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Regulation of Bcl-2 phosphorylation in response to oxidative stress in cardiac myocytes

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Abstract
Oxidative stress promotes cardiac myocyte death and has been implicated in the pathogenesis of many cardiovascular diseases. Bcl-2 family proteins are key regulators of the apoptotic response, while their functions can be regulated by post-transcriptional modifications including phosphorylation, dimerization or proteolytic cleavage. This study used adult cardiac myocytes to test the hypothesis that activation of specific kinase signalling pathways by oxidative stress may modulate either the expression or the phosphorylation of Bcl-2, with the resulting effect of a decrease or increase in its anti-apoptotic function. Stimulation of cardiac myocytes with 0.2 mM H_2O_2, which induces apoptosis, resulted in a marked down-regulation of Bcl-2 protein simultaneously with an increase in its phosphorylation. Inhibition of p38-MAPK resulted in attenuation of Bcl-2 phosphorylation, whereas inhibition of ERK1/2, JNKs or PI-3-K had no effect. These data suggest that activation of p38 MAPK by oxidative stress results in the phosphorylation and degradation of Bcl-2 and the inactivation of its anti-apoptotic activity.

Keywords: *Adult cardiac myocytes, Bcl-2, apoptosis, hydrogen peroxide, ROS, protein kinases*

Introduction

Cardiac myocytes are terminally differentiated cells that respond to an array of external stimuli leading to either adaptive growth or cell death. Oxidative stress is one of the principal insults that promote cardiac myocyte death and has been implicated in the pathogenesis of a variety of cardiovascular diseases including ischemic heart disease and heart failure [1,2]. Increased generation of reactive oxygen species (ROS) during both the ischemic and reperfusion phases may be a major contributing factor in the apoptosis observed in ischemia/reperfusion injury [3]. Mitochondrial damage, cytochrome *c* release and activation of caspase 9 and 3 have been implicated in apoptotic pathways [4]. However, it has been also reported that low levels of ROS may be cytoprotective or even have growth promoting effects [2,5]. Oxidative stress can trigger the activation of multiple signalling pathways including phosphorylation cascades as those involving mitogen activated protein kinases (MAPKs), protein kinase C (PKC) and Akt, which might regulate cellular injury [6–8]. The factors that modulate these signalling pathways have not been described fully in any system. However, increased understanding of the apoptotic process in the myocardium is clearly important as it may lead to the identification of novel therapeutic strategies.

The Bcl-2 family proteins, which contain both pro-apoptotic and anti-apoptotic members, are becoming increasingly recognized as important modulators of cardiac myocyte apoptosis as in other cells [4,9]. These proteins act at the mitochondria to regulate cytochrome *c* release. Bcl-2 family proteins act either as heterodimers or as homodimers and the dynamic equilibrium between such complexes appears to determine the predisposition to apoptosis [10]. Recent evidence indicates that the functions of Bcl-2 proteins can be regulated by post-translational modifications, including phosphorylation [10], dimerization [11]

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and/or proteolytic cleavage [12,13]. Post-translational modifications as a result of the activation of death or survival signalling can lead in altered stability or availability for complex formation.

Bcl-2, a 26 kDa mitochondrial membrane protein, inhibits apoptosis by forming a heterodimer with Bax and prevents oligomerization of Bax, which leads to loss of mitochondrial membrane integrity with cytosolic leakage of caspase activators including cytochrome *c* [14,15]. Phosphorylation may affect the function of Bcl-2 or Bax by altering the capacity of these proteins to form dimers or act independently of dimerization [4,16]. A number of studies have indicated that phosphorylation of Bcl-2 is closely associated with regulation of apoptosis [17–20]. Several kinases have been proposed to be responsible for phosphorylating Bcl-2, such as PKC, ERK1/2, p38-MAPK and JNK [21,22]. However, the mechanisms of Bcl-2 phosphorylation as well as the role of phosphorylated Bcl-2 in apoptosis remain poorly understood. In some instances, phosphorylation of Bcl-2 results in its inactivation, rendering the cells more sensitive to the induction of apoptosis [17,18,20], whereas other reports suggest that Bcl-2 phosphorylation can enhance its anti-apoptotic effect [19].

