Canadian Journal of Physiology and Pharmacology

Involvement of Akt-dependent CREB signaling pathway in hydrogen peroxide (H₂O₂)-induced early growth response protein-1 (Egr-1) expression in vascular smooth muscle cells

Journal:	Canadian Journal of Physiology and Pharmacology
Manuscript ID	cjpp-2019-0061.R1
Manuscript Type:	Article
Date Submitted by the Author:	07-Mar-2019
Complete List of Authors:	Rondeau, Vincent; CRCHUM Jain, Ashish; CRCHUM Truong, Vanessa; CRCHUM Srivastava, Ashok; Universite de Montreal Faculte de medecine
Is the invited manuscript for consideration in a Special Issue:	2018 IACS Slovakia
Keyword:	ROS, Egr-1, Akt, CREB, VSMC

SCHOLARONE™
Manuscripts

Involvement of Akt-dependent CREB signaling pathway in hydrogen peroxide (H₂O₂)-induced early growth response protein-1 (Egr-1) expression in vascular smooth muscle cells

Vincent Rondeau¹, Ashish Jain¹, Vanessa Truong¹ and Ashok K. Srivastava^{1, 2}

¹Laboratory of Cellular Signaling, Montreal Diabetes Research Center and Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM), 900 Rue St-Denis, Montreal QC H2X 0A9, Canada. ²Department of Medicine, Faculty of Medicine, University of Montreal, C.P. 6128, Succursale Centre-Ville, Montreal, QC H3C 3J7, Canada.

Address for correspondence:

Ashok K. Srivastava, Ph.D

CRCHUM

Tour Viger

900 rue St-Denis,

Montreal, Qc

H2X 0A9

Canada

Tel: 514-890-8000 ext 23604

Fax: 514-412-7648

E-mail: ashok.srivastava@umontreal.ca

Abstract

Increased generation of reactive oxygen species (ROS) is believed to play a key role in the pathophysiology of cardiovascular diseases. Excessive growth and proliferation of vascular smooth muscle cells (VSMC) have been suggested to be a major contributor to vascular dysfunction. A potential involvement of early growth response protein-1 (Egr-1), a zinc-finger transcription factor, in the development of vascular diseases has been suggested. Recent studies have shown that hydrogen peroxide (H_2O_2), a ROS, increases Egr-1 expression in VSMCs, however, signaling events leading to H_2O_2 -induced Egr-1 expression are not fully understood. Therefore, here we aimed to determine signaling pathways implicated in H_2O_2 -induced Egr-1 expression in VSMCs. We show that H_2O_2 enhanced the expression of Egr-1 which was associated with an increased phosphorylation of Akt. Pharmacological blockade of the phosphatidylinositol-3-kinase (PI3K)/Akt pathway by wortmannin or SC66 significantly inhibited the protein and mRNA levels of Egr-1 induced by H_2O_2 . H_2O_2 -induced Egr-1 expression was associated with an increased phosphorylation of cyclic AMP response element binding protein (CREB), and pharmacological inhibition or the silencing of Akt attenuated both H_2O_2 -induced CREB phosphorylation and Egr-1 expression. Moreover, RNA interference-mediated depletion of CREB almost completely abrogated the stimulatory effect of H_2O_2 on Egr-1 expression. In addition, pharmacological blockade or silencing of c-Src resulted in significant suppression of H_2O_2 -induced Egr-1 expression as well as Akt and CREB phosphorylation. These data suggest that H_2O_2 triggers its effects on Egr-1

expression through c-Src-mediated Akt and CREB –dependent signaling events in VSMCs.

Keywords: ROS, Egr-1, Akt, CREB, VSMC,

Introduction

Vascular diseases such as hypertension, atherosclerosis, and restenosis, are characterized by aberrant vessel remodeling promoted by exaggerated proliferative and migratory responses in vascular smooth muscle cells (VSMCs) (Dickinson et al. 2014; Jackson and Schwartz 1992). Accumulated evidence has suggested that heightened levels of growth factors and vasoactive peptides including angiotensin II (Ang-II), endothelin-1 (ET-1) and insulin-like growth factor-1 (IGF-1) stimulate growth-promoting cascades in VSMCs and thus contribute to vascular dysfunction (Bohm and Pernow 2007; Weiss et al. 2001; Youreva and Srivastava 2016). Interestingly, these peptides also promote oxidative stress via their ability to induce NAD(P)H oxidase activation and subsequent generation of reactive oxygen species (ROS) (Brown and Griendling 2015; Daou and Srivastava 2004; Vardatsikos et al. 2009; Youreva and Srivastava 2016). ROS has been suggested to contribute to vessel remodeling by activating the signaling events linked to enhanced cell growth and migration (Brown and Griendling 2015; Griendling and Ushio-Fukai 2000). Hydrogen peroxide (H_2O_2), a relatively more stable form of ROS, has been reported to be elevated in models of hypertension (Gao and Lee 2001). H_2O_2 has also been

