serum-starved quiescent A-10 VSMC were plated on glass coverslips and then treated in the absence (CTL) or presence of 6.5nmol/L IGF-1 for indicated time periods (C). Cells were fixed and stained with anti-Egr-1 antibody (green signal). Nuclei were stained with DAPI (4́,6-diamidino-2-phenylindole) (blue signal). Merged pictures show the DAPI-stained image superimposed on the Egr-1-stained image. Shown are representative images from 3 independent experiments. The images were taken using X-Cite series 120, TE2000-S fluorescence microscope.

fraction, was collected. The protein content in the nuclear fraction was determined by Bradford assay and equal amounts of protein were subjected to SDS-PAGE and immunoblotted using Egr-1 antibody.

RNA PREPARATION AND REVERSE TRANSCRIPTASE

Cells were washed twice with ice-cold PBS and total RNA was isolated with Trizol Reagent (Life Technologies, Burlington, ON). RNA concentration was quantified with the Biophotometer at 260 nm and the purity was determined by the A260/A280 ratio (Eppendorf, Mississauga, ON). cDNA was synthesized from 1 μg of total RNA using High Capacity RNA-to cDNA Kit (Applied Biosystems, Grand Island, NY) as per manufacturer's instructions. cDNA was stored at −20 until use.

qRT-PCR ANALYSIS

Real-time quantitative PCR was performed with SYBG (Life Technologies, Grand Island, NY) using 1 μl of cDNA in a final volume of 20 μl. Real-time PCR amplification was performed using 7500 fast RT-PCR system (Applied Biosystems, Grand Island, NY).

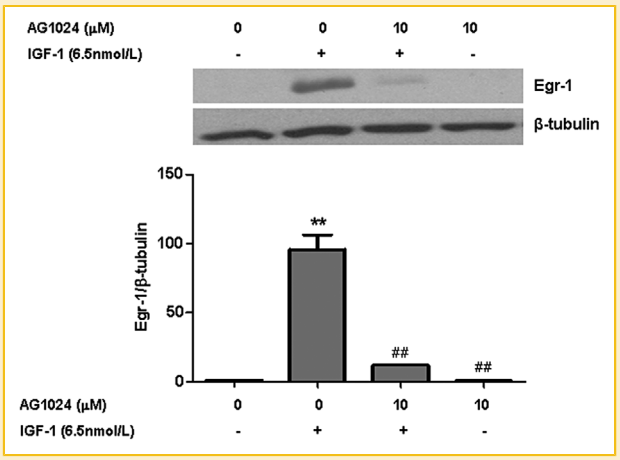

Fig. 2. Attenuation of Insulin-like growth factor-1 (IGF-1)-induced early growth response protein-1 (Egr-1) expression by AG1024 (IGF-1R-PTK inhibitor) in A-10 vascular smooth muscle cells (VSMC) in a dose-dependent fashion. Confluent, serum-starved quiescent A-10 VSMC were pre-treated with (10μM) or without (0) AG1024 for 30 min, followed by stimulation with 6.5nmol/L IGF-1 for 1 h. Nuclear fractions of the cell lysates were probed with Egr-1 antibody (top panel) and also analyzed for protein loading, using β-tubulin (middle panel). Bar diagrams (bottom panel) represent the densitometric quantifications of Egr-1 relative to β-tubulin. Values are the means ± SE of 3 independent experiments and expressed as a ratio where the control values are taken as 1. $^{**}P < 0.005$ compared with the control VSMC; $^{\#\#}P < 0.005$ compared to VSMC treated with IGF-1 alone.

The expression of Egr-1 mRNA was assessed using the following primers: forward 5′-CTGCTTCATCGTCTTCCTCTG-3′ and reverse 5′-GTCAGTGTTGGGAGTAGGAAAG-3′. Egr-1 mRNA expression was normalized with the β-actin mRNA level using primers: forward 5′-TCTTCCAGCCTTCCTTCCT-3′ and reverse 5′-CAGCACTGTGTTGGCATAGA-3′.

IMMUNOFLUORESCENCE

A-10 cells were grown on glass coverslips in 4-well culture plates to 80% confluence and made quiescent by incubating in FBS-free DMEM 5 h prior to the experimental treatment. Cells were pre-treated in the presence or absence of various agents for 30 min, followed by stimulation with 6.5 nmol/L IGF-1 for 1 h. Then cells were fixed with paraformaldehyde 4% for 30 min at 4°C, and permeabilized with 0.1% Triton X-100, 0.1% serum citrate pH 4.0 for 10 min at room temperature (RT). After fixation, slides were washed each for 5 min with PBS. Cells were incubated in blocking buffer (goat serum (15 μl/1ml PBS) for 1 h and incubated with Egr-1 (1:100), overnight at 4C. Cells were further incubated for 2 h at RT with Alexa Fluor 488 goat anti-rabbit IgG (1:150). The nuclei were stained with DAPI (2 μl/1.5 ml H_2O) and one drop of mounting buffer (30% Glycerol in PBS) was applied to each coverslip and mounted on slides. The images were obtained using X-Cite series 120, TE2000-S fluorescence microscope.

STATISTICS

Statistical analysis was performed by one-way, standard analysis of variance (ANOVA) followed by a Tukey post hoc test. All data are reported as means ± SE. The differences between means were considered significant at $P < 0.05$.