To our knowledge there are no studies that have addressed the functional role of Bcl-2 phosphorylation in cardiac myocytes. In this study, we used adult cardiac myocytes and H_2O_2 as a ROS generating system to test the hypothesis that activation of specific kinase signalling pathways by oxidative stress, in a concentration-dependent manner, may modulate either the expression or the phosphorylation of Bcl-2, with the resulting effect of a decrease or increase in its anti-apoptotic function.

Materials and methods

Isolation of adult rat cardiac myocytes and culture

Ventricular myocytes were isolated from adult male Wistar rats by cardiac retrograde aortic perfusion and collagenase (type II; Biochrom AG, Berlin, Germany) treatment as described previously [23]. Cells were transferred to M199 culture medium, supplemented with 100 U penicillin/streptomycin, 25 mM HEPES and 10% foetal bovine serum (PAA Laboratories GmBH, Pasching, Austria) and plated at a density of $2 \times 10^3/mm^2$ in culture dishes pre-coated with laminin (Becton Dickinson Hellas, Athens, Greece). Four hours after plating, dishes were washed with M199 medium to remove non-attached cells and attached myocytes were incubated in serum-free medium for 24 h in a 5% CO_2 humidified incubator at 37°C, prior to experimentation.

Cell treatments

Myocytes were exposed to various concentrations of H_2O_2 as indicated. When necessary, 10 min prior to this treatment, cells were exposed to protein kinase inhibitors. The following kinase inhibitors were used: 1 μM SB203580, the p38-MAPK inhibitor; 10 μM PD98059, the ERK 1/2 inhibitor; 10 μM SP600125, the JNKs inhibitor; 50 μM LY294002, the PI-3-K inhibitor. All the inhibitors were obtained from Calbiochem (La Jolla, CA). All experiments were performed in at least three repeats.

Cell viability and apoptosis detection

Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay which assesses mitochondrial activity in living cells. Cardiac myocytes were plated on a 96-well plate and exposed to various concentrations of H_2O_2 as described above. Tetrazolium bromide (Appli-Chem Gmbh, Darmstadt, Germany) at a concentration of 0.5 mg/ml was added 5 h before the end of incubation and the incubation was continued. At the end of incubation, 100 μl of DMSO was added for 10 min to dissolve formazan precipitate. Absorbance was measured at 570 nm using a microplate autoreader. Cell viability was calculated as the percentage of control OD.

Cardiac myocyte apoptosis was determined using the *in situ* Cell Death Detection kit (Roche Applied Science) according to the manufacturer's instructions. For this purpose cardiac myocytes were seeded on 4-well LAB-TEK Permanox slides (Nalge Nunc International, NY) starved and treated with H_2O_2. TUNEL reaction was carried out after 24 h of exposure to H_2O_2. Fluorescence staining was viewed with a fluorescent microscope (Nikon, Japan). Total nuclei were visualized by counterstaining with Hoechst 33342 (50 μg/ml, 15 min). At least 100 apoptotic nuclei were counted in randomly selected fields for a single experiment and three independent experiments were conducted. Results are expressed as the percentage of the total nuclei stained.

Immunoblot analysis

After treatments, cells were scraped off the culture dishes, collected in eppendorf tubes, lysed in a ice-cold buffer containing 20 mM β-glycerophosphate, 50 mM NaF, 2 mM EDTA, 10 mM benzamidine, 20 mM Hepes, 0.2 mM Na_3VO_4, 5 mM dithiothreitol (DTT), 0.2 mM leupeptin, 0.01 mM trans-epoxy succinyl-L-leucylamido-(4-guanidino)butane (E64), 0.3 mM phenyl methyl sulphonyl fluoride (PMSF) and 1% (w/v) Triton X-100 and extracted on ice for 30 min. Cell lysates were centrifuged (10 000 g, 5 min, 4°C) and the supernatants were boiled with