linked to hypercontractility and exaggerated growth responses in the vasculature (Clempus and Griendling 2006; Gao and Lee 2001). The signaling pathways activated by H_2O_2 include the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K)/Akt pathway as well as the receptor and non-receptor tyrosine kinases, IGF-1R and c-Src, respectively (Azar et al. 2006; Baas and Berk 1995; Blanc et al. 2004; Byon et al. 2016; Lyle and Griendling 2006; Mehdi et al. 2007). Hyperactivation of these signaling events triggers the induction of several transcription factors and genes linked to vascular remodelling (Clempus and Griendling 2006). Early growth response protein-1 (Egr-1), a zinc finger transcription factor, has emerged as an important regulator of vascular physiology due to its ability to modulate the expression of proinflammatory markers and cell cycle regulatory proteins that contribute to the pathogenesis of vascular proliferative diseases (Cheyou et al. 2014b; Khachigian 2006). Expression of Egr-1 has been shown to be elevated in tissues isolated from animal models of obesity and atherosclerosis as well as in response to vasoactive peptides and growth factors, supporting its role in the development of atherosclerotic lesions and neointimal thickening (Blaschke et al. 2004; Fahmy and Khachigian 2007; Han and Liu 2010; Lowe et al. 2001; McCaffrey et al. 2000). We recently demonstrated that IGF-1 enhances the expression of Egr-1 via a ROS-dependent pathway requiring PI3K/Akt signaling events in VSMCs (Youeva and Srivastava 2016). We have also reported that the cyclic AMP response element binding (CREB), a key transcription factor, is essential to trigger Ang-II-induced Egr-1 expression in VSMCs (Simo-Cheyou et al. 2017).

However, the involvement of Akt and CREB-dependent signaling in H₂O₂-induced Egr-1 expression in VSMCs remains unexplored. Therefore, here we have investigated the role of Akt in mediating the expression of Egr-1 in VSMCs.

Materials and Methods

Materials: H₂O₂ was obtained from Sigma-Aldrich (Saint-Louis, Missouri, USA) and cell culture reagents were procured by Invitrogen Corp. (Grand Island, New York, USA). PP2 (c-Src inhibitor), PP3 (inactive analog of PP2), wortmannin (PI3K inhibitor) and SC66 (Akt inhibitor), were purchased from Calbiochem (San Diego, California, USA). Rabbit monoclonal antibodies were used to detect Ser133 phosphorylated CREB (Cell Signaling Technology Cat# 9198, RRID: AB_2561044), total CREB (Cell Signaling Technology Cat# 4820, RRID:AB_1903940), Egr-1 (Cell Signaling Technology Cat# 4153, RRID: AB_2097038), total Akt (Cell Signaling Technology Cat# 4691, RRID:AB_915783), phosphorylated Akt (Ser473) (Cell Signaling Technology Cat# 4058, RRID:AB_331168), total c-Src (Cell Signaling Technology Cat#2108, RRID:AB_331137), and β -tubulin (Cell Signaling Technology Cat# 2146, RRID:AB_2210545). The enhanced chemiluminescence (ECL) detection system kit was procured from Perkin Elmer (Montreal, Quebec, Canada).

Cell culture: A-10 VSMCs derived from the thoracic aorta of embryonic rats (ATCC Cat# CRL-1476, RRID:CVCL_0130) 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% CO₂ exchange at 37°C. VSMCs between passages 3 and 8 were grown to 80-90% confluence in 60-mm culture plates and serum starved for 24 h prior to treatment with H₂O₂ or other agents. It should be noted that these cells exhibit similar characteristics as the primary cultured VSMC derived from normal adult rat aorta (Kimes and Brandt 1976) and have been extensively used to investigate vascular cellular events (Bassil et al. 2008; Youreva and Srivastava 2016). Moreover, A10 VSMC are relatively easy to culture and can be transfected with high efficiency with siRNAs.

Cell lysis and immunoblotting: Confluent, serum-starved A-10 VSMCs grown to 80% confluence in 60-mm culture plates were pre-treated in the presence of various agents for 30 min, followed by stimulation with 100 µM of H₂O₂ for indicated time points. Cells incubated in the absence or presence of various agents were washed three times with ice-cold phosphate buffer saline 1X (PBS 1X) and lysed on ice in 100 µl of RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0,5% Na-deoxycholate, 1% NP40, 0,1% sodium dodecyl sulphate (SDS), 2 mM EDTA and 50 mM NaF) containing a cocktail of three inhibitors; phenylmethylsulphonyl fluoride (PMSF), protease inhibitor cocktail (PIC) and orthovanadate (OV), all diluted 1:100. The cell lysates were clarified by centrifugation at 13 000g for 10 min at 4°C. Protein concentration was determined by Bradford assay. Equal amounts of protein were subjected to 7.5% or 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-

PAGE), transferred to Immobilon-P polyvinylidene difluoride (PVDF) membranes (Millipore, USA) and incubated overnight at 4°C with respective primary antibodies; Egr-1 (1:1000), p-Akt (1:1000), Akt (1:1000), p-CREB (1:1000), CREB (1:1000), c-Src (1:1000), and β -tubulin (1:5000). The antigen-antibody complex was detected by horseradish peroxidase-conjugated secondary antibody, dilution depending on primary antibody dilution, and protein bands were visualized by ECL. Equal loading of protein was confirmed by measuring protein levels of β -tubulin or total 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, USA) software programs.