RESULTS

IGF-1 INDUCES RAPID INCREASE IN Egr-1 EXPRESSION IN DOSE AND TIME-DEPENDENT FASHION THROUGH THE IGF-1R

Egr-1 is a zinc finger transcription factor that plays a critical role in vascular remodelling and cardiovascular pathobiology [Khachigian,

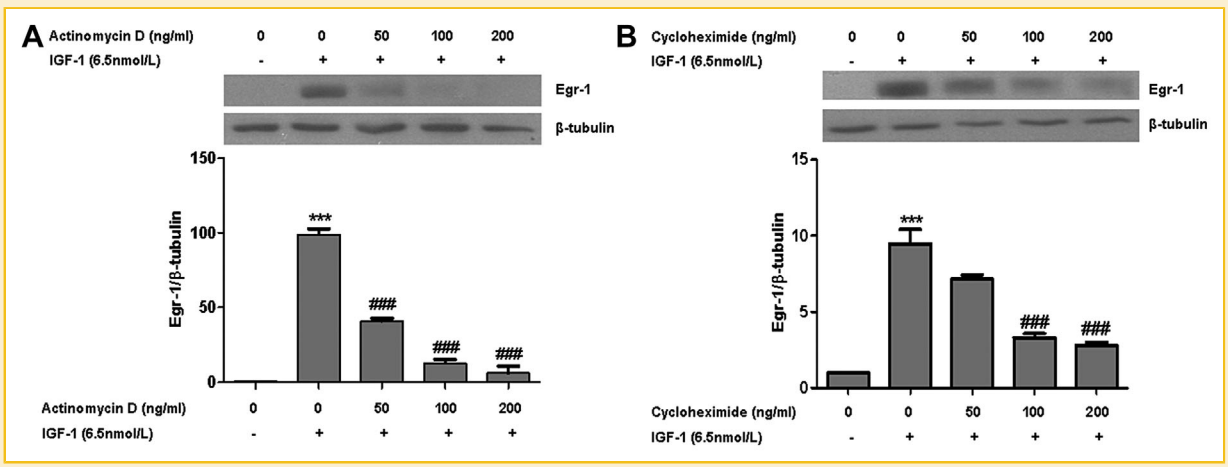

Fig. 3. Attenuation of Insulin-like growth factor-1 (IGF-1)-induced early growth response protein-1 (Egr-1) expression by Actinomycin D (RNA synthesis inhibitor) and Cycloheximide (protein synthesis inhibitor) in A-10 vascular smooth muscle cells (VSMC) in a dose-dependent fashion. Confluent, serum-starved quiescent A-10 VSMC were pre-treated with (50ng/ml, 100ng/ml and 200ng/ml) or without (0) Actinomycin D (mRNA synthesis inhibitor) (A) and Cycloheximide (protein synthesis inhibitor) (B) for 30 min, followed by stimulation with 6.5nmol/L IGF-1 for 1 h. Nuclear fractions of the cell lysates were probed with Egr-1 antibody (top panel) and also analyzed for protein loading, using β-tubulin (middle panel). Bar diagrams (bottom panel) represent the densitometric quantifications of Egr-1 relative to β-tubulin. Values are the means ± SE of 3 independent experiments and expressed as a ratio where the control values are taken as 1. $^{***}P < 0.0005$ compared with the control VSMC; $^{\#\#\#}P < 0.05$ compared to VSMC treated with IGF-1 alone.

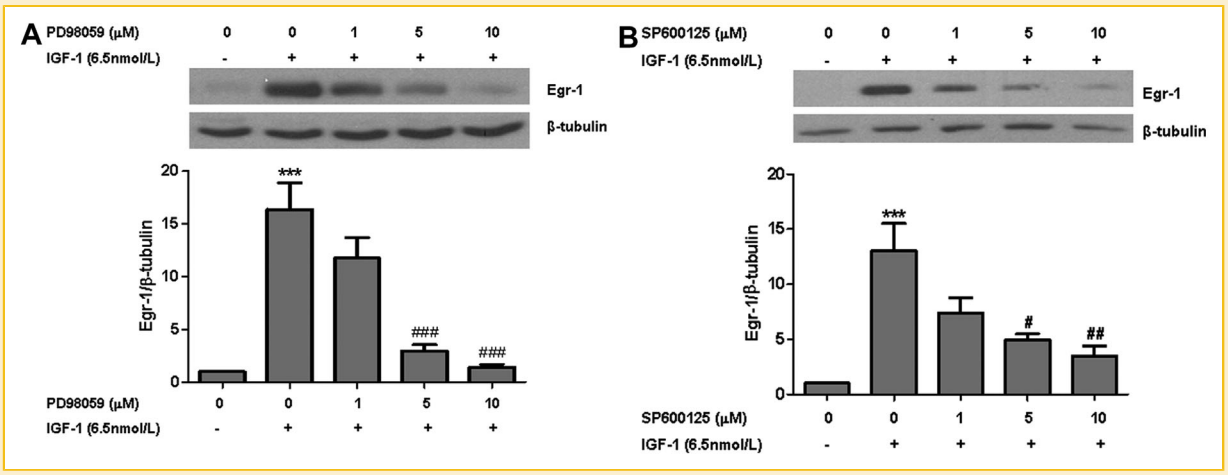

Fig. 4. Attenuation of Insulin-like growth factor-1 (IGF-1)-induced early growth response protein-1 (Egr-1) expression by PD98059 (MEK1/2 inhibitor) and SP600125 (JNK inhibitor) in A-10 vascular smooth muscle cells (VSMC) in a dose-dependent fashion. Confluent, serum-starved quiescent A-10 VSMC were pre-treated with (1 μM, 5 μM and 10 μM) or without (0) PD98059 (A) and SP600125 (B) for 30 min, followed by stimulation with 6.5 nmol/L IGF-1 for 1 h. Nuclear fractions of the cell lysates were probed with Egr-1 antibody (top panel) and also analyzed for protein loading, using β-tubulin (middle panel). Bar diagrams (bottom panel) represent the densitometric quantifications of Egr-1 relative to β-tubulin. Values are the means ± SE of 3 independent experiments and expressed as a ratio where the control values are taken as 1. $^{***}P < 0.0005$ compared with the control VSMC; $^{\#}P < 0.05$ compared to VSMC treated with IGF-1 alone.