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0.33 vol of SDS/PAGE sample buffer (0.33 M Tris/HCl, pH 6.8, 10% (w/v) SDS, 13% (v/v) glycerol, 133 mM DTT, 0.2% (w/v) bromophenol blue). Protein concentrations were determined using the BioRad Bradford assay. Proteins were separated by SDS-PAGE on 12% or 15% acrylamide, 0.275% (w/v) bis-acrylamide slab gels and transferred electrophoretically onto nitrocellulose membranes (0.45 μm). Membranes were blocked with TBS-T (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 0.1% (v/v) Tween 20) containing 5% (w/v) non-fat milk powder for 30 min at room temperature and then incubated with the appropriate antibody (in TBS-T containing 5% w/v BSA) at 4°C overnight. Antibodies used were: monoclonal Bcl-2 (Santa Cruz Biotechnology Inc., CA), monoclonal phospho-Bcl-2(Ser70) (Abcam, Cambridge, UK), polyclonal phospho-p38-MAPK and p38-MAPK, monoclonal phospho-ERK1/2, phospho-AKT(Ser473) and phospho-JNKs (Cell Signaling Technology, Beverly, MA). Proteins were detected with horseradish peroxidase (HRP) conjugated secondary antibody (1:5000 dilution in TBS-T containing 1% (w/v) non-fat milk powder, 1 h, room temperature) and were visualized by enhanced chemiluminescence (Chemicon International). Scanning densitometry was used for semiquantitative analysis of the data.

RNA preparation and quantitative PCR (qPCR)

Total RNA was extracted and cDNA synthesized as previously described [23]. qPCR analysis of Bcl-2 was performed using a Real-Time PCR System (Applied Biosystems) with forward primer 5′-ACGGTGGTGGAGGAACTCTTC-3′ and reverse 5′-CGCTCCCCACACACATGAC-3′ based on the published rat Bcl-2 sequence (EMBL accession number NM_016993.1). Each reaction mix contained 12.5 μl Sybr-Green Jump Start Taq Readymix (Sigma-Aldrich Co. Ltd.), 5 μl oligonucleotides (10 pmol each of forward and reverse primers) and 7.5 μl cDNA (diluted 1:10). qPCR analysis of glyceraldehyde-3-phosphate dehydrogenase (Gapdh) was performed as an endogenous control with forward primer 5′-GCTGGCATTGCTCTCAATGACA-3′ and reverse 5′-TCCACCACCCTGTTGCTGTA-3′ based on the published rat Gapdh sequence (EMBL accession number NM_017008). PCR conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Following qPCR, dissociation curve analysis was routinely performed to check for aberrant amplification products (e.g. primer-dimers).

Statistical analysis

All values are expressed as mean ± SEM. The differences between two groups were evaluated by Student's *t*-test. The data from more than two groups were evaluated by one-way analysis of variance (ANOVA) followed by Dunnet's multiple comparison test. A probability of 95% or more ($p < 0.05$) was considered significant.

Results

H_2O_2-induced death in cardiac myocytes

It is widely accepted that high concentrations of H_2O_2 or high levels of oxidative stress promote cardiac myocyte death [13,24], but some groups have reported that lower, non-toxic concentrations of H_2O_2 promote cytoprotection or growth [7]. We re-examined the concentration-dependent effects of H_2O_2 on viability (assessed by MTT assay) and on apoptosis (assessed by TUNEL staining of nuclei and by cleavage of caspase 3). Consistent with our previous study [13], H_2O_2 at concentrations higher than 0.05 mM decreased viability (Figure 1A) and increased the rate of apoptosis in cardiac myocytes (Figure 1C). The cleavage of caspase 3 was increased at 0.2 H_2O_2, whereas no effect was observed at 0.02 mM H_2O_2 (Figure 1B).

Effect of H_2O_2 on Bcl-2 family proteins

We examined the effects of non-toxic (0.02 mM) and apoptotic (0.2 mM) concentration of H_2O_2 on the expression of anti-apoptotic Bcl-2 and pro-apoptotic Bax proteins in cardiac myocytes. The ratio Bcl-2/Bax is widely used as a marker of apoptosis. Cardiac myocytes were exposed to H_2O_2 for 24 h and the protein levels of Bcl-2 and Bax were compared by immunoblotting. A profound down-regulation of Bcl-2 was observed when 0.2 mM H_2O_2 was used (Figure 2A). On the other hand, Bax was upregulated. The levels of these proteins did not change in cardiac myocytes exposed to 0.02 mM H_2O_2. To determine the time course of Bcl-2 down-regulation under these conditions, we examined its protein expression at several time points over 24 h (Figure 2B). It is evident that Bcl-2 expression is significantly decreased after 2 h of exposure to H_2O_2. In order to determine whether the down-regulation of Bcl-2 protein reflects changes at transcriptional level, we assessed Bcl-2 mRNA by qPCR. H_2O_2 had no significant effect on Bcl-2 mRNA levels when it was used at 0.2 mM, suggesting post-transcriptional regulation of Bcl-2. On the other hand, when H_2O_2 was used at 0.02 mM, Bcl-2 mRNA seemed to be up-regulated, although it did not reach statistical significance (Figure 2C).

