This unedited manuscript has been submitted for publication in the Annals of the NYAS.This paper has not been copyedited.

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Journal:	*Annals of the New York Academy of Sciences*
Manuscript ID:	draft
Volume Title:	Annals-1451 Apoptosis World 2008
Date Submitted by the Author:	n/a
Complete List of Authors:	Aggeli, Ioanna-Katerina; University of Athens, Animal & Human Physiology Beis, Isidoros; University of Athens, Animal & Human Physiology Gaitanaki, Catherine; University of Athens, Animal and Human Physiology
Keywords:	early growth response factor-1 (Egr-1), ERKs, JNKs, Oxidative stress, apoptosis, signaling, H9c2 cardiac myoblasts

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Running title: ERKs and JNKs regulate Egr-1 response to H_2O_2

Hydrogen peroxide upregulates Egr-1 expression and nuclear accumulation in H9c2 cells via ERKs and JNKs

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KEY WORDS: early growth response factor-1 (Egr-1); ERKs; JNKs; oxidative stress; apoptosis; signaling; H9c2 cardiac myoblasts

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Abstract

One of the most significant insults that jeopardize cardiomyocyte homeostasis is a surge in the levels of reactive oxygen species (ROS) i.e. during ischemia, reperfusion as well as in the failing myocardium. Early growth response factor-1 (Egr-1) has been found to act as a transcriptional regulator in a variety of biological processes protecting cells against apoptotic stimuli i.e. oxidative damage, known to exert deleterious-apoptotic effects on cardiac myocytes. We thus decided to investigate the signaling pathways involved in its regulation. Egr-1 mRNA levels were found to be maximally induced after 2h in H_2O_2-treated H9c2 cells. Blockade of ERKs and JNKs by appropriate pharmacological inhibitors significantly inhibited this response. Looking into Egr-1 respective response at the protein level, we found that the latter was maximally induced after 2 h of treatment with 200 μM H_2O_2, remaining elevated for 6 h, declining thereafter. H_2O_2-induced upregulation of Egr-1 protein levels was ablated in the presence of: PD98059, SP600125 and AS601245. In an effort to examine the subcellular distribution of Egr-1 under these conditions, immunofluorescent experiments revealed H_2O_2-induced Egr-1 nuclear sequestration to be also dependent on ERK and JNK cascades. Our results show for the first time the potential role of ERKs and JNKs in the mechanism regulating Egr-1 response to oxidative stress (a signal potentially causing apoptosis in cardiac cells) at multiple levels: mRNA, protein and subcellular localization. Further studies are essential so as to elucidate the specific effect of Egr-1 response on modulation of gene expression and determination of cell fate.

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INTRODUCTION

Apoptosis constitutes an extremely conserved throughout evolution form of programmed cell death, that is triggered by multiple stressful stimuli.[1,2] Oxidative stress has been shown to play a prominent role in triggering apoptosis, an effect that can be mediated by excessive reactive oxygen species (ROS) that compromise heart function having deleterious effects on cardiac myocytes.[3,4] Indeed, apoptotic mechanisms have been found to be triggered in a variety of cardiovascular pathologies including: atherosclerosis, ischemic episodes, myocardial infarction as well as in the case of ischemia/reperfusion injury.[5,6,7] Ample data from gene expression profiling experiments have led to the identification of a genetic survival program activated by ROS in the ischemic/reperfused myocardium or in other ROS-related cardiac disorders. The antiapoptotic genes that were demonstrated to be up-regulated mediating cell preservation under these adverse conditions include the early growth response factor-1 (Egr-1).[8]

The transcription factor Egr-1 (also termed NGF1-A, Zif/268, Krox-24) was originally identified as an immediate early response gene[9] bearing both growth inhibitory[10,11] or growth promoting properties.[12] It contains a DNA binding domain that consists of three zinc fingers[13,14] and binds to a GC-rich sequence in the promoter of its target genes that include growth factors and cytokines.[15] Egr-1 is rapidly induced by differentiation signals[16] as well as by heat shock, UV light[17] and ionizing radiation.[18] In terms of its tissue distribution, Egr-1 expression is highest in brain and heart.[19]

Containing both transactivation and repression domains this transcription factor has been found to either positively or negatively modulate gene transcription of its targets.[20] In particular, Egr-1 has been found to mediate transcriptional regulation of a variety of inflammatory and coagulant genes involved in atherosclerotic pathogenesis and restenosis following vascular injury. The latter include: transforming growth factor-β, intercellular adhesion molecule-1 (ICAM-1), plasminogen activator inhibitor-1 as well as platelet-derived growth factor A and B (PDGF-A and B).[21,22] Egr-1 is also expressed in endothelial and smooth muscle cells at atherosclerotic plaques[23] and mechanically injured carotid artery.[24] What is more, Okada et al. (2002) have noted the fundamental contribution of Egr-1 induction to the development of cardiac allograft vasculopathy;[25] eliciting salutary changes and mediating cardiac remodelling by altering the expression of genes like: atrial natriuretic factor (ANF) and α- or β-myosin heavy chain (α- or β-MHC), Egr-1 has also been shown to promote preservation of the heart contractile machinery.[26,27] Indeed, the levels of this transcription factor have been found to be induced in hypertrophied hearts.[27] In addition to this, Fahmy and Khachigian (2002) have marked that reduction of Egr-1 levels resulted in suppression of smooth muscle cell migration and proliferation limiting intimal hyperplasia in balloon-injured carotid.[28] This finding is indicative of Egr-1 fundamental role in modulation of vessel wall functional properties after injury. Previously, Muthukkumar et al. (1995) had disclosed Egr-1 central role in apoptotic pathways triggered in the context of melanoma cells,

