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ERKs and JNKs Mediate Hydrogen Peroxide-Induced Egr-1 Expression and Nuclear Accumulation in H9c2 Cells

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Summary

One of the most significant insults that jeopardize cardiomyocyte homeostasis is a surge of reactive oxygen species (ROS) in the failing myocardium. Early growth response factor-1 (Egr-1) has been found to act as a transcriptional regulator in multiple biological processes known to exert deleterious effects on cardiomyocytes. We thus investigated the signaling pathways involved in its regulation by H_2O_2. Egr-1 mRNA levels were found to be maximally induced after 2 h in H_2O_2-treated H9c2 cells. Egr-1 respective response at the protein level, was found to be maximally induced after 2 h of treatment with 200 μM H_2O_2, remaining elevated for 6 h, and declining thereafter. H_2O_2-induced upregulation of Egr-1 mRNA and protein levels was ablated in the presence of agents inhibiting ERKs pathway (PD98059) and JNKs (SP600125, AS601245). Immunofluorescent experiments revealed H_2O_2-induced Egr-1 nuclear sequestration to be also ERK- and JNK-dependent. Overall, our results show for the first time the fundamental role of ERKs and JNKs in regulating Egr-1 response to H_2O_2 treatment in cardiac cells at multiple levels: mRNA, protein and subcellular distribution. Nevertheless, further studies are required to elucidate the specific physiological role of Egr-1 regarding the modulation of gene expression and determination of cell fate.

Key words

Early growth response factor-1 (Egr-1) • ERKs • JNKs • H_2O_2 • Signaling

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Introduction

Oxidative stress mediated by excessive reactive oxygen species (ROS) has been shown to compromise heart function, having deleterious effects on cardiac myocytes (Byrne *et al.* 2003, Ferrari *et al.* 2004). Indeed, apoptotic mechanisms have been found to be triggered in a variety of cardiovascular pathologies including atherosclerosis, ischemic episodes, myocardial infarction as well as ischemia/reperfusion injury (Balla *et al.* 1991, Feuerstein and Young 2000, Flotats and Carrio 2003). Ample data from gene expression profiling experiments have led to the identification of a genetic survival program activated in numerous ROS-related cardiac disorders. The early growth response factor-1 (Egr-1) is included among the antiapoptotic genes mediating cell preservation under these adverse conditions (Depre *et al.* 2001).

The transcription factor Egr-1 (also termed NGF1-A, Zif/268, Krox-24) was originally identified as an immediate early response gene (Milbrandt 1987) bearing both growth inhibitory (Huang *et al.* 1997, Levin *et al.* 1995) or growth promoting properties (Eid *et al.* 1998). It contains a DNA binding domain that consists of three zinc fingers (Lemaire *et al.* 1988, Lim *et al.* 1987) and binds to a GC-rich sequence in the promoter of its target genes (Cao *et al.* 1993). Egr-1 is rapidly induced by differentiation signals (Sukhatme *et al.* 1988) as well as by heat shock, UV light (Lim *et al.* 1987) and ionizing radiation (Datta *et al.* 1992). In terms of its tissue distribution, Egr-1 expression is highest in brain and heart (Lanoix *et al.* 1998).

In particular, Egr-1 has been found to mediate

transcriptional regulation of a variety of inflammatory and coagulant genes involved in atherosclerotic pathogenesis following vascular injury, i.e. 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) (Khachigian *et al.* 1996, Yan *et al.* 2000). Egr-1 is also expressed in atherosclerotic plaques (McCaffrey *et al.* 2000) and mechanically injured carotid arteries (Santiago *et al.* 1999). Eliciting salutary changes and mediating cardiac remodeling by altering the expression of genes such as 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 (Bruneau *et al.* 1996, Saadane *et al.* 1999). In addition, Fahmy and Khachigian (2002) have observed that reduction of Egr-1 levels resulted in suppression of smooth muscle cell proliferation limiting intimal hyperplasia in balloon-injured carotid.