Effect of H_2O_2 on Bcl-2 phosphorylation

Several reports in other cell systems indicate that Bcl-2 may be phosphorylated and this phosphorylation affects the anti-apoptotic function of the protein,

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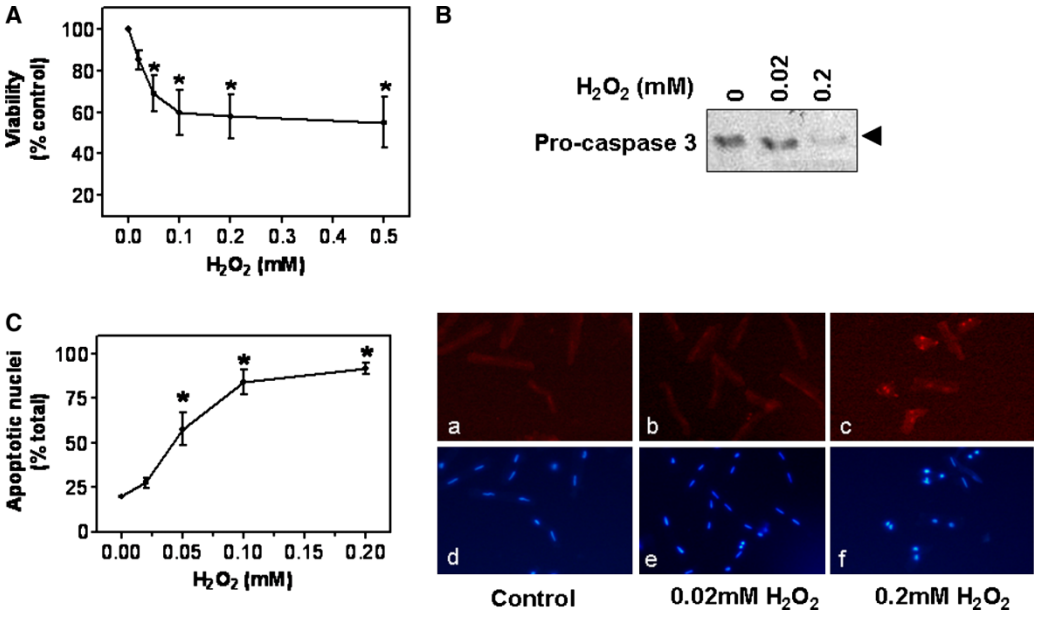

Figure 1. H_2O_2-induced death in cardiac myocytes. Cardiac myocytes were exposed to different concentrations of H_2O_2 for 24 h. (A) Cell viability was determined by the MTT assay. Results are means ± SEM from three independent experiments. $^*p < 0.05$ relative to control (one-way ANOVA with Dunnett post-test). (B) Pro-caspase 3 levels were assessed by immunoblotting. Blot is representative from three independent experiments. (C) Apoptotic nuclei were determined by TUNEL assay (a, b, c). Total nuclei were visualized with Hoechst 33342 (d, e, f). Results are expressed as the percentage of total nuclei stained and are means ± SEM from three independent experiments $^*p < 0.05$ compared to control (one-way ANOVA with Dunnett post-test).

either positively or negatively [19,25]. We, therefore, examined whether Bcl-2 is phosphorylated in response to increased ROS production in cardiac myocytes and whether this phosphorylation is related to the onset of apoptosis. Cells were stimulated with various concentrations of H_2O_2 (0–0.5 mM) for a time interval (15 min) shorter than that affecting total Bcl-2 protein levels. Phosphorylation of Bcl-2 was determined by immunoblotting using an antibody against phosphorylated (Ser70) Bcl-2. As seen in Figure 3, increased phosphorylation was observed with increasing concentration of H_2O_2. Maximal phosphorylation levels were attained around 0.1–0.2 mM, a concentration which induces apoptosis in cardiac myocytes (Figure 1).