further supporting the notion of its multifunctional role leading to specific phenotypes depending on the nature of the exogenous stimuli or cellular type.[29]

Numerous reports confirm that among the signal transduction pathways involved in Egr-1 regulation, MAPK subfamilies are included with compelling evidence reporting the crucial role of mainly extracellular signal-regulated kinases (ERKs) especially in cardiomyocytes.[30-32] MAPKs constitute a highly conserved family of serine/threonine protein kinases which are activated via dual phosphorylation of a specific threonine and tyrosine residue by members of the MAPK kinases (MKKs).[33] The three best-studied MAPKs subfamilies include ERKs, cJun-N-terminal kinases (JNKs) and p38-MAPK.[33,34] Upon activation, MAPKs can be found in both the cytoplasm and nucleus, where they interact with their substrates, i.e. other protein kinases, cytoskeletal proteins as well as transcription factors.[33-35]

Given the emerging importance of Egr-1 function in the myocardium under adverse conditions, as well as the fact that the mechanism regulating its expression remains elusive, this study was undertaken in an effort to decipher the redox signal transduction pathways involved in H_2O_2-induced Egr-1 response in the context of cardiac myocytes. Thus, we used H9c2 cardiomyoblasts as our experimental setting, a clonal cell line derived from embryonic heart ventricle,[36] retaining properties of signaling pathways of adult cardiomyocytes. This feature of H9c2 cell line accounts for their extensive use in studies investigating oxidative stress effects on the cardiomyocyte.[37,38] Overall, in the present study, we demonstrate for the first time, the involvement of both ERK and JNK signaling

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pathways in Egr-1 mRNA and protein levels upregulation along with its nuclear

sequestration in H_2O_2-treated cardiac cells.

MATERIALS AND METHODS

Materials

Hydrogen peroxide was purchased from Merck (Darmstadt, Germany). DMSO, leupeptin, trans-epoxy-succinyl-L-leucylamido-(4-guanidino) butane (E-64), dithiothreitol (DTT) and phenylmethylsulphonyl fluoride (PMSF) were obtained from Sigma-Aldrich (St Louis, Missouri, USA). SP600125, AS601245 and PD98059 were purchased from Calbiochem-Novabiochem (La Jolla, CA, USA) while SB203580 was from Alexis Biochemicals (Lausen, Switzerland). Nitrocellulose (0.45 μm) was obtained from Schleicher & Schuell (Keene NH, USA). Prestained molecular mass markers were from New England Biolabs (Beverly, MA, USA). Secondary antibodies were from DakoCytomation (Glostrup, Denmark). Primers for the detection of Egr-1 and GAPDH were synthesized by Invitrogen Life Technologies (California, USA). Super RX film was purchased from Fuji photo film GmbH (Dusseldorf, Germany). General laboratory reagents were purchased from Sigma-Aldrich or Merck.

Cell cultures and reagents

H9c2 cells (passage 18-25; American Type Culture Collection, Rockville, MD, USA) were cultured in DMEM (PAA Laboratories GmbH, Pasching, Austria) supplemented with 10% (v/v) heat inactivated fetal bovine serum (PAA Laboratories GmbH) and antibiotics, under an atmosphere of 95% air / 5% CO_2 at $37^{\circ}C$. Experiments were carried out using mononucleated myoblasts after serum had been withdrawn for 24 h. Hydrogen peroxide was added to the

medium for the times indicated. When pharmacological inhibitors were used, they were dissolved in DMSO and added to the medium 30 min prior to treatment with H_2O_2. Control experiments with DMSO alone were also performed for the same duration.