2006] and IGF-1 is a potent mitogen for VSMC [Clemmons, 2007; Meng et al., 2007; Meng et al., 2008; Vardatsikos et al., 2009]. However, the effect of IGF-1 on Egr-1 expression in VSMC has not been characterized in details; therefore, we investigated the response of IGF-1 on Egr-1 expression in A-10 aortic VSMC. As shown in Figure 1A, treatment of VSMC with IGF-1 induced the expression of Egr-1 in a dose-dependent manner and a significant increase in Egr-1 level was detected at 0.65 nmol/L IGF-1, the lowest dose tested. Further increasing the IGF-1 concentration led to a more robust increase in Egr-1 expression and at 6.5 nmol/L, there was about 25-fold increase in Egr-1 expression compared to control cells. Moreover, IGF-1-induced expression of Egr-1 was time-dependent; Egr-1 expression could be detected only after the stimulation of cells for 60 min which was significantly reduced after the treatment for 90 min (Fig. 1B). In parallel, we also performed immunofluorescence localization of Egr-1 in cells stimulated with IGF-1 with various time points (Fig. 1C). Similar to the immunoblotting data, there was an increased nuclear localization of Egr-1 at 60 min which declined to the basal level at 90 min. In order to confirm the involvement of IGF-1R in IGF-1-induced increase in Egr-1 expression, we used AG1024, a highly specific inhibitor that blocks IGF-1R tyrosine kinase activity. As depicted in Figure 2, a marked inhibition of Egr-1 expression was observed in cells treated with 10μM AG1024 compared to VSMC treated with IGF-1 alone. These results demonstrate that tyrosine kinase activity of IGF-1R is essential to trigger Egr-1 expression in VSMC.

IGF-1-INDUCED Egr-1 EXPRESSION INVOLVES BOTH RNA AND PROTEIN SYNTHESIS

Growth factors modulate protein expression by acting either on the transcriptional process leading to changes in mRNA synthesis or at translational level leading to protein synthesis. In order to test whether the upregulation of Egr-1 in response to IGF-1 is the result of enhanced transcription or translation we used Actinomycin D and Cycloheximide. Actinomycin D inhibits mRNA transcription by forming a stable complex with double stranded DNA via deoxyguanosine residues thereby preventing the elongation of RNA chains [Sobell, 1985]. Cycloheximide inhibits protein synthesis by blocking translation of mRNA on cytosolic 80S ribosomes [Quinton and Kramarcy, 1977]. As shown in Figure 3A, treatment of VSMC with Actinomycin D, prior to stimulation with IGF-1, resulted in a dose-dependent inhibition of Egr-1 expression. Similarly, Cycloheximide (Fig. 3B), also markedly attenuated IGF-1-induced Egr-1 expression in VSMC by about 60% at 100 ng/ml; however it appeared to be slightly less potent than Actinomycin D in inhibiting IGF-1-induced Egr-1 expression.

IGF-1-INDUCED Egr-1 EXPRESSION OCCURS VIA MAPK PATHWAYS

Since IGF-1 has been shown to activate MAPK signaling pathways [Bayes-Genis et al., 2000; Vardatsikos et al., 2009] and because MAPK pathways are known to regulate Egr-1 expression in different cell types of non-vascular origin [Hodge et al., 1998; Tsai et al., 2001], we hypothesized that IGF-1 upregulates Egr-1 expression through MAPK signaling cascades. To confirm this and in order to identify which of these MAPK are implicated in IGF-1-induced Egr-1 expression, PD98059 and SP600125 two specific pharmacological inhibitors of mitogen-extracellular-signal regulated kinase 1/2 (ERK1/2) kinase (MEK) and c-Jun N-terminal kinase (JNK) pathways, respectively were used. As shown in Figure 4A, Egr-1 expression was strongly enhanced by IGF-1, and PD98059 treatment dose-dependently attenuated this effect, with a marked attenuation of about 80% observed at 5 μM and almost complete inhibition of Egr-1 expression was detected at 10 μM. Using immunofluorescence analysis, the amount of Egr-1 in the nucleus was also reduced in cells pre-treated with PD98059 10μM (Fig. 5C). In addition, pre-treatment of A-10 VSMC with SP600125 prior to stimulation with IGF-1 also