RNA preparation and reverse transcriptase: Cells were washed three times 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 500 ng of total RNA using a High Capacity RNA-to cDNA Kit (Applied Biosystems, Grand Island, NY) as per manufacturer's instructions in a final volume of 20 μ l. cDNA was stored at -20°C until use.

qRT-PCR analysis: Real-time quantitative PCR was performed with SYBG (Life Technologies, Grand Island, NY) using 2 μ l of cDNA in a final volume of 10 μ l. Real-time PCR amplification was performed using a 7500 fast RT-PCR system (Applied Biosystems, Grand Island, NY). 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'.

siRNA transfection protocol: Transfection was performed using lipofectamine RNAi max (Life Technologies, Cat# 13778-075, Burlington, ON, Canada). A-10 VSMCs 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 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°C prior to stimulation with H₂O₂.

Statistics. Statistical analysis was performed by one-way, repeated-measures analysis of variance (ANOVA) followed by Tukey post hoc test using GraphPad Prism 5 (GraphPad Software, La Jolla, California, USA). All data are reported as means $\pm$ SEM of 3-5 independent experiments. The differences between means were considered significant at $p < 0.05$.

Results

H₂O₂-induced Egr-1 expression involves PI3K/Akt signaling

Since H_2O_2 has been reported to activate the PI3K/Akt pathway (Blanc et al. 2004; Bouallegue et al. 2009) and serves as an upstream regulator of IGF-1-induced Egr-1 expression in VSMCs (Youreva et al. 2013; Youreva and Srivastava 2016) and that IGF-1 mediates its responses via NADPH oxidase-mediated ROS generation (Griendling and Ushio-Fukai 2000), we investigated the involvement of PI3K/Akt signaling in H_2O_2 -induced Egr-1 expression. For these studies, wortmannin and SC66, specific pharmacological inhibitors of PI3K and Akt respectively, as well as siRNA of Akt, were utilized. As shown in Fig. 1, pharmacological blockade of PI3K or Akt (Figs. 1A and 1B) or silencing of Akt (Fig. 1C) significantly suppressed the expression of Egr-1 enhanced by H_2O_2 . In addition, both wortmannin and SC66 markedly suppressed the mRNA levels of Egr-1 induced by H_2O_2 (Fig. 1D). These data indicated that H_2O_2 upregulates the expression of protein as well as the mRNA levels of Egr-1 through the PI3K/Akt signaling pathway in VSMCs.

H_2O_2 -induced Egr-1 expression requires CREB

We have shown earlier that the cyclic AMP response element binding protein (CREB), is essential for the induction of Egr-1 expression by Ang-II (Simocheyou et al. 2017) and Ang-II is known to provoke its physiological responses through ROS generation (Griendling et al. 2000). Therefore, we next investigated if CREB also participates in the signaling events leading to H_2O_2 -stimulated Egr-1 expression. As shown in Fig. 2A, depletion of CREB by siRNA resulted in almost complete inhibition of Egr-1 expression induced by H_2O_2 in VSMCs. Since Akt plays a critical role in Egr-1 expression (Fig. 1) and an increased

phosphorylation of CREB on Ser¹³³ is essential for its activation (Montminy 1997), we next explored the role of Akt in inducing the phosphorylation of this motif in CREB in response to H₂O₂. As shown in Figs. 2B and 2C, stimulation of VSMCs with H₂O₂ potently induced the phosphorylation of the Ser¹³³ residue in CREB, and inhibition of Akt activity either by SC66 or by siRNA, significantly attenuated this increase in CREB phosphorylation suggesting that Akt mediates its effect on Egr-1 expression via CREB-dependent pathways in VSMCs.

H₂O₂-induced Egr-1 expression, Akt and CREB phosphorylation is dependent on c-Src

Tyrosine kinases play a critical role in activating many signaling pathways implicated in cardiovascular diseases (Knock 2015) and we have shown earlier that ET-1-induced Egr-1 expression is mediated via a c-Src-dependent pathway (Simo-Cheyou et al. 2016), therefore, we investigated if H₂O₂-induced Egr-1 expression also requires c-Src. To probe the involvement of the c-Src family of tyrosine kinases in H₂O₂-induced responses, we utilized PP2, a selective inhibitor of the c-Src family tyrosine kinase, and PP3, a negative control of PP2. As shown in Fig. 3A, PP2 almost completely attenuated H₂O₂-induced Egr-1 expression while PP3 did not exert any inhibitory effect on Egr-1 expression. To further confirm the participation of c-Src in this process, we assessed whether siRNA-induced silencing of c-Src would exert a similar effect as PP2 in inhibiting the expression of Egr-1. As shown in Fig. 3B, a depletion of c-Src markedly reduced the expression of Egr-1 in response to H₂O₂ in VSMCs demonstrating that c-Src is required to induce the expression of Egr-1 in response to H₂O₂ in VSMCs. In

order to determine a role of c-Src in activating the Akt pathway, we next investigated whether siRNA-mediated silencing of c-Src would also block the phosphorylation of Akt induced by H_2O_2 in VSMCs. As shown in Fig. 3C, depletion of c-Src inhibited the phosphorylation of Akt induced by H_2O_2 . To further confirm the involvement of the c-Src/Akt pathway in CREB phosphorylation, we assessed whether the decreased phosphorylation of Akt in c-Src-depleted cells was associated with a decrease in CREB phosphorylation. As shown in Fig. 3D, siRNA-mediated depletion of c-Src almost completely suppressed the phosphorylation of CREB on Ser¹³³, suggesting that c-Src is an upstream regulator of Akt-mediated CREB signaling leading to Egr-1 expression in response to H_2O_2 in VSMCs.