Preparation of nuclear extracts

Nuclear extracts were prepared as previously described.[39] Cells were harvested into buffer A (10 mM Tris-HCl pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.3 mM Na_3VO_4, 200 μM leupeptin, 10 μM E-64, 5 mM DTT, 300 μM PMSF). Samples were centrifuged (10,000 rpm, 5 min, 4°C), and the supernatants discarded. Pellets were re-suspended in buffer A containing 0.1% (v/v) Nonidet P40 (10 min, 4°C). After centrifugation (10,000 rpm, 5 min, 4°C), pellets were re-suspended in buffer C [20 mM Hepes pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% (v/v) glycerol, 0.3 mM Na_3VO_4, 200 μM leupeptin, 10 μM E-64, 5 mM DTT, 300 μM PMSF]. After centrifugation (12,000 rpm, 5 min, 4°C) supernatants (nuclear extract) were boiled with 0.33 vol. of SDS-PAGE sample buffer [SB4X: 0.33 M Tris-HCl (pH 6.8), 10% (w/v) SDS, 13% (v/v) glycerol, 20% (v/v) 2-mercaptoethanol, 0.2% (w/v) bromophenol blue]. Protein concentrations were determined using the BioRad Bradford assay reagent (Bio-Rad, Hercules, California, USA).

Immunoblotting

Protein samples (30 µg) from nuclear fraction extracts were separated by SDS-PAGE on 8% (w/v) polyacrylamide gels and transferred electrophoretically onto nitrocellulose membranes. Nonspecific binding sites were blocked with 5% (w/v) nonfat milk powder in TBST [20 mM Tris-HCl pH 7.5, 137 mM NaCl, 0.1% (v/v) Tween 20] for 30 min at room temperature. Subsequently, membranes were incubated overnight with the appropriate primary antibody [1:1000 anti-Egr-1 (sc-110, Santa Cruz Biotechnology, Inc. California, USA) or 1:2000 anti-actin (A2103, Sigma-Aldrich St Louis, Missouri, USA)] at 4^oC. After washing in TBST (3 x 5 min) blots were incubated with the respective horseradish peroxidase-conjugated secondary antibody 1:5000 in TBST containing 1% (w/v) nonfat milk powder (60 min). After washing in TBST (3 x 5 min), bands were detected using enhanced chemiluminescense (ECL) (Amersham Biosciences, Uppsala, Sweden) and quantified by scanning densitometry (Gel Analyzer v. 1.0).

RNA preparation, cDNA synthesis and ratiometric Reverse transcription PCR (RT-PCR)

The expression of endogenous Egr-1 was determined by ratiometric reverse transcription of total RNA followed by PCR analysis. Total RNA was extracted from cells using Trizol (Invitrogen Life Technologies), according to the manufacturer's instructions. For cDNA synthesis, 2 µg of total RNA was denatured in the presence of 5 p mole oligo-dT primer in a reaction volume of 13.5 µl at 65^oC for 5 min. Reverse transcription was performed with M-MLV

Reverse Transcriptase (Invitrogen Life Technologies), first strand buffer (Promega, Madison, USA), dithiothreitol (Promega) and deoxy-nucleotide triphosphates (dNTPs) (Promega). The first strand reaction was incubated at $37^{o}C$ for 1 h. Termination of the reaction was achieved by inactivation of the reverse transcriptase at $70^{o}C$ for 5 min. PCR for Egr-1 was performed using 1.5 Units Taq (Bioron GmbH, Ludwigshafen, Germany) with sense 5'-GTG CGA GTG GAG ATC GGA AT-3' and antisense 5'- GTA ACC GCA GCA TTC AAC CT-3' primers, based on the sequence of rat Egr-1 [Genbank accession no. **NM012551**]. These primers amplify a 205-base pair PCR product. After a 5 sec denaturation at $95^{o}C$, PCR was carried out for 25 cycles ($95^{o}C$ for 30 sec, $59^{o}C$ for 30 sec and $72^{o}C$ for 30 sec), and then a final extension was done at $72^{o}C$ for 4 min. PCR (25 cycles) for GAPDH was performed using the following primers: sense 5'-ACC ACA GTC CAT GCC ATC AC-3' and antisense 5'-TCC ACC ACC CTG TTG CTG TA-3' [Genbank accession no. **X02231**]. cDNA samples derived from "control" and treated cells were always amplified simultaneously. PCR products were separated on a 2% (w/v) agarose gel supplemented with ethidium bromide (EtBr) at a final concentration of 100 μg/l. Band intensities were determined using an appropriate image analysis programme (Gel Analyzer v. 1.0). All values were normalized for the amount of GAPDH mRNA and estimation of fragment band size (Egr-1 205 bp, GAPDH 452 bp) was performed by comparison with GeneRuler 100bp DNA ladder (Fermentas Life Sciences Inc., Hanover, USA).

Immunofluorescence staining

Cells were grown on appropriate chamber slides in plating medium and were treated after serum had been withdrawn for 24 h. Subsequently, cells were fixed with 4% (v/v) formaldehyde in phosphate buffer saline (PBS) pH 7.4 for 15 min at R_T, washed in PBS (x3) and incubated (5 min, R_T) with 1% (w/v) BSA in PBS containing 0.3% (v/v) Triton X-100. Incubation with the primary antibody against Egr-1 (1:100, 1 h, $37^{o}C$) was followed by 0.5 h incubation at $37^{o}C$ with an Alexa Fluor 488-conjugated anti-rabbit secondary antibody (1:250) (green fluorescense). After washing, cell nuclei were stained using TO-PRO-3 iodide (642/661) (1 µM in DMSO) (red fluorescence). Following mounting, chamber slides were visualized under a laser scanning confocal Zeiss Axiovert BioRad Radience 2100 microscope.

