Differential regulation of Bcl-2, AP-1 and NF-κB on cardiomyocyte apoptosis during myocardial ischemic stress adaptation

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Abstract Acute ischemia followed by prolonged reperfusion has been shown to induce cardiomyocyte apoptosis. In this report, we demonstrate that myocardial adaptation to ischemia induced by repeated cyclic episodes of short-term ischemia each followed by another short duration of reperfusion reduced cardiomyocyte apoptosis and DNA fragmentation. This was associated with the induction of the expression of Bcl-2 mRNA and translocation and activation of NF-κB. Another transcription factor, AP-1, remained unaffected by repeated ischemia and reperfusion, but exhibited significant upregulation by a single episode of 30 min ischemia followed by 2 h of reperfusion. This activation of AP-1 was inhibited by a scavenger of oxygen free radicals, DMTU. Thirty minutes ischemia and 120 min reperfusion downregulated the induction of the expression of Bcl-2 mRNA, but moderately activated NF-κB binding activity. This was associated with an increased number of apoptotic cells and DNA fragmentation in cardiomyocytes which were attenuated by DMTU. The results of this study indicate that Bcl-2, AP-1 and NF-κB differentially regulate cardiomyocyte apoptosis mediated by acute ischemia and prolonged reperfusion.

Key words: Bcl-2; AP-1; Nuclear factor κB; Ischemia; Reperfusion; Heart; Apoptosis; DNA fragmentation; Ischemic adaptation

1. Introduction

Recent studies have demonstrated that myocardial ischemia and reperfusion result in apoptotic cell death in addition to tissue necrosis [1–4]. Studies from our laboratory indicated translocation of phosphatidyl serine and phosphatidyl ethanolamine, a hallmark for apoptosis, occurs during ischemia, but apoptosis does not become apparent until hearts are reperfused following an ischemic insult [5]. Myocardial adaptation to ischemia induced by repeated cyclic episodes of reversible short periods of ischemia each followed by another short period of reperfusion was found to be effective in reducing apoptotic cell death [6].

Reactive oxygen species serve as a trigger for apoptosis in a variety of cell types [7,8]. A study from our laboratory demonstrated a role of oxygen free radicals in apoptotic cell death associated with ischemia and reperfusion [9,10]. Another recent study documented that ischemic adaptation translocated and increased the binding of nuclear transcription factor NF-κB in heart [11]. NF-κB is a member of the Rel transcription factor family which is involved in the regulation of stress defense mechanisms. Since ischemic adaptation was also found to reduce apoptosis, we speculated a direct role of NF-κB in apoptosis. AP-1 is another redox-sensitive signaling molecule which also plays an important regulatory role in cellular responses to stress induced by external factors including UV radiation, phorbol esters, and tumor necrosis factor α [12]. AP-1 is composed of either homodimers of c-Jun or heterodimers of c-Jun and c-Fos. The AP-1 binding site is the TRE (12-*O*-tetradecanoyl phorbol 13-acetate response element), and initiates transcription of target genes [13]. Induction of apoptosis by elevated levels of c-Jun is a crucial event in growth factor-deprived nerve cells [14]. Stress induced by ischemia/reperfusion was previously shown to induce the activation of c-Jun [15,16].

Bcl-2 is an oncogene which inversely regulates apoptosis [17,18]. A preliminary study from our laboratory showed upregulation of BCl-2 by ischemic adaptation [19]. Induction of Bcl-2 was found to be associated with reduction of apoptotic cell death and DNA fragmentation. In this study, we investigated the role of two redox-sensitive transcription factors, NF-κB and AP-1, and the anti-apoptotic gene, Bcl-2, in myocardial adaptation to ischemia and how these transacting factors regulate apoptosis during the stress induced by ischemia/reperfusion.

2. Materials and methods

2.1. Chemicals

p65 antibody was obtained from Santa Cruz Biotechnological Co., CA. AP-1 and NF-κB consensus oligonucleotides were purchased from Promega, Madison, WI. cDNA probe for Bcl-2 was obtained from Oncor, Gaithersburg, MD. All other chemicals were of high purity and obtained from Sigma Chemical Co., St. Louis, MO.