Discussion

ROS has been implicated in the pathogenesis of vascular diseases such as atherosclerosis and hypertension (Dhalla et al. 2000; Fearon and Faux 2009). Remodelling of the vasculature, due to an aberrant proliferation, hypertrophy and migration of VSMCs has been suggested to play a key role in this process (Gomez and Owens 2012). Among various ROS, H_2O_2 , which is relatively more stable and cell permeable, has been demonstrated to activate signaling pathways that regulate cell proliferation, hypertrophy and migration of VSMCs (Brown and Griendling 2015; Byon et al. 2016). In the present studies, we have demonstrated that H_2O_2 potently activates the expression of Egr-1, a zinc finger transcription factor implicated in the pathogenesis of vascular diseases

(Khachigian 2016). By using specific pharmacological inhibitors of the PI3K and Akt pathway and siRNA-induced silencing, we have also demonstrated that Akt signaling plays an essential role in triggering Egr-1 expression in response to H₂O₂. We have also provided evidence suggesting that CREB is a critical component of the signaling cascade that mediates the enhanced expression of Egr-1. CREB is an important transcription factor that regulates the expression of several genes linked to proliferation and hypertrophy in VSMCs (Funakoshi et al. 2002; Molnar et al. 2014; Montminy 1997; Shaywitz and Greenberg 1999). An involvement of CREB in Ang-II- induced proliferation of VSMCs and angioplasty-induced neointimal hyperplasia has been reported (Molnar et al. 2014). A role of CREB in Ang-II and lysophosphatidic acid- induced Egr-1 expression via ERK1/2-dependent pathways has been demonstrated (Cui et al. 2006; Simo-Cheyou et al. 2017). MAP kinase pathways have also been implicated in ROS and prostaglandin-induced CREB phosphorylation and VSMC hypertrophy (Fukuyama et al. 2005; Ichiki et al. 2003), however, our data have revealed that in addition to ERKs, Akt can also induce the phosphorylation of CREB and promote Egr-1 expression in VSMCs stimulated with H₂O₂. Recent studies have suggested an important role of Akt in vascular injury and disease (Abeyrathna and Su 2015; Jin et al. 2017; Yu et al. 2015). An upregulation of Akt in VSMCs isolated from the aorta of spontaneously hypertensive rats (SHR) has been reported (Jain and Anand-Srivastava 2018) and pharmacological blockade of PI3K/Akt signaling by wortmannin was found to suppress the exaggerated expression of cell cycle regulatory proteins in SHR VSMCs (El Andalousi et al.

2013). Since Egr-1 has been suggested to modulate the expression of cell cycle regulatory proteins, promotes hyperproliferative events in vasculature and is upregulated in models of vascular injury (Cheyou et al. 2014a; Khachigian 2016), our results showing that Akt is essential to induce Egr-1 expression in response to H₂O₂ in VSMCs further support a critical role of Akt in regulating vascular functions.

Our findings that either pharmacological blockade or silencing of c-Src attenuates both Akt and CREB phosphorylation suggest that c-Src functions upstream of Akt and CREB in the signaling cascade responsible to induce Egr-1 expression in response to H₂O₂ in VSMCs. c-Src is a prominent member of the SRC family of non-receptor tyrosine kinases and has been reported to propagate H₂O₂-induced signaling events (Mehdi et al. 2007; Mehdi et al. 2005). We have also recently demonstrated that ET-1-induced Egr-1 expression is mediated via the c-Src-dependent ERK1/2 pathway (Simo-Cheyou et al. 2016). An involvement of c-Src in Ang-II-induced ROS generation and signaling responses in VSMCs has also been documented (Touyz et al. 2001). A role of c-Src in enhanced ERK1/2 phosphorylation and Gq alpha protein expression in VSMCs isolated from SHR have also been reported (Atef and Anand-Srivastava 2016) and pharmacological blockade of c-Src has been shown to improve cardiovascular function and reduce the development of hypertension in Ang-II-infused animal models of hypertension (Callera et al. 2016; Qin and Zhou 2015). Our studies are consistent with a key role of c-Src in mediating the signaling

pathways induced by H_2O_2 and demonstrate that the Akt-dependent phosphorylation of CREB is crucial to trigger Egr-1 expression in VSMCs.

Acknowledgments

This work was supported by a grant from the Canadian Institutes of Health Research (CIHR). The authors wish to thank Dr. John Chan's laboratory for the use of the 7500 fast RT-PCR system.