Statistical evaluations

All data are presented as means ± S.E.M. Comparisons between control and treatment were performed using Student's paired t-test. A value of at least $P<0.05$ was considered to be statistically significant.

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RESULTS

H_2O_2 *stimulates Egr-1 mRNA levels in H9c2 cells in a JNK- and ERK-dependent manner.*

There is emerging evidence revealing Egr-1 diverse biological and cytoprotective effects against oxidative damage.[40] Given the importance of the triggered cellular responses by oxidative insults in the myocardium, the present study was undertaken in order to characterize the mechanism regulating Egr-1 transcriptional upregulation under oxidative stress conditions. To this end, H9c2 cells were treated with 200 μM H_2O_2, a concentration used routinely for gene expression studies in cardiomyocyte experimental settings exposed to oxidative stress.[41] Thus, Egr-1 mRNA was found to be induced from 1 h (3.04±0.52 fold relative to control), maximized at 2 h (6.03±0.16 fold relative to control) (Figure 1A upper panel) decreasing thereafter. GAPDH (glyceraldehydes-3-phosphate dehydrogenase) mRNA levels were also assayed as a housekeeping gene (Figures 1A, C bottom panels). Data shown (Figures 1B, D) represents densitometric analysis of Egr-1 PCR product bands normalized for the respective GAPDH values. Subsequently, to probe into the actual pathways transducing this effect, we tried to determine the signaling cascades involved in stimulation of Egr-1 transcript levels by H_2O_2. To this end, various pharmacological inhibitors were used: PD98059 (25 μM): that blocks the ERK1/2 pathway, SP600125 (10 μM) and AS601245 (1 μM): selective JNKs inhibitors and SB203580 (10 μM): a p38-MAPK inhibitor. The effect of cycloheximide (20 μM) which is known to suppress *de novo* protein synthesis as well as actinomycin D (5 μg/ml) which is a

widely used transcription inhibitor, were also examined. Cells were left untreated (control) or incubated with either DMSO or the inhibitors alone or with the inhibitors followed by exposure to 200 μM H_2O_2 for 1 h. DMSO as well as the inhibitors alone had no effect on Egr-1 mRNA levels (data not shown). As shown in Figure 1C (upper panel) and Figure 1D, we observed that pre-treatment of H9c2 cells with PD98059 and SP600125 as well as AS601245 almost abrogated H_2O_2-stimulated Egr-1 response. These results also indicate that ERKs and JNKs participate in H_2O_2-induced Egr-1 mRNA upregulation in H9c2 cells. In contrast, there is no apparent intermediacy of p38-MAPK in the observed response. Furthermore, the latter was abolished in the presence of actinomycin D indicative of Egr-1 regulation at the transcriptional level. H_2O_2-induced Egr-1 levels were markedly enhanced in the presence of cycloheximide, an effect that confirms Egr-1 to function as an immediate early response gene.

ERKs and JNKs are involved in Egr-1 protein upregulation by H_2O_2 in H9c2 cells.

Subsequently, an effort was made, to examine the time-dependent profile of Egr-1 response to H_2O_2 at the protein level. As shown in Figure 2A and 2C, a sustained upregulation of Egr-1 protein levels was observed in samples from nuclear extracts at 1 h after the onset of stimulation (2.17±0.17 fold relative to control) with maximal values being attained at 2 h (3.71±0.15 fold relative to control) and being sustained for at least 6 h, declining thereafter. Additionally, using various inhibitors, the contribution of a number of signaling pathways to

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Egr-1 protein upregulation was assessed. In particular, cells were left untreated (control) or incubated with either DMSO, or the inhibitors alone, or with the inhibitors followed by exposure to 200 μM H_2O_2 for 2 h. DMSO as well as the inhibitors alone had no effect on Egr-1 protein levels (data not shown). Our results suggest that the latter are upregulated via a mechanism involving ERKs and JNKs, since the respective inhibitors almost ablated the observed response (Figure 2B, D). SB203580, a p38-MAPK inhibitor, had no effect. Equal protein loading was verified by reprobing the membranes with a specific anti-actin antibody (Figures 2A, B bottom panels).