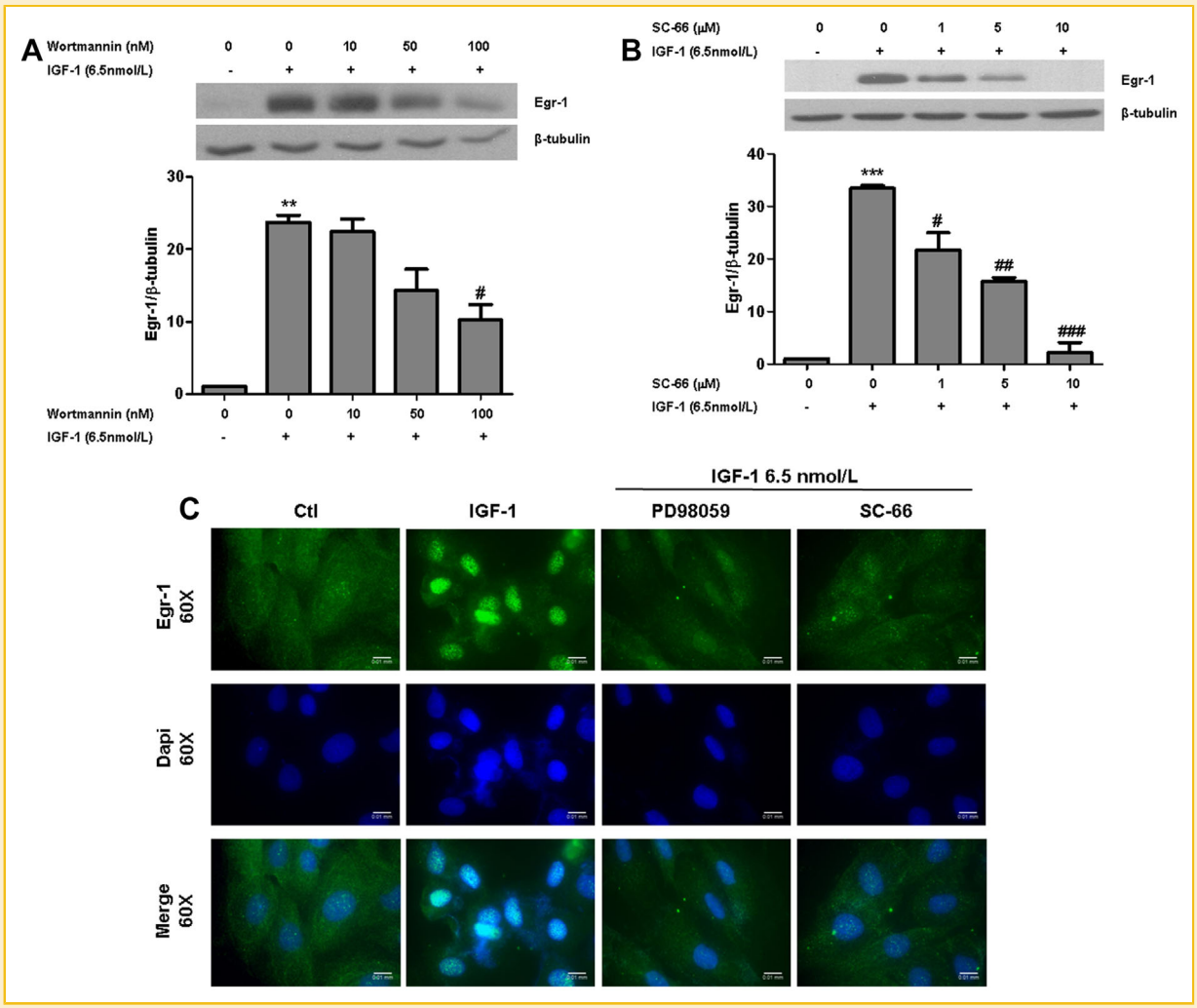

Fig. 5. Attenuation of Insulin-like growth factor-1 (IGF-1)-induced early growth response protein-1 (Egr-1) expression by Wortmannin (PI3-K inhibitor) and SC-66 (PKB inhibitor) in A-10 vascular smooth muscle cells (VSMC) in a dose-dependent fashion. Confluent, serum-starved quiescent A-10 VSMC were pre-treated with either Wortmannin (10 nM, 50 nM and 100 nM) (A) or SC-66 (1 μM, 5 μM and 10μM) (B) for 30 min, followed by stimulation with 6.5 nmol/L IGF-1 for 1 h. Nuclear fractions of the cell lysates were probed with Egr-1 antibody (top panel) and also analyzed for protein loading, using β-tubulin (middle panel). Bar diagrams (bottom panel) represent the densitometric quantifications of Egr-1 relative to β-tubulin. Values are the means ± SE of 3 independent experiments and expressed as a ratio where the control values are taken as 1. $^{**}P < 0.005$ compared to CTL; $^{\#}P < 0.05$ compared to VSMC treated with IGF-1 alone. For immunofluorescence assays, confluent, serum-starved quiescent A-10 VSMC were plated on glass coverslips and then treated with or without PD98059 (10 μM) or SC-66 (10 μM) for 30 min followed by stimulation with IGF-1 for 1 h (C). Cells were fixed and stained with anti-Egr-1 antibody (green signal). Nuclei were stained with DAPI (4́,6-diamidino-2-phenylindole) (blue signal). Merged pictures show the DAPI-stained image superimposed on the Egr-1-stained image. Shown are representative images from 3 independent experiments. The images were taken using X-Cite series 120, TE2000-S fluorescence microscope.

dose-dependently decreased Egr-1 expression (Fig. 4B). This indicated the involvement of both MEK/ERK and JNK pathways in IGF-1-induced Egr-1 expression in VSMC.

ACTIVATION OF PI3-K/PKB PATHWAYS IS REQUIRED FOR IGF-1-INDUCED Egr-1 EXPRESSION

The implication of PI3-K/PKB pathways in triggering some signaling responses of IGF-1 has been well documented [Radhakrishnan et al., 2008; Jia et al., 2010]. In addition, PKB pathway is known to modulate the protein synthesis and activity of the transcriptional factors through its multiple downstream targets, including p70S6K and elF4E in multiple cell types [Georgakis and Younes, 2006]. However, involvement of the PKB in mediating IGF-1-induced Egr-1 expression in VSMC has not been examined. Therefore, to investigate whether PI3-K/PKB pathway participates in IGF-1-induced Egr-1 expression, we used Wortmannin (PI3-K inhibitor) [Wipf and Halter, 2005] and SC-66 (PKB inhibitor) [Jo et al., 2011]. As shown in Figure 5A, 100 nM of Wortmannin caused a significant reduction in Egr-1 expression induced by IGF-1. Consistent with the results shown in Figure 5A, pre-treatment of A10 VSMC with SC-66 also dose-dependently inhibited IGF-1-induced Egr-1 expression. In fact, a significant inhibitory effect of SC-66 could be detected at 1 μM, representing 40% of inhibition; however, higher concentrations of this antagonist exerted a more robust inhibitory effect