We next examined the time course of Bcl-2 phosphorylation. Cardiac myocytes were stimulated with 0.2 mM H_2O_2 for 0–120 min and the levels of total and phosphorylated Bcl-2 were determined (Figure 4A and B). As total Bcl-2 levels are down-regulated over the 120 min period, the ratio of the phosphorylated to the total protein was determined in order to assess the net phosphorylation. Increased phosphorylation was observed after 5 min of H_2O_2 stimulation and it was maintained over 90 min (Figure 4B). The phosphorylation of Bcl-2 was also determined after stimulation with the non-toxic concentration of H_2O_2 0.02 mM. It was confirmed that this concentration of H_2O_2 does not have any effect either on the expression or the phosphorylation of Bcl-2 protein (Figure 4C).

The role of kinases in Bcl-2 phosphorylation

A variety of protein kinases, including the MAPK family members, have been reported to induce Bcl-2 phosphorylation in other cell systems [20,21,26]. In addition, several kinases are known to be activated upon oxidative stress and to be involved in the apoptotic process [4]. Thus, the question was raised whether these signalling pathways contributed to the H_2O_2-induced Bcl-2 phosphorylation in cardiac myocytes. We first analysed the activation of Akt, JNKs, ERK1/2 and p38-MAPK following exposure of cardiac myocytes to 0.02 or 0.2 mM H_2O_2 (Figure 5A). No activation of JNKs was observed after stimulation of cardiac myocytes with either the low or high concentration of H_2O_2, whereas Akt was activated (~4-fold) in response to 0.2 mM H_2O_2 only. ERK1/2 exhibited a 3–4-fold increase in phosphorylation at both H_2O_2 concentrations. Phosphorylation of p38-MAPK was more robust and particularly at the high concentration of H_2O_2 (4.6-fold at 0.02 mM vs 7.16-fold at 0.2 H_2O_2, $p < 0.05$)

To determine whether these kinases are involved in Bcl-2 phosphorylation observed during cardiac myocyte apoptosis, cells were pre-treated with inhibitors of the pathways before exposure to H_2O_2 and phosphorylated Bcl-2 was determined. As seen in Figure 5B, pre-treatment of cardiac myocytes with the p38-MAPK inhibitor, SB203580, resulted in attenuation of the H_2O_2-induced phosphorylation of

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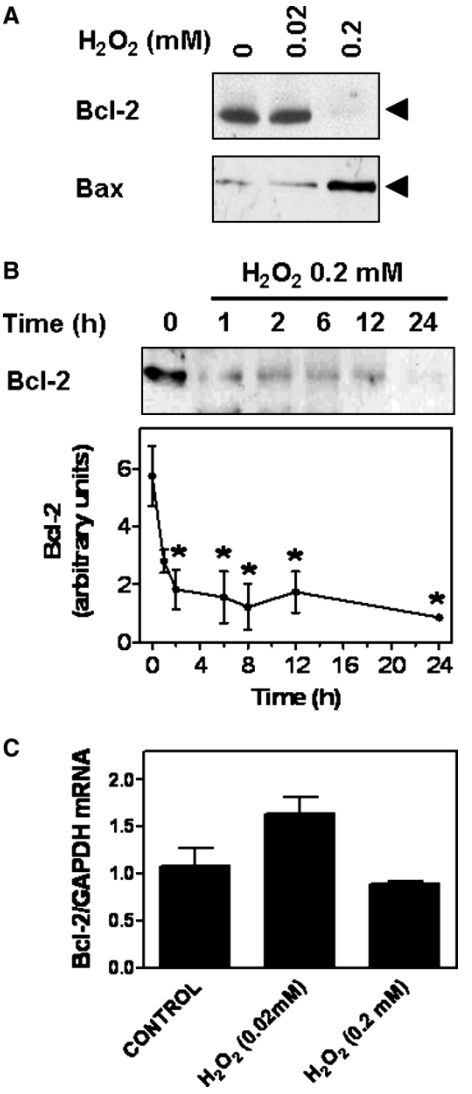

Figure 2. Effect of H_2O_2 on Bcl-2 family proteins. (A) Cardiac myocytes were exposed to 0.02 or 0.2 mM H_2O_2 for 24 h. Cell extracts were immunoblotted for Bcl-2 (upper panel) or Bax (lower panel). Representative blots are shown. The experiment was repeated three times with similar results. (B) Cardiac myocytes were exposed to 0.2 mM H_2O_2 for the times indicated. Cell extracts were immunoblotted for Bcl-2 (upper panel) and blots were quantified by scanning densitometry (lower panel). (C) Cardiac myocytes were exposed to 0.02 or 0.2 mM H_2O_2 for 24 h. mRNA was extracted and qPCR was performed for Bcl-2 and Gapdh. Results are means ± SEM from three independent experiments. $^*p < 0.05$ compared to control (one-way ANOVA with Dunnett post-test).