Distribution pattern of Egr-1 in H_2O_2-treated H9c2 cells

Our aforementioned findings prompted us to look into Egr-1 distribution pattern as well as into the mechanism modulating the latter. To our knowledge, this is the first report describing regulation of Egr-1 subcellular localization in the context of cardiac cells exposed to oxidative stress. Effectively, monitoring the distribution profile of this transcription factor, we observed that in untreated cells (Figure 3-control) the basal-minimal immunofluorescent signal detected was located in both the cytoplasm and nucleus. Interestingly, after exposure of H9c2 cells for 2 h to 200 μM H_2O_2, there was a significant enhancement of Egr-1 staining which was accumulated exclusively in the nucleus (Figure 3- H_2O_2). In the presence of PD98059 as well as SP600125, ERKs and JNKs selective inhibitors respectively, there was a marked decrease in Egr-1 immunostaining nearly to basal levels, which was once more distributed in the nucleus as well as in the cytoplasm

(Figure 3- PD/H_2O_2 and SP/H_2O_2, respectively). Thus, it appears that under oxidative stress conditions, one observes the nuclear sequestration of enhanced Egr-1 protein levels, a process found to be both ERK- and JNK-dependent.

DISCUSSION

Several cardiac pathological conditions have as their prime cause exposure to increased levels of reactive oxygen species (ROS).[3,4] Oxidative stress may lead cardiac myocytes to apoptotic death as has been observed in various pathological heart conditions such as: ischemic heart failure, arrhythmias or myocardial infarction.[6,7] Hydrogen peroxide constitutes one of the most widely investigated ROS that has been found to exert a dual effect by either stimulating proliferation or triggering apoptosis.[42-44] Early growth response factor-1 (Egr-1) was originally characterized as an immediate early gene (IEG) that is induced by stimuli implicated in vascular pathology (i.e. growth factors, cytokines, hypoxia, hyperoxia, hemorrhagic shock injury).[45,46] Given the fact that Egr-1 constitutes a major regulator of cellular stress response, modulating both proliferative and apoptotic pathways, it was of interest to probe into its regulation under oxidative

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stress conditions. The experimental setting used in the present work were H9c2

cardiac cells, a model used extensively in studies investigating stress-induced

signal transduction mechanisms in the cardiac myocyte.[47-49]

Noticeably, accumulating reports, account for a key role of Egr-1 and its

targets in orchestrating cellular response following oxidative stress.[50]

Accordingly, in our hands, exposure of H9c2 cells to 200 µM H_2O_2 resulted in the

upregulation of Egr-1 mRNA that was maximal after 2 h declining thereafter

(Figure 1A, B). Nevertheless, in aortic smooth muscle cells Jin et al. (2000) have

shown H_2O_2-induced Egr-1 mRNA to peak within 1 h and to be regulated by a

tyrosine kinase-dependent mechanism.[50] In our study, induction of Egr-1 mRNA

levels was mediated via ERKs and JNKs signaling cascades, as evidenced by

abrogation of the observed effect in the presence of their respective

pharmacological inhibitors: PD98059 and SP600125 or alternatively AS601245

(Figure 1C, D). On the contrary, SB203580, a p38-MAPK inhibitor, did not block

H_2O_2-induced Egr-1 mRNA stimulation. Accordingly, numerous reports have

shown ERKs to be involved in EGR-1 mRNA upregulation by diverse stimuli in a

plethora of cell types and tissues. In particular, Hasan and Schafer (2008) have

marked ERK-dependent Egr-1 mRNA stimulation by hemin in vascular smooth

muscle cells.[51] In addition to this, in astrocytes treated with endothelin-3, Egr-1

transcription has been shown to be regulated through an ERK-related

mechanism.[52] This was also the case in primary neonatal cardiomyocytes treated

with estrogen, with Egr-1 mRNA once more upregulated in an ERK-dependent

manner[32] as well as in RAW macrophages exposed to hypoxia.[53] However, in

NIH3T3 cells, Lim et al. (1998) have shown various forms of stress (UV radiation, heat shock) to induce Egr-1 gene via a mechanism independent of ERKs, involving p38-MAPK and JNKs.[17] Only a few investigators have observed the participation of JNKs in Egr-1 mRNA levels stimulation. In particular, Chung et al. (2007) have reported amitriptyline (an antidepressant inhibiting neurotransmitter reuptake) to induce Egr-1 gene expression in rat C6 glial cells via ERKs and JNKs, using their respective selective inhibitors.[54] Given the differential mechanisms implicated in each setting, one can postulate that involvement of MAPKs in the transcriptional regulation of Egr-1 depends on the nature of the stimulus applied and on cell type. The observed abrogation of H_2O_2-induced Egr-1 mRNA by actinomycin D, a known inhibitor of gene transcription,[55,56] confirmed this response to be regulated at the transcriptional level, while the additive effect of cycloheximide on induction of Egr-1 mRNA by H_2O_2, substantiated that the latter constitutes an immediate-early response, underscoring Egr-1 function as an IEG (Figure 1C, D).