Numerous reports confirm that among the signal transduction pathways involved in Egr-1 regulation, MAPK subfamilies are included, with compelling evidence reporting the particularly crucial role of extracellular signal-regulated kinases (ERKs) especially in cardiomyocytes (Chiu *et al.* 1999, deHager *et al.* 2001, Hodge *et al.* 1998). MAPKs constitute a highly conserved family of serine/threonine protein kinases which are activated *via* dual phosphorylation of a specific threonine and tyrosine residue (Goedert *et al.* 1997). The three best-studied MAPKs subfamilies include: ERKs, cJun-N-terminal kinases (JNKs) and p38-MAPK (Goedert *et al.* 1997, Kyriakis and Avruch 1996). 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 (Bogoyevitch 2000, Kyriakis and Avruch 1996).

Given the emerging importance of Egr-1 function in the myocardium under oxidative stress conditions (Ross 1998), 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 of cardiac myocytes. Thus, we used H9c2 cardiomyoblasts as our experimental setting. This clonal cell line derived from embryonic heart ventricle retains properties of signaling pathways of adult cardiomyocytes (Kimes and Brandt 1976) which accounts for its extensive use in studies investigating signal transduction mechanisms in cardiomyocytes (Han *et al.* 2004, Su *et al.* 1999, Tanaka *et al.* 2003, Turner *et al.* 1998). In the present study, we demonstrate for the first time, the involvement of both ERK and JNK signaling pathways in Egr-1 mRNA and protein levels upregulation along with its nuclear sequestration in H_2O_2-treated cardiac cells.

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, treatments 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 ^{o}C. The experiments were carried out using mononucleated myoblasts after serum had been withdrawn for 24 h. Hydrogen peroxide (200 μM) was added to the medium for the times indicated. This concentration of hydrogen peroxide is used routinely for gene expression studies in cardiomyocyte experimental settings exposed to oxidative stress (Kemp *et al.* 2003). When pharmacological inhibitors were used, they were dissolved in DMSO and added to the medium 30 min prior to treatment with 200 μM H_2O_2 as follows: PD98059 (25 μM), SP600125 (10 μM), AS601245 (1 μM), SB203580 (10 μM), cycloheximide (20 μM) and actinomycin D (5 μg/ml). 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 (mRNA studies) or 2 h (protein studies), respectively. Control experiments with DMSO alone were performed for the respective duration (1.5 h for Egr-1 mRNA studies and 2.5 h for Egr-1 protein studies).

Preparation of nuclear extracts

Nuclear extracts were prepared as previously described (Aggeli *et al.* 2006). 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 g, 5 min, 4 °C) in a BR4i Jouan centrifuge, 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 g, 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 g, 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 °C. 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 pmol oligo-dT primer in a reaction volume of 13.5 μl at 65 °C 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 °C for 1 h. Termination of the reaction was achieved by inactivation of the reverse transcriptase at 70 °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 CAA 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 s denaturation at 95 °C, PCR was carried out for 25 cycles (95 °C for 30 s, 59 °C for 30 s and 72 °C for 30 s), and then a final extension was done at 72 °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 program (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 100 bp 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