Figure Legends

Figure 1. Activation of the PI3K/Akt pathway is required for H₂O₂-induced EGR-1 expression in A10 VSMCs. Serum-starved cells were pre-treated with either wortmannin, 100 nM (PI3K inhibitor) (A) or SC66, 10 μ M (Akt inhibitor) (B) for 30 minutes or transfected with 10 nM Akt siRNA or 10 nM scrambled siRNA for 48h (C) prior to stimulation with H₂O₂, 100 μ M for 1 hr. Cell lysates were subjected to immunoblotting with antibodies for Egr-1 (top panels), total Akt (middle panel in C) and β -tubulin (bottom panels). Bar diagrams represent the quantification of Egr-1 relative to β -tubulin. Values are expressed as a ratio where the control (-) values are taken as 1. *** p <0.001 compared with the CTL VSMCs and ### p <0.001 compared to VSMCs treated with H₂O₂ alone. Shown are representative blots from 3 independent experiments. (D) Quiescent A-10 cells were pre-treated with either wortmannin, 100 nM or SC66, 10 μ M for 30 minutes followed by stimulation with H₂O₂, 100 μ M for 1 hr. Analysis of relative Egr-1 mRNA levels was performed by qRT-PCR. Relative level of Egr-1 mRNA is measured as a fold variation compared to the control and normalized with β -actin level taken as a standard. *** p <0.001 compared to control VSMCs and ### p <0.001 compared to VSMCs treated with H₂O₂ alone. Representative bar diagram from 3 independent experiments are shown.

Figure 2. Akt and CREB phosphorylation are required for H₂O₂-induced Egr-1 expression in A10 VSMCs. Cells were transfected with 10 nM CREB siRNA (A) or 10 nm Akt siRNA (C) or 10 nM control siRNA for 48 h or pre-treated with

SC66, 10 μ M (Akt inhibitor) (B) for 30 minutes prior to stimulation with 100 μ M H_2O_2 for 1 hr. Cell lysates were subjected to immunoblotting with Egr-1 (top panel in A), p-CREB (top panels B and C), total CREB (middle panels in A, B and C), p-Akt (middle panel in A), total Akt (middle panel in C) and β -tubulin (bottom panels) antibodies. Bar diagrams represent the quantifications of Egr-1 relative to β -tubulin and p-CREB relative to total CREB. Values are expressed as a ratio where control values are taken as 1. *** p <0.001 compared to control VSMCs and ### p <0.001 compared to VSMCs treated with H_2O_2 alone. Representative blots from 3 independent experiments are shown.

Figure 3. c-Src is required for H_2O_2 -induced Egr-1 expression in A10 VSMCs.

Serum-starved A10 VSMCs were pre-treated with (+) or without (-) PP2 10 μ M (selective c-Src non-receptor PTK inhibitor) or PP3 10 μ M (inactive analog of PP2) (A) for 30 mins or transfected with 10 nM c-Src siRNA or 10 nM scrambled siRNA for 48h (B, C, D) prior to stimulation with H_2O_2 , 100 μ M for 1 hr. Cell lysates were subjected to immunoblotting with Egr-1, p-Akt, p-CREB, total c-Src, total Akt, total CREB and β -tubulin antibodies. Bar diagrams represent the quantifications of Egr-1 relative to β -tubulin, p-Akt relative to total Akt and p-CREB relative to total CREB. Values are expressed as a ratio where control values are taken as 1. *** p <0.001 compared to control VSMCs and ### p <0.001 compared to VSMCs treated with H_2O_2 alone. Representative blots from 3 independent experiments are shown.

Figure 4: **Schematic model showing the signaling pathway involved in H₂O₂-induced Egr-1 expression in VSMCs.** In this pathway, c-Src appears to act upstream of Akt and CREB since its pharmacological inhibition by PP2 or silencing by siRNA decreased both Akt and CREB phosphorylation. Akt-induced phosphorylation of CREB is essential to trigger the expression of Egr-1.