Subsequently, taking into account the fact that in skeletal muscle cells induction of Egr-1 mRNA by various stimuli (including endothelin 1, angiotensin II and alpha adrenergic agonists) was followed by a translational block,[57] it appeared of interest to elucidate the mechanism modulating expression of Egr-1 protein under oxidative stress in our experimental model. Correlating with studies reporting Egr-1 coordinated upregulation of mRNA and protein levels, we found Egr-1 protein to be maximally induced at 2 h of H_2O_2 treatment, returning to basal levels after 6 h (Figure 2A, C). Supporting our findings, Shamin et al. (1999)

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reported Egr-1 upregulation at both mRNA and protein levels, in neonatal cardiomyocytes exposed to endothelin-1, angiotensin II or norepinephrine,[58] with Hasan and Schafer (2008) also reporting a similar effect in vascular smooth muscle cells exposed to hemin.[51] What is more, regarding our observation of Egr-1 protein upregulation by H_2O_2 in an ERK- and JNK-dependent manner, numerous reports have also pointed to the involvement of ERKs i.e. in estrogen-treated neonatal cardiac myocytes[32] and prostaglandin-treated cardiac myocytes.[59] Additionally, in accordance with our findings, Ahn et al. (2007) have observed that Egr-1 protein upregulation in phorbol myristate-treated human glioma cells was ERK- and JNK-dependent.[60] To underline the diverse role of MAPKs depending on the stimulus involved and contradicting our results, Wang et al. (2005) have found p38-MAPK rather than ERKs or JNKs to mediate isoproterenol-induced Egr-1 protein expression in H9c2 cells.[61]

In terms of Egr-1 subcellular localization, we have shown H_2O_2 to induce its protein levels and to cause its sequestration (Figure 3 - H_2O_2 vs. control). To our knowledge, our study is also the first to report the involvement of both ERKs (Figure 3 – PD/H_2O_2) and JNKs (Figure 3 – SP/H_2O_2) in the enhanced expression and nuclear accumulation of Egr-1 protein in cardiac myocytes exposed to oxidative stress conditions, potentially mediating this transcription factor's interaction with its substrates, thus allowing for any modulation of gene expression. Correlating with our findings, Moon et al. (2007) have observed that in human intestinal epithelial cells exposed to sulindac sulfide, a non-steroidal

anti-inflammatory drug, the promoted expression and nuclear translocation of Egr-1 was blocked in the presence of an ERK cascade inhibitor.[62]

Egr-1 is the primary member of a zinc-finger transcription factors family and has been found to play a significant role in the pathogenesis of vascular diseases, first reported by Khachigian et al. (1996) in endothelial cells.[22] Modulating transcription of several genes involved in pathogenesis of the vasculature (i.e. PDGF-A, PDGF-B, TNF-α, interleucin-2 etc.) it has been characterized as a key mediator of compensatory physiological adjustments, indispensable for preservation of cardiac function.[45] Involvement of Egr-1 in regulation of sodium-calcium exchanger-1 (NCX1) as well as in fibroblast growth factor-2 (FGF-2) gene expression in cardiac myocytes, further substantiates Egr-1 cardioprotective, anti-apoptotic properties.[61,63] In this context, it becomes evident that Egr-1 holds a fundamental role in cardiovascular homeostasis, contributing to preservation of heart function. Therefore, elucidating the signal transduction pathways mediating its regulation under oxidative stress conditions becomes compelling. Overall, our data disclose the role of ERKs and JNKs in the regulation of Egr-1 response in H_2O_2-treated cardiac cells. The diagram in Figure 4 constitutes a schematic representation of our results. Further studies are nevertheless required so as to decipher the precise repertoire of effects of this immediate responsive transcription factor in the complex context of the myocardium, justifying Egr-1 characterization as a primary regulator of cell fate under stressful conditions.

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LEGENDS

FIGURE 1. Time course analysis of H_2O_2–induced Egr-1 mRNA upregulation in H9c2 cardiomyoblasts; a JNK- and ERK–mediated response. (**A**) H9c2 cells were exposed to 200 µM H_2O_2 for the times indicated. (**C**) H9c2 cells were left untreated (control) or pre-incubated with 10 µM PD98059 (PD), 10 µM SP600125 (SP), 1 µM AS601245 (AS), 10 µM SB203580 (SB), 20 µM cycloheximide (CLX), and 5 mg/ml actinomycin D (ActD) for 30 min, then exposed to 200 µM H_2O_2 for 1 h in the absence or presence of the inhibitors. RNA was extracted and expression of Egr-1 (**A** and **C** upper panels) as well as GAPDH (**A** and **C** lower panels) mRNA was analyzed by ratiometric RT-PCR. The positions of the 500, 400, 300 and 200bp markers are indicated on the left of the panels. After densitometric analysis of the PCR products, results were normalized for GAPDH and the data is presented (**B** and **D**) as fold stimulation. Results are means ± SEM for at least three independent experiments. ***** p<0.001 compared to control values; ****** p<0.001 compared to identically treated cells in the absence of inhibitors.