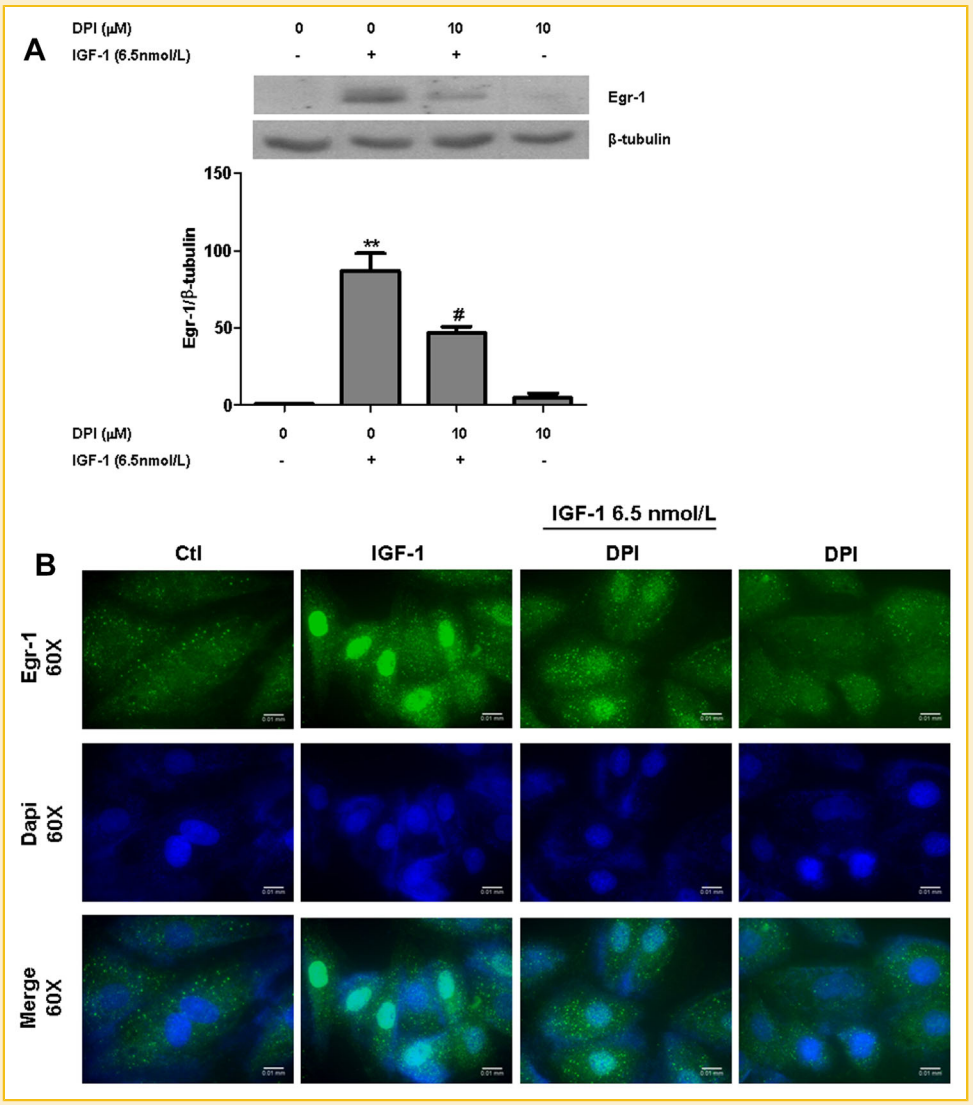

Fig. 6. Attenuation of Insulin-like growth factor-1 (IGF-1)-induced early growth response protein-1 (Egr-1) expression by DPI (NADPH oxidase inhibitor) in A-10 vascular smooth muscle cells (VSMC) in a dose-dependent fashion. Confluent, serum-starved quiescent A-10 VSMC were pre-treated with DPI (10μM) for 30 min, followed by stimulation with 6.5nmol/L IGF-1 for 1 h (A). Nuclear fractions of the cell lysates were probed with Egr-1 antibody (top panel) and also analyzed for protein loading, using β-tubulin (middle panel). Bar diagrams (bottom panel) represent the densitometric quantifications of Egr-1 relative to β-tubulin. For immunofluorescence assays, confluent, serum-starved quiescent A-10 VSMC were plated on glass coverslips and then treated with DPI (10 μM) for 30 min followed by stimulation with IGF-1 for 1 h (B). Cells were fixed and stained with anti-Egr-1 antibody (green signal). Nuclei were stained with DAPI (4́,6-diamidino-2-phenylindole) (blue signal). Merged pictures show the DAPI-stained image superimposed on the Egr-1-stained image. Shown are representative images from 3 independent experiments. The images were taken using X-Cite series 120, TE2000-S fluorescence microscope Values are the means ± SE of 3 independent experiments and expressed as a ratio where the control values are taken as 1. $^{**}P < 0.05$ compared with the control VSMC; $^{\#}P < 0.05$ compared to VSMC treated with IGF-1 alone.

and almost completely attenuated the Egr-1 expression induced by IGF-1 (Fig. 5B). Similarly, immunofluorescence microscopy showed that the blockade of PKB by SC-66 inhibited IGF-1-induced nuclear localization of Egr-1 (Fig. 5C). These data demonstrate that PI3-K/PKB pathways are critical upstream regulators of IGF-1-induced Egr-1 expression in VSMC.