Draft

REFERENCES

- Abeyrathna, P., and Su, Y. 2015. The critical role of Akt in cardiovascular function. *Vascul Pharmacol* **74**: 38-48. doi: 10.1016/j.vph.2015.05.008.
- Atef, M.E., and Anand-Srivastava, M.B. 2016. Oxidative stress contributes to the enhanced expression of Gqalpha/PLCbeta1 proteins and hypertrophy of VSMC from SHR: role of growth factor receptor transactivation. *Am J Physiol Heart Circ Physiol*: ajpheart 00659 02015. doi: 10.1152/ajpheart.00659.2015.
- Azar, Z.M., Mehdi, M.Z., and Srivastava, A.K. 2006. Activation of insulin-like growth factor type-1 receptor is required for H₂O₂-induced PKB phosphorylation in vascular smooth muscle cells. *Can J Physiol Pharmacol* **84**(7): 777-786. doi: 10.1139/y06-024.
- Baas, A.S., and Berk, B.C. 1995. Differential activation of mitogen-activated protein kinases by H₂O₂ and O₂⁻ in vascular smooth muscle cells. *Circ Res* **77**(1): 29-36.
- Bassil, M., Li, Y., and Anand-Srivastava, M.B. 2008. Peroxynitrite inhibits the expression of G(i)alpha protein and adenylyl cyclase signaling in vascular smooth muscle cells. *American journal of physiology. Heart and circulatory physiology* **294**(2): H775-784. doi: 10.1152/ajpheart.00841.2007.
- Blanc, A., Pandey, N.R., and Srivastava, A.K. 2004. Distinct roles of Ca²⁺, calmodulin, and protein kinase C in H₂O₂-induced activation of ERK1/2, p38 MAPK, and protein kinase B signaling in vascular smooth muscle cells. *Antioxidants and Redox Signaling* **6**(2): 353-366.
- Blaschke, F., Bruemmer, D., and Law, R.E. 2004. Egr-1 is a major vascular pathogenic transcription factor in atherosclerosis and restenosis. *Rev Endocr Metab Disord* **5**(3): 249-254. doi: 10.1023/B:REMD.0000032413.88756.ee.
- Bohm, F., and Pernow, J. 2007. The importance of endothelin-1 for vascular dysfunction in cardiovascular disease. *Cardiovasc Res* **76**(1): 8-18. doi: 10.1016/j.cardiores.2007.06.004.
- Bouallegue, A., Vardatsikos, G., and Srivastava, A.K. 2009. Role of insulin-like growth factor 1 receptor and c-Src in endothelin-1- and angiotensin II-induced PKB phosphorylation, and hypertrophic and proliferative responses in vascular smooth muscle cells. *Can J Physiol Pharmacol* **87**(12): 1009-1018. doi: 10.1139/Y09-056.
- Brown, D.I., and Griendling, K.K. 2015. Regulation of signal transduction by reactive oxygen species in the cardiovascular system. *Circ Res* **116**(3): 531-549. doi: 10.1161/CIRCRESAHA.116.303584.

- Byon, C.H., Heath, J.M., and Chen, Y. 2016. Redox signaling in cardiovascular pathophysiology: A focus on hydrogen peroxide and vascular smooth muscle cells. *Redox Biol* **9**: 244-253. doi: 10.1016/j.redox.2016.08.015.
- Callera, G.E., Antunes, T.T., He, Y., Montezano, A.C., Yogi, A., Savoia, C., and Touyz, R.M. 2016. c-Src Inhibition Improves Cardiovascular Function but not Remodeling or Fibrosis in Angiotensin II-Induced Hypertension. *Hypertension* **68**(5): 1179-1190. doi: 10.1161/HYPERTENSIONAHA.116.07699.
- Cheyoun, E.R., Youreva, V., and Srivastava, A.K. 2014a. Involvement of the early growth response protein 1 in vascular pathophysiology: an overview. *Indian J.Biochem.Biophys.* **51**(6): 457-466.
- Cheyoun, E.R., Youreva, V., and Srivastava, A.K. 2014b. Involvement of the early growth response protein 1 in vascular pathophysiology: an overview. *Indian J Biochem Biophys* **51**(6): 457-466.
- Clempus, R.E., and Griendling, K.K. 2006. Reactive oxygen species signaling in vascular smooth muscle cells. *Cardiovasc Res* **71**(2): 216-225. doi: 10.1016/j.cardiores.2006.02.033.
- Cui, M.Z., Laag, E., Sun, L., Tan, M., Zhao, G., and Xu, X. 2006. Lysophosphatidic acid induces early growth response gene 1 expression in vascular smooth muscle cells: CRE and SRE mediate the transcription. *Arterioscler Thromb Vasc Biol* **26**(5): 1029-1035. doi: 10.1161/01.ATV.0000214980.90567.b5.
- Daou, G.B., and Srivastava, A.K. 2004. Reactive oxygen species mediate Endothelin-1-induced activation of ERK1/2, PKB, and Pyk2 signaling, as well as protein synthesis, in vascular smooth muscle cells. *Free Radical Biology and Medicine* **37**(2): 208-215.
- Dhalla, N.S., Temsah, R.M., and Netticadan, T. 2000. Role of oxidative stress in cardiovascular diseases. *J Hypertens* **18**(6): 655-673.
- Dickinson, M.G., Kowalski, P.S., Bartelds, B., Borgdorff, M.A., van der Feen, D., Sietsma, H., Molema, G., Kamps, J.A., and Berger, R.M. 2014. A critical role for Egr-1 during vascular remodelling in pulmonary arterial hypertension. *Cardiovasc Res* **103**(4): 573-584. doi: 10.1093/cvr/cvu169.
- El Andalouji, J., Li, Y., and Anand-Srivastava, M.B. 2013. Natriuretic peptide receptor-C agonist attenuates the expression of cell cycle proteins and proliferation of vascular smooth muscle cells from spontaneously hypertensive rats: role of Gi proteins and MAPkinase/PI3kinase signaling. *PloS one* **8**(10): e76183. doi: 10.1371/journal.pone.0076183.