2.2. Isolated perfused heart preparation

Sprague Dawley rats weighing about 300 g were anesthetized with pentobarbital (80 mg/kg, i.p.). After intravenous administration of heparin (500 IU/kg), the chests are opened, the hearts were rapidly excised and mounted on a non-recirculating Langendorff perfusion apparatus [5,20]. Retrograde perfusion was established at a pressure of 100 cm H_2O with oxygenated normothermic Krebs-Henseleit bicarbonate (KHB) buffer with the following ion concentrations (in mM): 118.0 NaCl, 24.0 $NaHCO_3$, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 1.7 $CaCl_2$, and 10.0 glucose. The KHB buffer had been previously equilibrated with 95% O_2/5% CO_2, pH 7.4 at 37°C. The hearts were randomly divided into five groups. All hearts were allowed to stabilize for 15 min before any intervention. In group I, hearts were perfused for 240 min with the KHB buffer while for group II, hearts were perfused for 90 min with the same buffer followed by 30 min of ischemia and 120 min reperfusion. For group III, hearts were preperfused for 15 min with KHB buffer containing 10 mM DMTU, a hydroxyl radical scavenger. Hearts of groups IV and V were adapted by subjecting them a single cycle of 5 min of ischemia followed by 10 min of reperfusion (1×PC) (group IV) or four cyclic episodes of 5 min ischemia and 10 min reperfusion (4×PC) (group V) prior to

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subjecting them to a 30 min ischemia/120 min reperfusion protocol [11]. At the end of each experiment, hearts were frozen in liquid nitrogen and stored at −70°C until further analysis.

2.3. Determination of Bcl-2, NF-κB and AP-1 activities

2.3.1. AP-1. The oligonucleotide used for AP-1 consisted of the following sequence: 5′-CGCTTGATGAGTCAGCCGAA-3′. Gel shift assay was performed according to the manufacturer's (Promega) protocol with slight modification. ^{32}P end labeled oligonucleotide was incubated in a 10 μl reaction mixture containing 10 mmol/l Tris-HCl, pH 7.5, 0.5 mmol/l EDTA, 0.5 mmol/l DTT, 4% glycerol, 50 mmol/l NaCl, 1 mmol/l $MgCl_2$ and 0.5 μg poly(dI-dC) and 4.5 μg of nuclear extracts for 30 min at room temperature. Parallel competition experiments were also performed using unlabeled oligonucleotide (10–100 molar) which was added to the binding reaction mixture. After incubation, dye was added to the reaction mixture and the complex formed was separated in 4% polyacrylamide gel (acrylamide:bisacrylamide 30:1) by electrophoresis. The gel was subsequently dried and exposed to Kodak film at −70°C.

2.3.2. NF-κB. To determine NF-κB binding activity, nuclear proteins were isolated from the heart according to the method described previously [11]. In short, about 150 mg of left ventricle from heart tissue was homogenized in ice-cold Tris-buffered saline (TBS) and centrifuged at 3000×*g* for 5 min at 4°C. The pellet was resuspended by gentle pipetting in 1.5 ml of ice-cold hypotonic buffer containing 10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, and 1 μM each of aprotinin, pepstatin and leupeptin. The solution was allowed to swell on ice for 15 min. After addition of 100 μl of 10% Nonidet P-40, the tube was vigorously vortexed for 45 s. This homogenate was centrifuged for 30 s at 4°C in a microcentrifuge tube. The supernatant contained the cytoplasmic protein. The nuclear pellet was resuspended in a solution containing 20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF and 1 μM each of aprotinin, pepstatin and leupeptin. The tubes were vigorously shaken at 4°C for 30 min on a shaking platform. The extracts were then centrifuged and the supernatants were stored at −70°C. Protein concentration was estimated using the Pierce Protein Assay kit (Pierce Chemical Co., Rockford, IL).

NF-κB oligonucleotide (AGTTGAGG-GGACTTTCCCAGG) (2.5 μl [20 ng/μl]) was labeled using T4 polynucleotide kinase as previously described [11]. The binding reaction mixture contained in a total volume of 20.2 μl, 0.2 μl DTT (0.2 M), 1 μl BSA (20 mg/ml), 4 μl poly(dI-dC) (0.5 μg/μl), 2 μl buffer D^+, 4 μl buffer F, 2 μl ^{32}P-oligo (0.5 ng/μl) and 7 μl extract containing 10 μg protein. The composition of buffer D^+ was 20 mM HEPES, pH 7.9, 20% glycerol, 100 mM KCl, 0.5 mM EDTA, 0.25% Nonidet P-40 while buffer F contained 20% Ficoll 400, 100 mM HEPES, pH 7.9, and 300 mM KCl. Incubation was carried out for 20 min at room temperature. 10 μl of the solution was loaded onto a 4% acrylamide gel and separated at 80 V until the dye hit the bottom. After electrophoresis, gels were dried and exposed to Kodak X-ray film at −70°C. Autoradiographic results were evaluated quantitatively by an image analyzer. The binding signal from each sample was measured in the same sized area. The non-specific binding was determined by adding at least 60-fold excess of unlabeled DNA probes to the assay. Therefore, the specific binding was calculated by subtracting non-specific binding from total binding.