ACTIVATION OF ROS PATHWAYS IS REQUIRED FOR IGF-1-INDUCED SIGNALING AND Egr-1 EXPRESSION IN A-10 VSMC

ROS are believed to contribute to the pathogenesis of vascular abnormalities and IGF-1 has been reported to mediate many of their responses through the activation of NAD(P)H oxidase derived generation of ROS [Meng et al., 2008; Xi et al., 2013]. However, a role of ROS in IGF-1-induced Egr-1 expression in VSMC remains unexplored. Therefore by using diphenyleneiodonium (DPI), a NAD(P)H oxidase inhibitor, we investigated whether ROS are involved in Egr-1 expression in response to IGF-1. As depicted in Figure 6A, pre-treatment of VSMC with DPI markedly reduced IGF-1-induced Egr-1 expression in VSMC. Similarly, immunofluorescence analysis showed a significant attenuation of Egr-1 nuclear localisation in cells pre-treated with DPI 10 μM (Fig. 6B). Interestingly, DPI treatment of VSMC also resulted in the attenuation of IGF-1

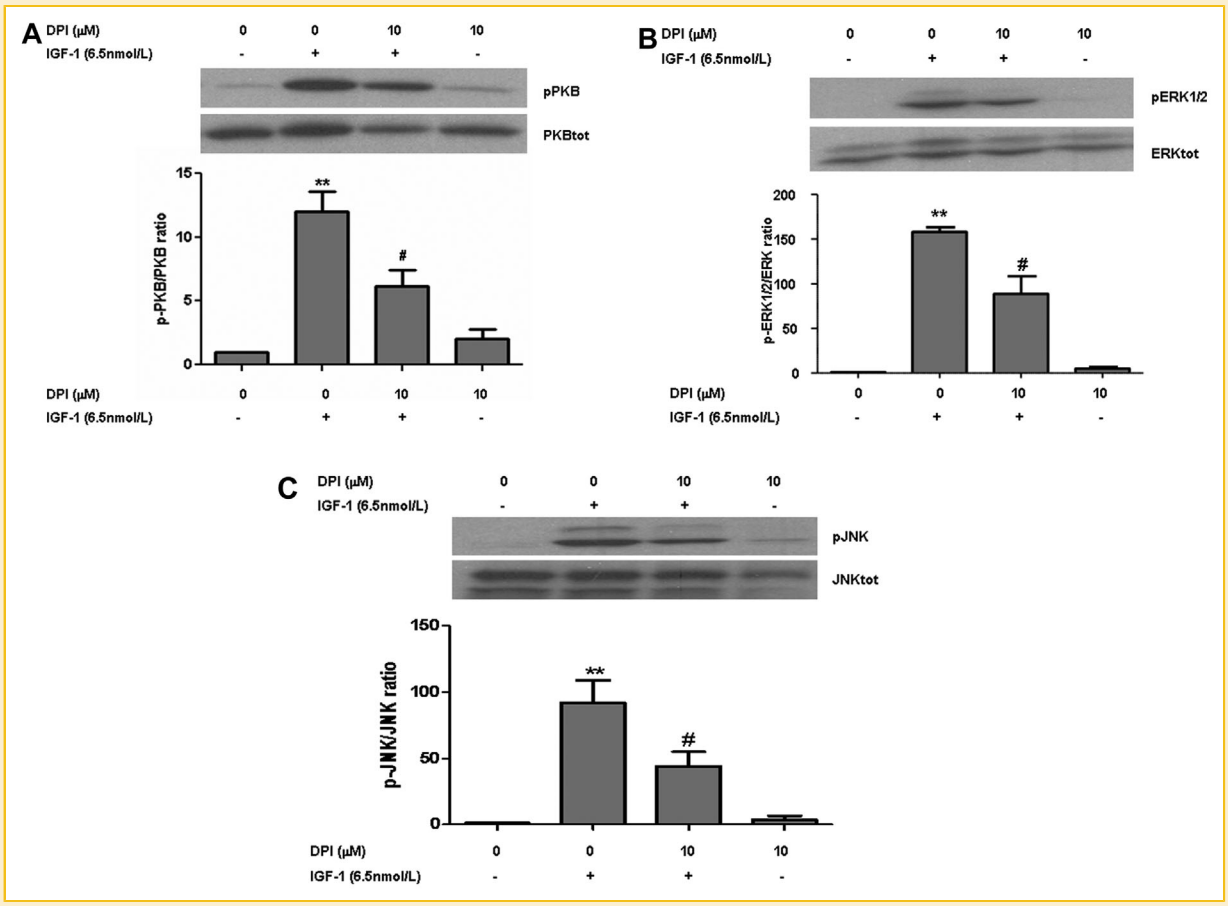

Fig. 7. Effect of the DPI (NAD(P)H oxidase inhibitor) on Insulin-like growth factor-1 (IGF-1)-induced PKB, ERK1/2 and JNK phosphorylation in A-10 vascular smooth muscle cells (VSMC). Confluent, serum-starved quiescent A-10 VSMC were pre-treated with DPI (10 μM) for 30 min, followed by stimulation with 6.5nmol/L IGF-1 for 15 min. Cell lysates were probed with phospho-specific-Ser^{473}-PKB antibody (A), phospho-specific Thr^{202}/Tyr^{204}-ERK1/2 antibody (B) or phospho-specific Thr^{183}/Tyr^{185}-JNK antibody (C), (top panel). Blots were also analyzed for total PKB, ERK1/2 or JNK (middle panel). Bar diagrams (bottom panel) represent the quantifications of phosphorylated p-PKB relative to total PKB, phosphorylated p-ERK1/2 relative to total ERK or phosphorylated p-JNK relative to total JNK. Values are the means ± SE of 3 independent experiments and expressed as a ratio where the control values are taken as 1. $^{**}P < 0.005$ compared with the control VSMC; $^{\#}P < 0.5$ compared to VSMC treated with IGF-1 alone.

induced phosphorylation of PKB (Fig. 7A), ERK1/2 (Fig. 7B), and JNK (Fig. 7C) in VSMC. These data demonstrate the involvement of ROS generation in IGF-induced signaling, as well as in IGF-1-induced Egr-1 expression in A-10 VSMC.