Bcl-2, whereas none of the other inhibitors tested had any effect.

Discussion

H_2O_2, as a physiologically relevant form of oxidative stress, induces cell death in neonatal and adult cardiac myocytes [13,24,27,28]. Whereas concentrations of $H_2O_2 > 0.05$ mM promote cardiac myocyte apoptosis, at high concentrations (> 1 mM) the process becomes unregulated and myocyte death

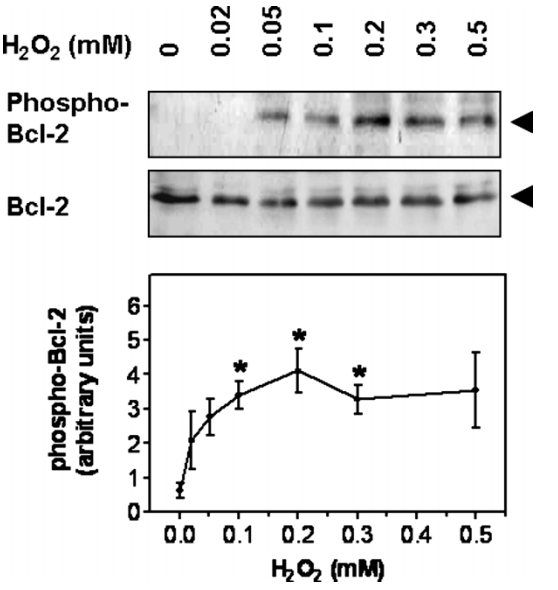

Figure 3. Bcl-2 is phosphorylated by increasing concentrations of H_2O_2. Cardiac myocytes were exposed to increasing concentrations of H_2O_2 for 15 min. Cell extracts were immunoblotted using antibodies for phospho-Bcl-2(Ser70) (upper panel) and Bcl-2 (middle panel). Blots were quantified by scanning densitometry (lower panel). Results are means ± SEM from four independent experiments. $^*p < 0.05$ compared to control (one-way ANOVA with Dunnett post-test).

becomes necrotic [7]. In contrast, low levels of H_2O_2 or other forms of oxidative stress may be involved in the cytoprotection afforded by ischemic pre-conditioning [5] or even have growth-promoting effects [2,7]. Although the exact mechanisms have not been fully elucidated, it is increasingly recognized that mitochondrial dysfunction and the Bcl-2 family proteins are key components of the apoptotic process in cardiac myocytes [4,13,24,28]. Bcl-2 proteins can be regulated either by transcription, heterodimerization or proteolytic cleavage, whereas their phosphorylation status plays important role in those events [4,29]. In this study, we employed two different levels of oxidative stress (0.02 mM and 0.2 mM H_2O_2) in order to investigate the regulation of the anti-apoptotic Bcl-2 protein in adult cardiac myocytes.

Induction of apoptosis by oxidative stress in adult cardiac myocytes had no effect on Bcl-2 mRNA, but resulted in down-regulation of Bcl-2 protein levels concomitant with an increase in the phosphorylation levels (Figure 1–3). Consistent with this, loss of Bcl-2 protein during oxidative stress was also observed in neonatal cardiac myocytes exposed to H_2O_2 [24,30]; however, the latter studies have not examined the phosphorylation of the protein. The mechanisms involved in the down-regulation of Bcl-2 protein were not investigated in the present study; increased protein degradation and/or reduced protein synthesis may be involved. However, the increased phosphorylation observed led us to speculate that