FIGURE 2. (**A**) Kinetics of Egr-1 protein expression levels in H_2O_2-treated H9c2 cardiomyoblasts. H9c2 cells were left untreated (control) or were exposed to 200 µM H_2O_2 for the times indicated. (**B**) Effect of PD98059, SP600125, AS601245 and SB203580 on Egr-1 response. H9c2 cells were left untreated or were pre-incubated with 10 µM PD98059 (PD), 10 µM SP600125 (SP), 1 µM AS601245 (AS) and 10 µM SB203580 (SB) for 30 min, then exposed to 200 µM H_2O_2 for 2 h

in the absence or presence of the inhibitors. Nuclear cell extracts (30 μg) were

subjected to SDS-PAGE and immunoblotted with an antibody for total Egr-1

protein levels (**A** and **B** upper panels). To verify equal loading, the membranes

were then stripped and re-incubated with a specific anti-actin antibody (**A** and **B**

lower panels). Bands were quantified by laser scanning densitometry (**C** and **D**).

Blots and results shown are representative of at least three independent

experiments. Results are means ± SEM for at least three independent

experiments. ***** p<0.001 compared to control values; ****** p<0.001 compared to

identically treated cells in the absence of inhibitors.

FIGURE 3. Localization profile of Egr-1 protein levels in H9c2 cardiomyoblasts

left untreated (control) or exposed to 200 μM H_2O_2 for 2 h. Cells were subjected

to immunocytochemical analysis with an antibody directed against total Egr-1

protein levels (green fluorescence). To reveal nuclear morphology nuclei were

stained with TO-PRO-3 iodide (642/661) (red fluorescence). Following mounting,

chamber slides were visualized under a laser scanning confocal Zeiss Axiovert

BioRad Radience 2100 microscope. Representative images are shown,

indicative of at least three independent experiments.

FIGURE 4. A hypothetical model of the mechanism regulating H_2O_2-induced

Egr-1 expression at the transcriptional and translational levels as well as its

subcellular distribution profile. → activation, ⊤ inhibition.

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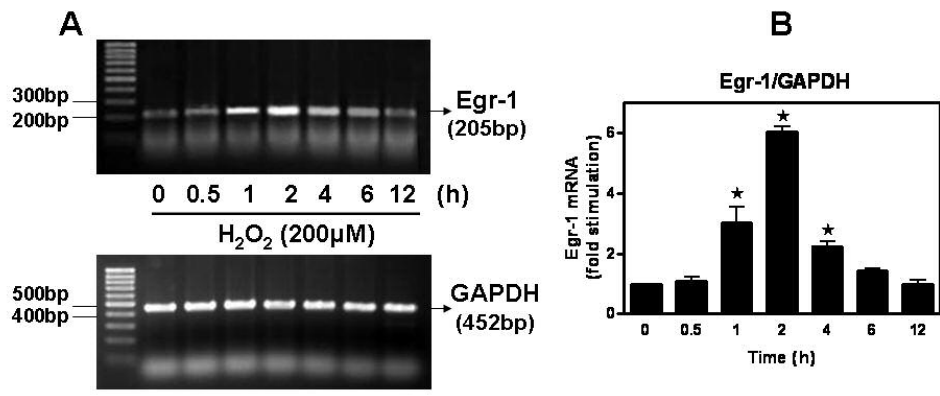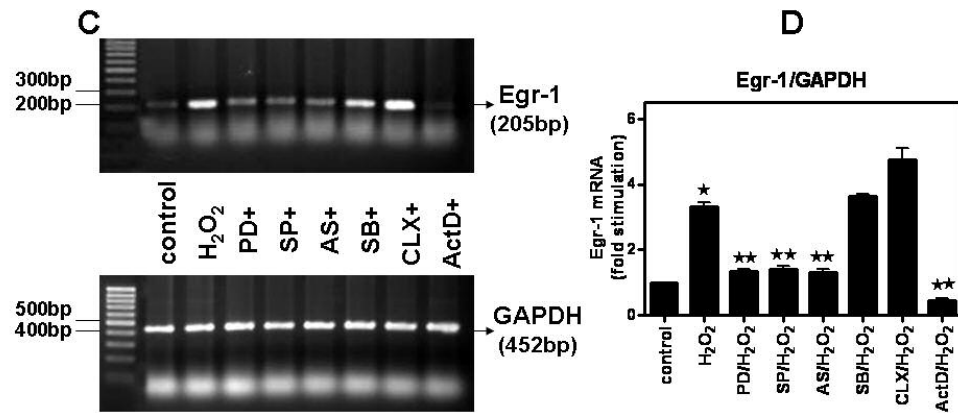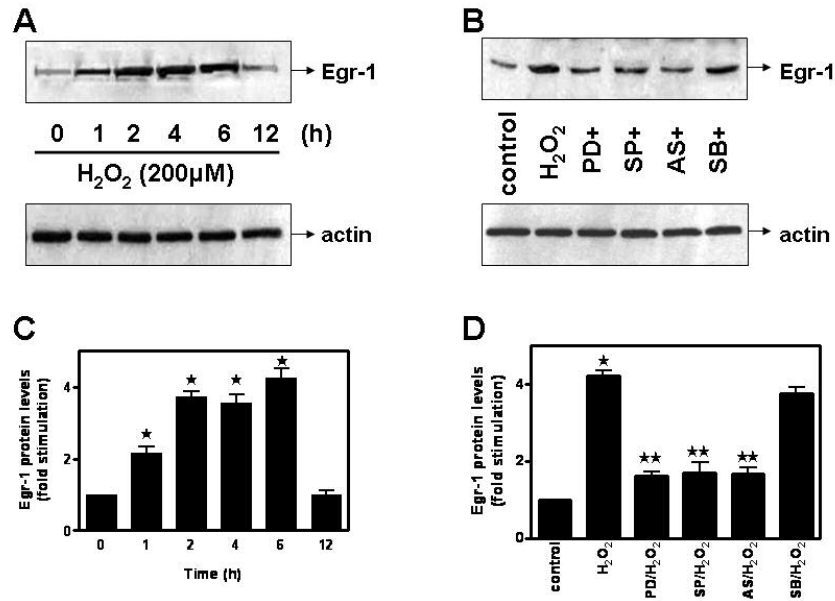
Fig. 1 Aggeli et al. 2008