- Fahmy, R.G., and Khachigian, L.M. 2007. Suppression of growth factor expression and human vascular smooth muscle cell growth by small interfering RNA targeting EGR-1. *J Cell Biochem* **100**(6): 1526-1535. doi: 10.1002/jcb.21145.
- Fearon, I.M., and Faux, S.P. 2009. Oxidative stress and cardiovascular disease: novel tools give (free) radical insight. *J Mol Cell Cardiol* **47**(3): 372-381. doi: 10.1016/j.yjmcc.2009.05.013.
- Fukuyama, K., Ichiki, T., Ono, H., Tokunou, T., Iino, N., Masuda, S., Ohtsubo, H., and Takeshita, A. 2005. cAMP-response element-binding protein mediates prostaglandin F₂ α -induced hypertrophy of vascular smooth muscle cells. *Biochemical and biophysical research communications* **338**(2): 910-918. doi: 10.1016/j.bbrc.2005.10.019.
- Funakoshi, Y., Ichiki, T., Takeda, K., Tokuno, T., Iino, N., and Takeshita, A. 2002. Critical role of cAMP-response element-binding protein for angiotensin II-induced hypertrophy of vascular smooth muscle cells. *The Journal of biological chemistry* **277**(21): 18710-18717. doi: 10.1074/jbc.M110430200.
- Gao, Y.J., and Lee, R.M. 2001. Hydrogen peroxide induces a greater contraction in mesenteric arteries of spontaneously hypertensive rats through thromboxane A₂ production. *Br J Pharmacol* **134**(8): 1639-1646. doi: 10.1038/sj.bjp.0704420.
- Gomez, D., and Owens, G.K. 2012. Smooth muscle cell phenotypic switching in atherosclerosis. *Cardiovasc Res* **95**(2): 156-164. doi: 10.1093/cvr/cvs115.
- Griendling, K.K., Sorescu, D., Lassegue, B., and Ushio-Fukai, M. 2000. Modulation of protein kinase activity and gene expression by reactive oxygen species and their role in vascular physiology and pathophysiology. *Arterioscler Thromb Vasc Biol* **20**(10): 2175-2183.
- Griendling, K.K., and Ushio-Fukai, M. 2000. Reactive oxygen species as mediators of angiotensin II signaling. *Regul Pept* **91**(1-3): 21-27.
- Han, W., and Liu, G.N. 2010. EGR-1 decoy ODNs inhibit vascular smooth muscle cell proliferation and neointimal hyperplasia of balloon-injured arteries in rat. *Life Sci* **86**(7-8): 234-243. doi: 10.1016/j.lfs.2009.12.005.
- Ichiki, T., Tokunou, T., Fukuyama, K., Iino, N., Masuda, S., and Takeshita, A. 2003. Cyclic AMP response element-binding protein mediates reactive oxygen species-induced c-fos expression. *Hypertension* **42**(2): 177-183. doi: 10.1161/01.HYP.0000079791.26014.04.
- Jackson, C.L., and Schwartz, S.M. 1992. Pharmacology of smooth muscle cell replication. *Hypertension* **20**(6): 713-736.

- Jain, A., and Anand-Srivastava, M.B. 2018. Natriuretic peptide receptor-C-mediated attenuation of vascular smooth muscle cell hypertrophy involves Gqalpha/PLCbeta1 proteins and ROS-associated signaling. *Pharmacol Res Perspect* **6**(1). doi: 10.1002/prp2.375.
- Jin, Y., Xie, Y., Ostriker, A.C., Zhang, X., Liu, R., Lee, M.Y., Leslie, K.L., Tang, W., Du, J., Lee, S.H., Wang, Y., Sessa, W.C., Hwa, J., Yu, J., and Martin, K.A. 2017. Opposing Actions of AKT (Protein Kinase B) Isoforms in Vascular Smooth Muscle Injury and Therapeutic Response. *Arteriosclerosis, thrombosis, and vascular biology* **37**(12): 2311-2321. doi: 10.1161/ATVBAHA.117.310053.
- Khachigian, L.M. 2006. Early growth response-1 in cardiovascular pathobiology. *Circulation research* **98**(2): 186-191. doi: 10.1161/01.RES.0000200177.53882.c3.
- Khachigian, L.M. 2016. Early growth response-1 in the pathogenesis of cardiovascular disease. *J Mol Med (Berl)* **94**(7): 747-753. doi: 10.1007/s00109-016-1428-x.
- Kimes, B.W., and Brandt, B.L. 1976. Characterization of two putative smooth muscle cell lines from rat thoracic aorta. *Experimental cell research* **98**(2): 349-366.
- Knock, G.A. 2015. Tyrosine kinases as key modulators of smooth muscle function in health and disease. *J Physiol* **593**(17): 3805-3806. doi: 10.1113/JP271023.
- Lowe, H.C., Fahmy, R.G., Kavurma, M.M., Baker, A., Chesterman, C.N., and Khachigian, L.M. 2001. Catalytic oligodeoxynucleotides define a key regulatory role for early growth response factor-1 in the porcine model of coronary in-stent restenosis. *Circ Res* **89**(8): 670-677.
- Lyle, A.N., and Griendling, K.K. 2006. Modulation of vascular smooth muscle signaling by reactive oxygen species. *Physiology (Bethesda)* **21**: 269-280. doi: 10.1152/physiol.00004.2006.
- McCaffrey, T.A., Fu, C., Du, B., Eksinar, S., Kent, K.C., Bush, H., Jr., Kreiger, K., Rosengart, T., Cybulsky, M.I., Silverman, E.S., and Collins, T. 2000. High-level expression of Egr-1 and Egr-1-inducible genes in mouse and human atherosclerosis. *J Clin Invest* **105**(5): 653-662. doi: 10.1172/JCI8592.
- Mehdi, M.Z., Azar, Z.M., and Srivastava, A.K. 2007. Role of receptor and nonreceptor protein tyrosine kinases in H₂O₂-induced PKB and ERK1/2 signaling. *Cell Biochem Biophys* **47**(1): 1-10.
- Mehdi, M.Z., Pandey, N.R., Pandey, S.K., and Srivastava, A.K. 2005. H₂O₂-induced phosphorylation of ERK1/2 and PKB requires tyrosine kinase activity of insulin receptor and c-Src. *Antioxid Redox Signal* **7**(7-8): 1014-1020. doi: 10.1089/ars.2005.7.1014.