IGF-1-INDUCED Egr-1 mRNA EXPRESSION IN A-10 VSMC

Since we have demonstrated that IGF-1 induces a rapid increase in Egr-1 protein expression, we wanted to examine whether this change correlates with changes in the levels of Egr-1 mRNA. As shown in Figure 8, SYBR Green qRT-PCR analysis demonstrated a significant induction of Egr-1 mRNA following 1 h incubation with IGF-1. We next evaluated the effects of ERK1/2, JNK, PI3-K, and PKB inhibitors on IGF-1-induced Egr-1 mRNA level. Consistent with the data on Egr-1 protein expression, pre-treatment of VSMC with PD98059, SP600125, Wortmannin, and SC-66 respective inhibitors of these pathways, markedly attenuated IGF-1 inducible expression of Egr-1 mRNA in these cells (Fig. 8).

DISCUSSION

In the present work, we have identified the intracellular signaling pathways involved in IGF-1-induced Egr-1 expression. By using PD98059 and SP600125, specific pharmacological inhibitors of ERK1/2 and JNK respectively, we demonstrate the involvement of the MAPK in IGF-1-induced Egr-1 expression in VSMC. We show that inhibition of any of these MAPK is sufficient to turn-off the stimulatory effect of IGF-1 on Egr-1 expression in A-10 VSMC. A similar role of ERK1/2 and JNK pathways in inducing hydrogen-peroxide (H_2O_2)-, lipopolysaccharide (LPS)-, and lysophophatidic acid (LPA)-induced Egr-1 expression in cardiomyocytes, human monocytic cells and VSMC respectively, has been reported [Guha et al., 2001; Aggeli et al., 2010; Iyoda et al., 2012]. A role of ERK1/2 pathway in the upregulation of Egr-1 expression by Apelin-13, a ligand for G protein-coupled receptor, as well as by TNF-α, has also been demonstrated in VSMC [Goetze et al., 2001; Liu et al., 2013]. An involvement of ERK/JNK-dependent mechanism in mediating Egr-1

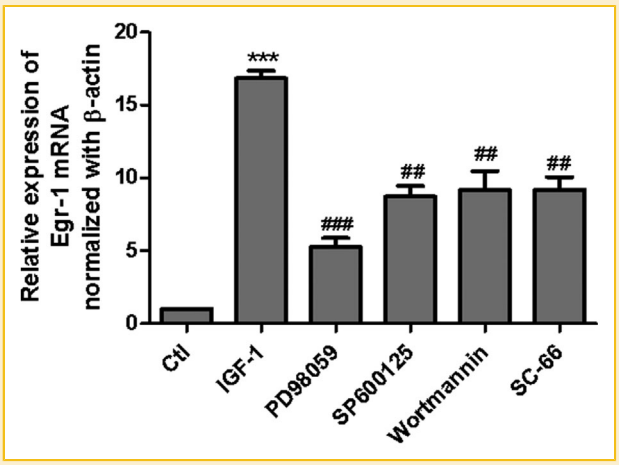

Fig. 8. Effect of Insulin-like growth factor-1 (IGF-1) on early growth response protein-1 (Egr-1) mRNA level in A-10 vascular smooth muscle cells (VSMC). Confluent, serum-starved quiescent A-10 VSMC were pre-treated with either PD98059 (10 μM), SP600125 (10μM), SC-66 (10 μM) or Wortmannin (100 nM) for 30 min, followed by stimulation with 6.5 nmol/L IGF-1 for 1 h. Analysis of relative Egr-1 mRNA levels was performed by using qRT-PCR. Values are the means ± SE of 3 independent experiments. 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. $^{*}P < 0.05$ compared to CTL; $^{\#}P < 0.5$ compared to VSMC treated with IGF-1 alone.

expression in non-vascular cells in response to epidermal growth factor (EGF) [Gregg and Fraizer, 2011] and silica dust has also been reported [Chu et al., 2013]. These findings underscore the importance of MAPK pathways in transducing the signals of various stimuli leading to the enhanced expression of Egr-1.

In addition, our data showing that pharmacological blockade of PI3-K by Wortmannin or PKB by SC-66 almost completely attenuated the enhanced expression of Egr-1 in response to IGF-1 implicates the PI3-K/PKB signaling cascade as an upstream regulator of Egr-1 expression in VSMC. Previous data have shown a role of PI3-K in insulin-induced Egr-1 expression in rat fibroblasts [Harada et al., 2001] and in integrin-induced Egr-1 expression in endothelial cells [Cabodi et al., 2009]. However, to the best of our knowledge, the work presented here is the first to report an involvement of MAPK and PI3-K/PKB signaling events in modulating Egr-1 expression in response to IGF-1 in VSMC.

Our studies showing that pharmacological blockade of NAD(P)H oxidase by DPI significantly attenuated Egr-1 expression in response to IGF-1 in VSMC, support the notion that ROS generation is a key trigger to induce Egr-1 expression in response to IGF-1. The fact that DPI treatment also inhibited the phosphorylation of ERK1/2/JNK and PKB, indicates that ROS act upstream of these signaling molecules in IGF-1-induced pathways leading to Egr-1 expression in VSMC. Consistent with these studies, curcumin, a polyphenolic compound with anti-oxidant properties [Huang et al., 1992; Ak and Gulcin, 2008; Wilken et al., 2011; Hu et al., 2012] has also been shown to attenuate IGF-1-induced signlaing responses and Egr-1 expression in VSMC [Youreva et al., 2013]. A role of ROS generated by NAD(P)H oxidase 4 (NOX4) in inducing the signaling responses as well as the proliferative and migratory responses of IGF-1 in VSMC has been reported earlier [Meng et al., 2007]. In accordance with the present results, previous studies have also shown the involvement of ROS generation in Egr-1 induction in response to xanthine oxidase (XO) in pulmonary artery smooth muscle cells [Hartney et al., 2011]. Hemin has also been reported to induce Egr-1 expression via the ROS/ERK pathways in human aortic VSMC [Hasan and Schafer, 2008]. Taken together, these data demonstrate that ROS generation are required to induce Egr-1 expression in response to IGF-1 in VSMC.