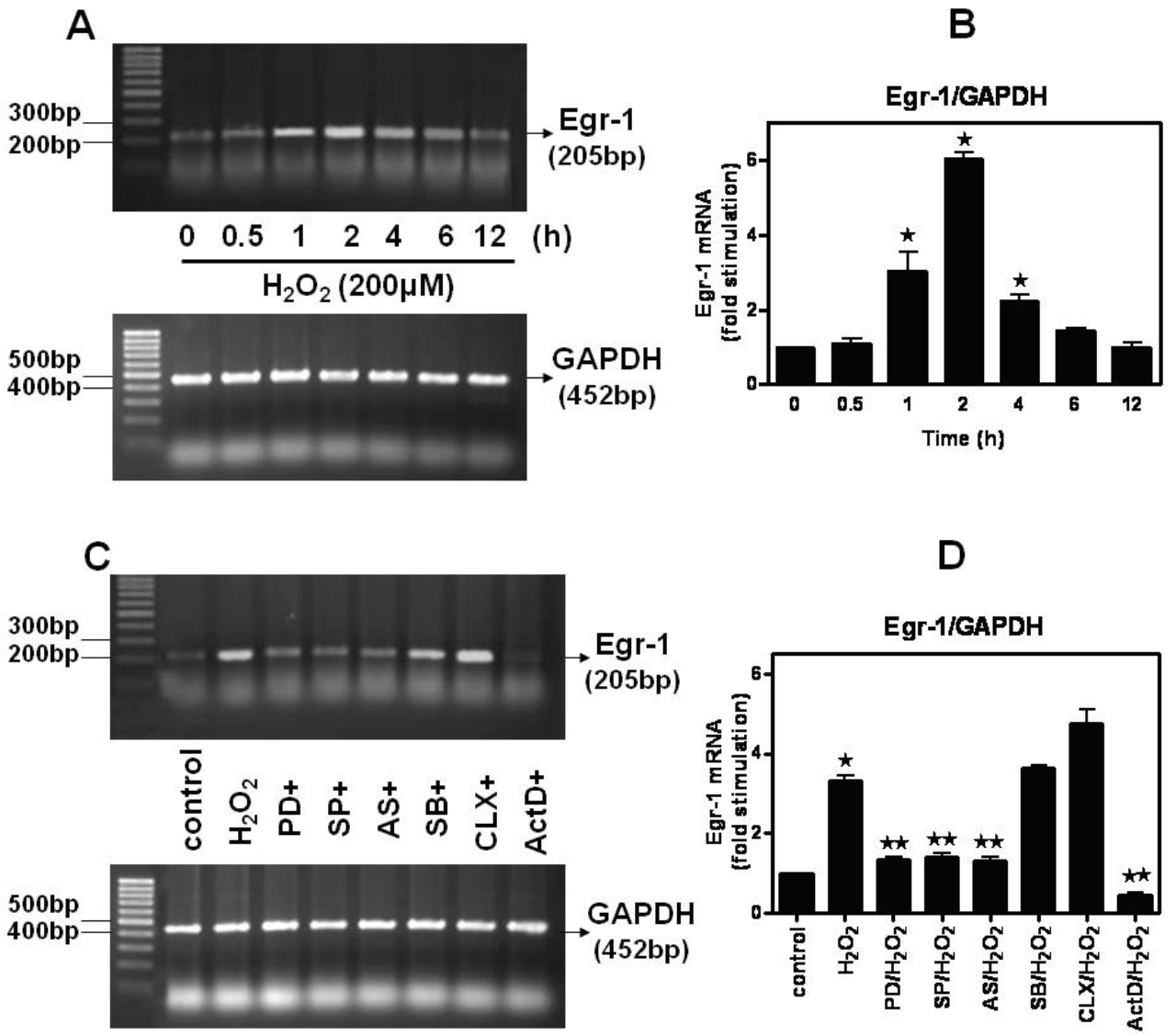

Fig. 1. Time course analysis of H_2O_2-induced Egr-1 mRNA upregulation in H9c2 cardiomyoblasts; a JNK- and ERK-mediated response. (**A**) H9c2 cells were exposed to 200 μM H_2O_2 for the times indicated. (**C**) H9c2 cells were left untreated (control) or pre-incubated with 10 μM PD98059 (PD), 10 μM 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.

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 °C) was followed by 0.5 h incubation at 37 °C with an Alexa Fluor 488-conjugated anti-rabbit secondary antibody (1:250) (green fluorescence). 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.