- Molnar, P., Perrault, R., Louis, S., and Zahradka, P. 2014. The cyclic AMP response element-binding protein (CREB) mediates smooth muscle cell proliferation in response to angiotensin II. *Journal of cell communication and signaling* **8**(1): 29-37. doi: 10.1007/s12079-013-0215-5.
- Montminy, M. 1997. Transcriptional regulation by cyclic AMP. *Annu Rev Biochem* **66**: 807-822. doi: 10.1146/annurev.biochem.66.1.807.
- Qin, B., and Zhou, J. 2015. Src Family Kinases (SFK) Mediate Angiotensin II-Induced Myosin Light Chain Phosphorylation and Hypertension. *PLoS One* **10**(5): e0127891. doi: 10.1371/journal.pone.0127891.
- Shaywitz, A.J., and Greenberg, M.E. 1999. CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. *Annual review of biochemistry* **68**: 821-861. doi: 10.1146/annurev.biochem.68.1.821.
- Simo-Cheyou, E.R., Tan, J.J., Grygorczyk, R., and Srivastava, A.K. 2017. STIM-1 and ORAI-1 channel mediate angiotensin-II-induced expression of Egr-1 in vascular smooth muscle cells. *J Cell Physiol* **232**(12): 3496-3509. doi: 10.1002/jcp.25810.
- Simo-Cheyou, E.R., Vardatsikos, G., and Srivastava, A.K. 2016. Src tyrosine kinase mediates endothelin-1-induced early growth response protein-1 expression via MAP kinase-dependent pathways in vascular smooth muscle cells. *International journal of molecular medicine* **38**(6): 1879-1886. doi: 10.3892/ijmm.2016.2767.
- Touyz, R.M., Wu, X.H., He, G., Park, J.B., Chen, X., Vacher, J., Rajapurohitam, V., and Schiffrin, E.L. 2001. Role of c-Src in the regulation of vascular contraction and Ca²⁺ signaling by angiotensin II in human vascular smooth muscle cells. *J Hypertens* **19**(3): 441-449.
- Vardatsikos, G., Sahu, A., and Srivastava, A.K. 2009. The insulin-like growth factor family: molecular mechanisms, redox regulation, and clinical implications. *Antioxid Redox Signal* **11**(5): 1165-1190. doi: 10.1089/ARS.2008.2161.
- Weiss, D., Kools, J.J., and Taylor, W.R. 2001. Angiotensin II-induced hypertension accelerates the development of atherosclerosis in apoE-deficient mice. *Circulation* **103**(3): 448-454.
- Youreva, V., Kapakos, G., and Srivastava, A.K. 2013. Insulin-like growth-factor-1-induced PKB signaling and Egr-1 expression is inhibited by curcumin in A-10 vascular smooth muscle cells. *Can J Physiol Pharmacol* **91**(3): 241-247. doi: 10.1139/cjpp-2012-0267.

Youeva, V., and Srivastava, A.K. 2016. 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. *Journal of cellular biochemistry* **117**(1): 152-162. doi: 10.1002/jcb.25260.

Yu, H., Littlewood, T., and Bennett, M. 2015. Akt isoforms in vascular disease. *Vascul Pharmacol* **71**: 57-64. doi: 10.1016/j.vph.2015.03.003

Draft

Figure 1A

Figure 1A

338x190mm (300 x 300 DPI)

Figure 1B

Figure 1B

338x190mm (300 x 300 DPI)

Figure 1C

Figure 1C

338x190mm (300 x 300 DPI)

Figure 1D

Figure 1D

338x190mm (300 x 300 DPI)

Figure 2A

Figure 2A

338x190mm (300 x 300 DPI)

Figure 2B

Figure 2B

338x190mm (300 x 300 DPI)

Figure 2C

Figure 2C

338x190mm (300 x 300 DPI)

Figure 3A

Figure 3A

338x190mm (300 x 300 DPI)

Figure 3B

Figure 3B

338x190mm (300 x 300 DPI)

Figure 3C

Figure 3C

338x190mm (300 x 300 DPI)

Figure 3D

Figure 3D

338x190mm (300 x 300 DPI)

Figure 4

Figure 4

338x190mm (300 x 300 DPI)