Recent studies have reported a role of c-Jun in shear stress and injury-induced Egr-1 expression in VSMC [Ni et al., 2010]. Since c-Jun is a downstream target of JNK, it may be suggested that IGF-1-induced phosphorylation of JNK contributes to enhanced transcription of Egr-1 via c-Jun in VSMC. Moreover, shear stress and injury-induced enhanced expression c-Jun was also demonstrated to require the activation of ERK1/2 and JNK dependent pathways to stimulate Egr-1 expression [Ni et al., 2010]. Elk-1 is a downstream effector of ERK1/2 [Hodge et al., 1998] and a role of Elk-1 in Hemin-induced expression of Egr-1 has been suggested [Hasan and Schafer, 2008]. It is thus possible that IGF-1 induced ERK1/2 phosphorylation via Elk-1 activation is also responsible for IGF-1-induced Egr-1 expression in VSMC.

Egr-1 is an important transcription factor involved in the pathogenesis of several cardiovascular disorders [Khachigian, 2006; Cheyou et al., 2014]. Its ability to regulate the transcription of several genes linked with cell growth, proliferation and

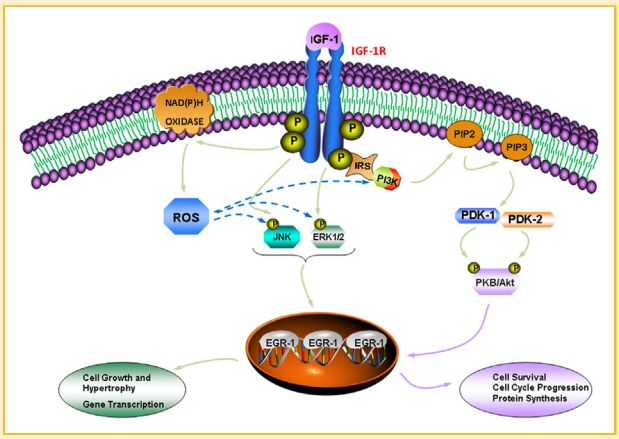

Fig. 9. Schematic model of signaling pathways involved in Insulin-like growth factor-1 (IGF-1)-induced early growth response protein 1 (Egr-1) expression in A-10 vascular smooth muscle cells (VSMC). Activation of IGF-1R stimulates multiple signaling pathways, such as ERK1/2, JNK, as well as PI3-K/PKB. ROS generation via an upregulated NAD(P)H oxidase system serves as a key trigger in this process. ERK1/2 pathway contributes to gene transcription, cell growth and hypertrophy, whereas PI3-K/PKB signaling pathway increases protein synthesis and regulates cell survival and proliferation. Both ERK1/2, JNK and PI3-K/PKB-induced signaling events contribute to the upregulation of Egr-1 by IGF-1. Early growth response protein-1 (Egr-1); Insulin-like growth factor-1(IGF-1); Insulin-like growth factor-1 receptor (IGF-1R); Extracellular regulated kinase 1 and 2 (ERK1/2); c-Jun N-terminal kinase (JNK); Protein kinase B (PKB); Phosphoinositide 3- kinase (PI3-K); Reactive Oxygen Species (ROS); p (phosphorylated).

inflammation has been attributed towards its pathogenic roles [Blaschke et al., 2004]. Some of these genes include cyclin-dependent kinase 1 (Cyclin-D1), proliferating cell nuclear antigen, adhesion molecules such as the intercellular and vascular cell adhesion molecules [McCaffrey et al., 2000; Han and Liu, 2010]. It should be noted that expression of many of these genes is upregulated in atherosclerosis and in animal models restenosis [Khachigian, 2006], and silencing of Egr-1 expression either by ASO or DNAnzymes has been reported to attenuate restenotic lesions in these models, suggesting its critical role in vessel damage [Lowe et al., 2001; Fahmy and Khachigian, 2007; Chen et al., 2009; Han and Liu, 2010; Wu et al., 2010; Pagel et al., 2012].

IGF-1 system has been implicated in vascular pathologies and the levels of IGF-1R or signaling has been reported to be heightened in aorta of hypertensive animal models [Gomez Sandoval and Anand-Srivastava, 2011; Liu et al., 2011). Moreover, dominant negative IGF-1R or ASO of IGF-1R has been shown to inhibit neointimal formation in an injured carotid artery rat model [Lim et al., 2004]. It is thus possible that the ability of IGF-1 to enhance the expression of Egr-1 in VSMC may be one of the mechanisms by which it contributes to vascular remodelling associated with vascular dysfunction.

In summary, our data have demonstrated the involvement of ERK1/2, JNK and PI3-K/PKB signaling pathways as upstream regulators of Egr-1 expression in response to IGF-1. The fact that pharmacological blockade of NAD(P)H oxidase system attenuated the signaling pathways as well as Egr-1 expression suggests a key role of ROS generation in triggering Egr-1 expression in VSMC (Fig. 9). Since MAPK and PI3-K/PKB signaling pathways have been shown to be upregulated in vascular pathologies, and ROS generation contributes to vascular abnormalities, it may be suggested that exaggerated expression of Egr-1 detected in vascular diseases such as atherosclerosis, may be caused by hyperactivation of these signaling cascades.

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