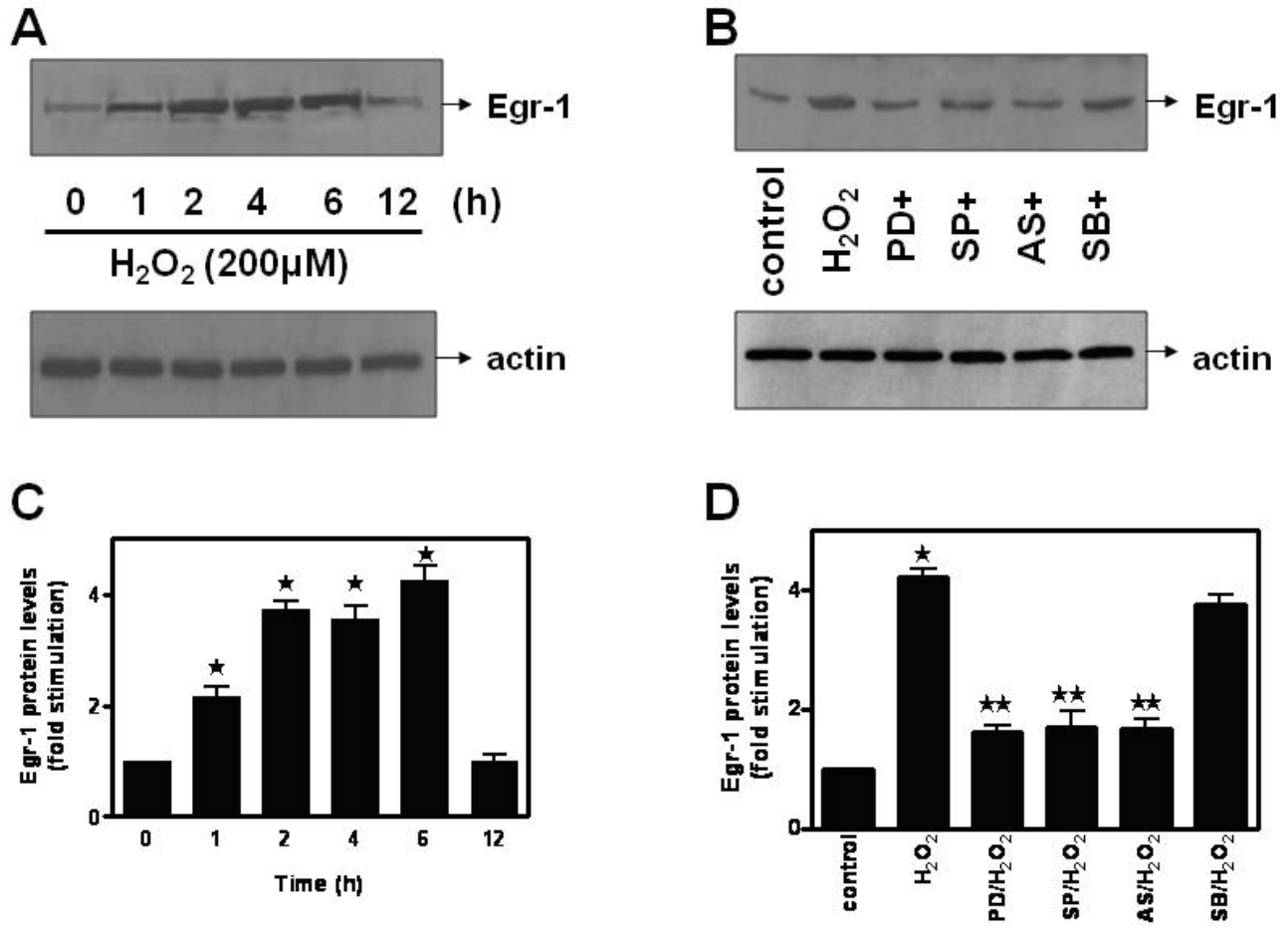

Fig. 2. (**A**) Kinetics of Egr-1 protein expression levels in H_2O_2-treated H9c2 cardiomyoblasts. H9c2 cells were left untreated (control) or were exposed to 200 μM H_2O_2 for the times indicated. (**B**) Effect of PD98059, SP600125, AS601245 and SB203580 on Egr-1 response. H9c2 cells were left untreated 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.