254x190mm (96 x 96 DPI)

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254x190mm (96 x 96 DPI)

Fig. 2 Aggeli et al. 2008

A

→ Egr-1

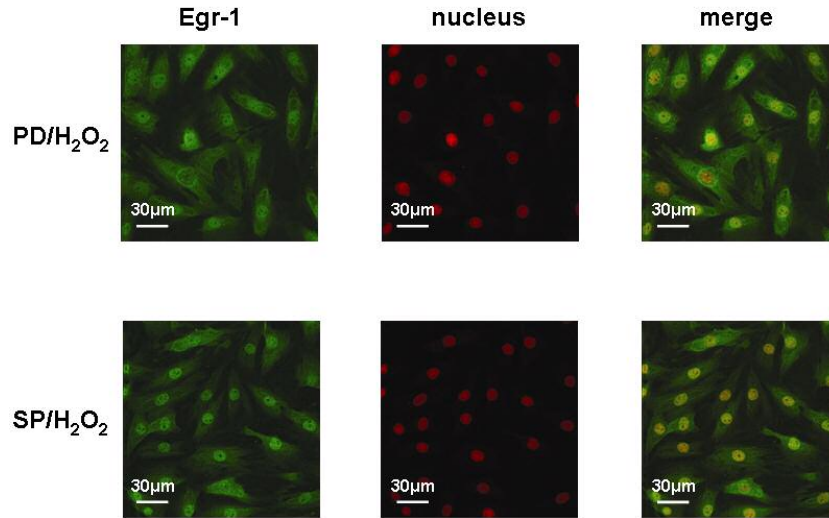0   1   2   4   6   12   (h)

H_2O_2 (200μM)

→ actin

B

→ Egr-1

control H_2O_2 PD+ SP+ AS+ SB+

→ actin

C

Egr-1 protein levels (fold stimulation)

Time (h)
0   1   2   4   6   12

D

Egr-1 protein levels (fold stimulation)

control H_2O_2 PD/H_2O_2 SP/H_2O_2 AS/H_2O_2 SB/H_2O_2

254x190mm (96 x 96 DPI)
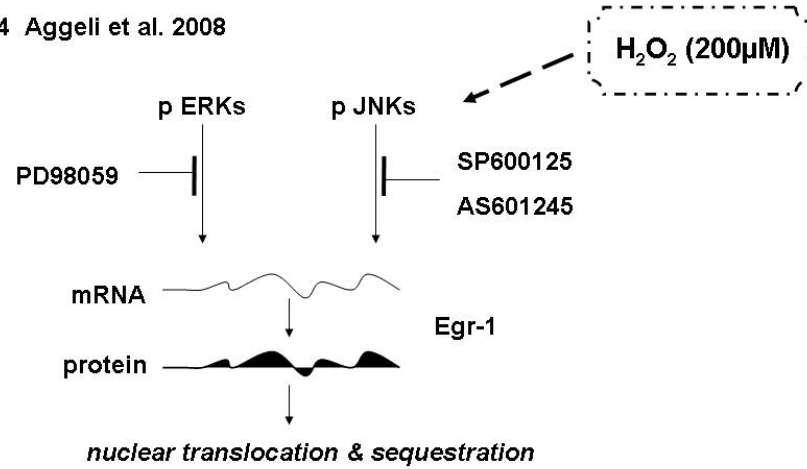
Fig. 3 Aggeli et al. 2008

254x190mm (96 x 96 DPI)

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254x190mm (96 x 96 DPI)

Fig. 4 Aggeli et al. 2008

H$_2$O$_2$ (200µM)

p ERKs   p JNKs

PD98059   SP600125

AS601245

mRNA

Egr-1

protein

nuclear translocation & sequestration

254x190mm (96 x 96 DPI)