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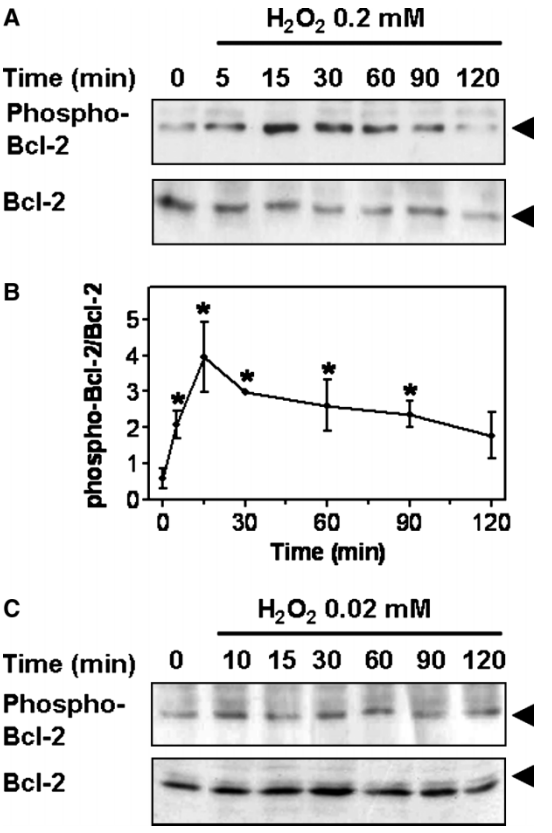

Figure 4. Time course of Bcl-2 phosphorylation in response to high and low doses of H_2O_2. (A) Cardiac myocytes were exposed to 0.2 mM H_2O_2 for the times indicated. Cells extracts were immunoblotted for phospho-Bcl-2(Ser70) (upper panel) and Bcl-2 (lower panel). (B) Blots were quantified by scanning densitometry and the ratio of phospho-Bcl-2/Bcl-2 was determined. Results are means ± SEM from three independent experiments. $^{\star}p < 0.05$ compared to control (one-way ANOVA with Dunnett post-test). (C) Cardiac myocytes were treated with 0.02 mM H_2O_2 for the times indicated. Cells extracts were immunoblotted for phospho-Bcl-2 (upper panel) and Bcl-2 (lower panel). Representative blots are shown. The experiment was repeated four times with similar results.

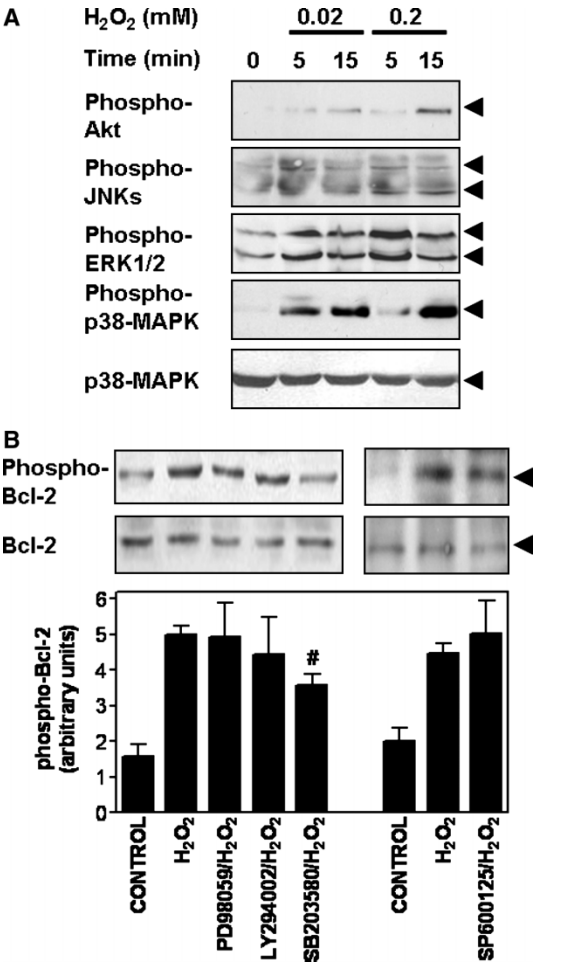

Figure 5. The role of protein kinases in Bcl-2 phosphorylation. (A) Cells were exposed to H_2O_2 (0.2 or 0.02 mM) for the times indicated. Extracts were immunoblotted with antibodies against phosphorylated Akt, JNKs, ERK1/2 and p38-MAPK. Equal protein loading was verified by immunoblotting for total p38-MAPK. Blots are representative from three-to-five independent experiments. (B) Cardiac myocytes were either not exposed to inhibitors or they were pre-treated with PD98059 (10 μM), LY294002 (50 μM), SB203580 (1 μM) or SP600125 (10 μM) for 10 min and then they were exposed to H_2O_2 (0.2 mM) for 15 min. Cell extracts were immunoblotted using antibodies for phospho-Bcl-2(Ser70) (upper panel) or Bcl-2 (middle panel). Blots were quantified by scanning densitometry (lower panel). Results are means ± SEM from four independent experiments. $\#p < 0.05$ compared to H_2O_2 (two-tailed Student's *t*-test).

phosphorylation of Bcl-2 decreases protein stability and facilitates protein degradation. It has been previously reported that phosphorylation of Bcl-2 targets the protein to the proteasome pathway [31].