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 effects under stressful conditions. Given the importance of the triggered cellular responses by oxidative insults in the myocardium, Egr-1 transcriptional response to hydrogen peroxide treatment was examined in the present study by exposure of H9c2 cells to 200 μM H_2O_2. 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) (Fig. 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 (Figs 1B and 1D) 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 that blocks the ERK1/2 pathway, SP600125 and AS601245 (both selective JNKs inhibitors) and SB203580 (a p38-MAPK inhibitor). The effect of cycloheximide which is known to suppress *de novo* protein synthesis as well as actinomycin D which is a widely used transcription inhibitor, were also examined. 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 pretreatment of H9c2 cells with PD98059 and SP600125 as well as with 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, indicating Egr-1 regulation at the transcriptional level. Additionally, 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 Figures 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 Egr-1 protein upregulation was assessed. 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 (Figs 2B and 2D). SB203580, a p38-MAPK inhibitor, had no effect. Equal protein loading was verified by reprobing the membranes with a specific anti-actin antibody (Figs 2A and 2B, 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 cardiac cells exposed to hydrogen peroxide. Effectively, monitoring the distribution profile of this transcription factor, we observed that in untreated cells (Fig. 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 (Fig. 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 almost reaching basal levels, which was once more distributed in the nucleus as well as in the cytoplasm (Fig. 3 – PD/H_2O_2 and SP/H_2O_2, respectively). A minimal immunofluorescent signal was detected when the inhibitors were used alone in both the cytoplasmic and nuclear compartments (data not shown). Thus, it appears that after treatment with H_2O_2, 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 the exposure to increased levels of reactive oxygen species (ROS) which induce the apoptotic death of cardiac myocytes (Byrne *et al.* 2003, Feuerstein and Young 2000). 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 (Kanno *et al.* 2000, Sundaresan *et al.* 1995, Wei *et al.* 2000). 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 (Gess *et al.* 1997, Silverman and Collins T 1999). Given the fact that accumulating reports account for a key role of Egr-1 and its targets in orchestrating cellular response following oxidative stress (Jin *et al.* 2000), it was of interest to probe into its regulation by H_2O_2 in H9c2 cardiac cells.

Accordingly, in our hands, the exposure of H9c2 cells to 200 μM H_2O_2 resulted in the transient upregulation of Egr-1 mRNA (Figs 1A and 1B). In aortic smooth muscle cells, Jin *et al.* (2000) have shown H_2O_2-induced Egr-1 mRNA to peak within 1 h and its regulation by a tyrosine kinase-dependent mechanism. 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 (Figs 1C and 1D).

On the contrary, SB203580 (a p38-MAPK inhibitor) did not block H_2O_2-induced Egr-1 mRNA stimulation. In line with our findings, 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. In addition to this, Egr-1 transcription has also been shown to be regulated through an ERK-related mechanism, in astrocytes treated with endothelin-3 (Biesiada *et al.* 1996) as well as in primary neonatal cardiomyocytes treated with estrogen (deJager *et al.* 2001) and RAW macrophages exposed to hypoxia (Mishra *et al.* 2006). However, using NIH3T3 cells Lim *et al.* (1998) have shown that various forms of stress (UV radiation, heat shock) induce Egr-1 gene *via* a mechanism independent of ERKs, involving p38-MAPK and JNKs.

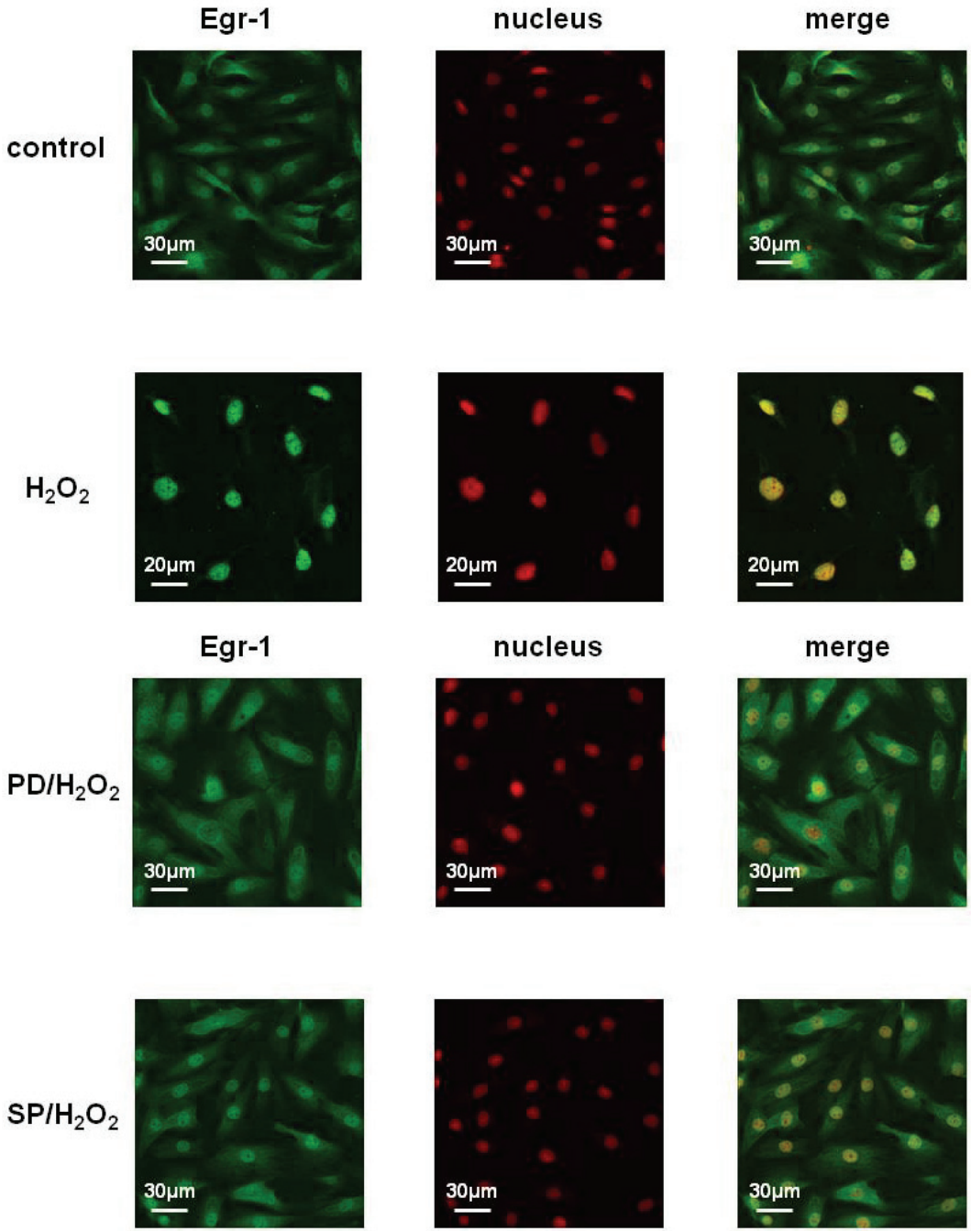

Fig. 3. Localization profile of Egr-1 protein levels in H9c2 cardiomyoblasts left untreated (control) or exposed to 200 μM H_2O_2 for 2 h. Cells were subjected to immunocytochemical analysis with an antibody directed against total Egr-1 protein levels (green fluorescence). To reveal nuclear morphology 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.

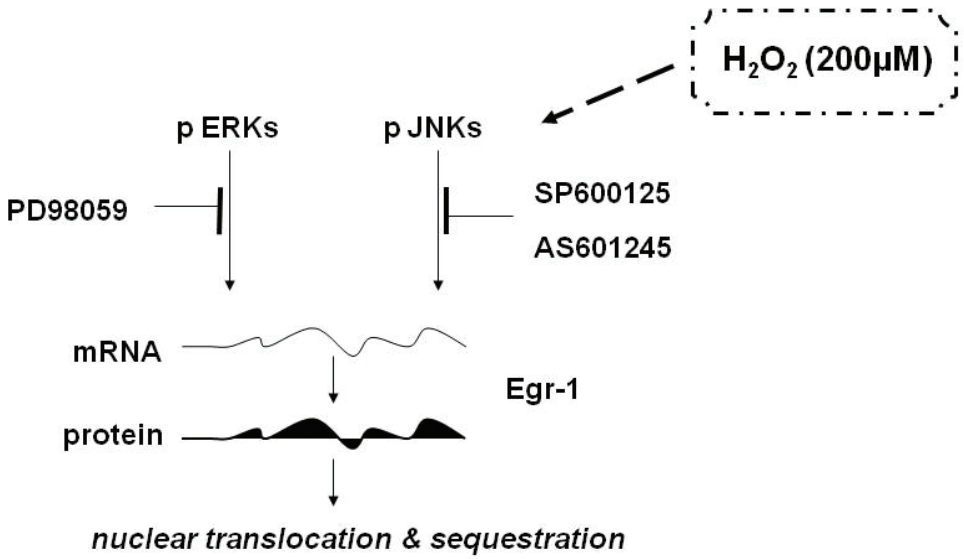

Fig. 4. A hypothetical model of the mechanism regulating H_2O_2-induced Egr-1 expression at the transcriptional and translational levels as well as its subcellular distribution profile.
→ activation, ⊤ inhibition.