The up-regulation of Bcl-2 mRNA by the low levels of oxidative stress (Figure 2C) in the absence of protein upregulation (Figure 2A) may represent an attempt to promote myocyte survival under conditions where global protein synthesis is inhibited [32]. Consistent with this, an increase of Bcl-2 mRNA has been also observed in isolated rat hearts subjected to ischaemia/reperfusion (A. Lazou, P.H. Sugden, A. Clerk, unpublished data).

The functional role of Bcl-2 phosphorylation has not been investigated before in cardiac myocytes. In other cell systems, many interpretations for the purpose of Bcl-2 phosphorylation have been proposed; for example, it has been suggested that Bcl-2 phosphorylation is involved in the inactivation of anti-apoptotic function [17,18,20,25], that is a marker of M phase events [33] and that promotes anti-apoptotic mechanisms [19]. Here, we demonstrated that Bcl-2 is phosphorylated in response to increased generation of ROS, which induce apoptosis in cardiac myocytes. These observations indicate that the phosphorylation of Bcl-2 results in the inactivation of its anti-apoptotic function; thus, the present findings are consistent with those of previous reports in other cell systems [17,18,20,25].

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Furthermore, increased Bcl-2 phosphorylation and Bcl-2 down-regulation was correlated with cardiac myocyte apoptosis after myocardial infarction [34] and ischemia/reperfusion [35] in rabbit heart. However, our findings remain in contrast with the observations obtained in certain cell lines in which unphosphorylated Bcl-2 displays less protection [19,36]. Thus, it is likely that the regulation of the anti-apoptotic function of Bcl-2 by phosphorylation is dependent on cell-type specific factors that are as yet undefined.

Increased oxidative stress may activate multiple cell signalling pathways, including the MAP kinase pathways. Oxidative stress has been shown to activate all three MAP kinase sub-families in neonatal rat ventricular myocytes [6] and isolated perfused rat hearts [37]. The dynamic balance among the effects of ERK1/2, JNKs and p38-MAPK is important in determining whether a cell will survive or undergo apoptosis [38]. In adult rat cardiac myocytes, p38-MAPK was more potently activated, as compared to the other MAPK sub-families, by low or high doses of H_2O_2 (Figure 5A). Furthermore, the p38-MAPK inhibitor attenuated Bcl-2 phosphorylation, indicating that this kinase mediates Bcl-2 phosphorylation (Figure 5B). In this context, p38-MAPK is activated by both concentrations of H_2O_2 (0.02 mM or 0.2 mM, Figure 5), whereas Bcl-2 is phosphorylated only in response to high levels of H_2O_2 (Figure 4). This could be due to the different amplitude of p38-MAPK phosphorylation induced by the two concentrations of H_2O_2 and/or different compartmentalization of the kinase. Correlation of p38-MAPK activation and Bcl-2 phosphorylation was also shown in rabbit heart after myocardial infarction which was associated with increased oxidative stress [34]. The close association between p38-MAPK activation and Bcl-2 phosphorylation has been shown previously in the cultured lymphoblastoid cells, mouse embryonic fibroblasts and neuronal cells [21,26,39]. Other studies have implicated ERK in the regulation of Bcl-2(S87) phosphorylation in human blood cells [20]. In addition, JNKs seem to mediate Bcl-2 phosphorylation in drug-induced apoptosis of carcinoma cells [18,22]. On the whole, these data support the hypothesis that several kinases can phosphorylate specific Bcl-2 residues and evoke different biologic events in different cell systems.

In conclusion, we have shown that induction of apoptosis by oxidative stress in adult cardiac myocytes results in down-regulation of Bcl-2 protein levels in parallel with an increase in its phosphorylation. It seems that p38-MAPK mediates this Bcl-2 phosphorylation. Our data would fit with the model where activation of p38-MAPK by the increased levels of ROS results in the phosphorylation and degradation of Bcl-2 and the inactivation of its anti-apoptotic activity.

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