Interestingly, only a few investigators have observed the participation of JNKs in stimulation of Egr-1 mRNA levels. 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. Similarly, Choi *et al.* (2008) have demonstrated Egr-1 induction by curcumin in U-87MG human glioblastoma cells to involve ERKs and JNKs. Given the different mechanisms implicated in each setting, one can postulate that the involvement of MAPKs in the transcriptional regulation of Egr-1 is stimulus- and cell type-specific. The observed abrogation of H_2O_2-induced Egr-1 mRNA by actinomycin D, a known inhibitor of gene transcription (McConkey *et al.* 1989a, 1989b), confirmed that this response is 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 – immediate early gene (Milbrandt 1987) (Figs 1C and 1D).

Subsequently, taking into account the fact that in skeletal muscle cells the induction of Egr-1 mRNA by various stimuli (including endothelin-1, angiotensin II and alpha-adrenergic agonists) was followed by a translational block (Maass *et al.* 1994), it appeared of interest to elucidate the mechanism modulating expression of H_2O_2-induced Egr-1 protein levels in our experimental model. In agreement 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 (Figs 2A and 2C). Similarly to our findings, Shamin *et al.* (1999) reported Egr-1 upregulation at both mRNA and protein levels in neonatal cardiomyocytes exposed to endothelin-1, angiotensin II or norepinephrine, with Hasan and Schafer (2008) reporting a similar effect in vascular smooth muscle cells exposed to hemin. What is more, related to 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 (deJager *et al.* 2001) and prostaglandin-treated cardiac myocytes (Xu *et al.* 2008). 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. However, contrary to 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.

In terms of Egr-1 subcellular localization, we have shown H_2O_2 to cause its nuclear sequestration (Fig. 3 – H_2O_2 vs. control). To our knowledge, our study is the first to report the involvement of both ERKs (Fig. 3 – PD/H_2O_2) and JNKs (Fig. 3 – SP/H_2O_2) in the enhanced expression and spatial distribution, i.e. nuclear accumulation of Egr-1 protein in cardiac myocytes exposed to H_2O_2, potentially mediating this transcription factor's interaction with its substrates, allowing thereafter for any modulation of gene expression. In accordance 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.

Egr-1 has been found to play a significant role in preservation of cardiac function and pathogenesis of vascular diseases, because Okada *et al.* (2002) noted the fundamental contribution of Egr-1 induction to the development of cardiac allograft vasculopathy. Furthermore, the 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 properties (Jimenez *et al.* 2004, Wang *et al.* 2005). However, recent evidence also denotes the possible implication of Egr-1 in the pathogenesis of myocardial ischemia/reperfusion injury, with Egr-1 inhibition leading to amelioration of hemodynamics *in vivo* and to a relief of myocardial injuries in morphology and structure as evidenced by an increase of cell viability (Zhang *et al.* 2008). The controversy concerning Egr-1 physiological role is further enhanced by the report of Kasneci *et al.* (2009) who demonstrated Egr-1 to act as a transcriptional repressor of calsequestrin (CSQ) resulting in its downregulation, with negative effects on cardiac function. This is due to the fact that CSQ constitutes the major calcium storage protein that links excitation–contraction coupling in the cardiac sarcoendoplasmic reticulum (Chopra *et al.* 2007).

One can deduce from the above that elucidating the signal transduction pathways mediating Egr-1 response to hydrogen peroxide appears compelling, particularly in cardiac myocytes. Overall, our data disclose the role of ERKs and JNKs in the regulation of Egr-1 temporal and spatial expression pattern in H_2O_2-treated cardiac cells. Figure 4 shows a schematic representation of our results. Nevertheless, further studies are required to elucidate the precise 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.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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Abbreviations

Early growth response factor-1 (Egr-1); Extracellular signal-regulated kinases (ERKs); cJun-N-terminal kinases (JNKs); Mitogen-activated protein kinases (MAPKs); glyceraldehyde-3-phosphate dehydrogenase (GAPDH); Dimethyl-sulfoxide (DMSO); Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE); polymerase chain reaction (PCR)

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