2.3.3. Bcl-2. Total RNA was extracted from the heart tissues by the acid-guanidinium thiocyanate-phenol-chloroform method as described previously [15]. For Northern blot analysis, total RNA was electrophoresed in 1% agarose formaldehyde-formamide gel and transferred to Gene Screen Plus hybridization transfer membrane (Biotech Systems, NEN Research products, Boston, MA). The membrane was then baked under vacuum at 80°C for 1 h. Each hybridization was repeated at least three times using different membranes. After each hybridization the residual cDNA was removed and rehybridized with GAPDH cDNA probe, the results of which served as a loading control. The autoradiograms were quantitatively evaluated by computerized β scanner. The results of densitometric scanning were normalized relative to the signal obtained using GAPDH cDNA.

2.4. Measurement of malonaldehyde (MDA) formation in heart

MDA was estimated in heart muscle to determine the development of oxidative stress and free radical generation as described previously [21]. In short, weighed heart biopsies were homogenized in 2 ml of 20% trichloroacetic acid, 5.3 mM sodium bisulfite, kept on ice for 10 min, centrifuged at 3000×*g* for 10 min, and then supernatants were collected, derivatized with 2,4-dinitrophenylhydrazine (DNPH), and extracted with pentane. Aliquots of 25 μl in acetonitrile were injected onto a Beckman Ultrasphere C_{18} (3 mm) column. The products were eluted isocratically with a mobile phase containing acetonitrile-water-acetic acid (40:60:0.1, v/v/v) and measured at three different wavelengths (307 nm, 325 nm and 356 nm) using a Waters M-490 multichannel UV detector. The peak for MDA was identified by co-chromatography with DNPH derivative of the authentic standard, peak addition, UV pattern of absorption at the three wavelengths, and by GC-MS.

2.5. Evaluation of apoptosis

To examine apoptosis, cardiomyocytes were obtained by established methods [5]. Following experiments, hearts (control, ischemic, reperfused) were quickly placed into a chilled dissociation buffer containing (mM): NaCl 137, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.0, KH_2PO_4 0.44, Na_2HPO_4 0.34, dextrose 5.6, HEPES buffer (pH 7.5) 20 N, penicillin 50 U/ml, and streptomycin 50 μg/ml. The ventricles were cut into 1–2 mm cubes and dissociated by trypsinization (0.05% trypsin-EDTA at 37°C for 10 min). Unfreed cells from the first treatment were discarded, and the sequence was repeated until all tissue was dissociated (approximately five times). Freed cells were collected in cold Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Gaithersburg, MD) supplemented with 0.5% fetal calf serum (FCS) and 0.002% DNase, and washed in the same medium. The isolated primary cardiac myocytes were used to evaluate DNA fragmentation.

Apoptotic cell death was evaluated using the TUNEL method using APOP TAG kit (Oncor, Gaithersburg, MD). In brief, control, ischemic/reperfused, and adapted heart tissues were immediately put in 10% formalin and fixed in an automatic tissue fixing machine. The tissues were carefully embedded in molten paraffin in metallic blocks, covered with flexible plastic moulds and kept under freezing plates to allow the paraffin to solidify. The metallic containers were removed and tissues became embedded in paraffin on the plastic moulds. Ten sections were made from each sample in order to make a clear judgement for each experiment. Prior to analyzing tissues for apoptosis, tissue sections were deparaffinized with xylene and washed in succession with different concentrations of ethanol (absolute, 95%, 70%). Tissues were then treated with proteinase K for 15 min at room temperature, excess liquids carefully blotted around the sections, 1× equilibrium buffer was applied directly on the specimens, and the specimens were placed in a humidified chamber for 5 min at room temperature. Specimens were then treated with terminal deoxynucleotidyl transferase (TdT) at 37°C for 1 h in a humidified chamber. After 1 h, coverslips were removed, and the specimens were placed in a Coplin jar containing stop/wash buffer (supplied in kit) for 10 min at room temperature. 52 μl of working strength anti-digoxigenin-fluorescein was added to the slides and incubated for 30 min at room temperature, washed in PBS, and counterstained with propidium iodide/antifade (supplied with kit) directly on the slide. Apoptotic cells were visualized by direct fluorescence detection of digoxigenin-labeled genomic DNA by epifluorescence using standard fluorescein excitation and emission filters with an Axiovert 100 TV microscope. This method was based on the new 3′-OH DNA end generated by DNA fragmentation and typically localized in morphologically identifiable nuclei and apoptotic bodies. In contrast, normal nuclei, which had relatively insignificant numbers of DNA 3′-OH ends, were not stained with this reagent.

To further characterize apoptosis, DNA was isolated from cardiomyocytes by standard techniques. In brief, myocytes were pelleted in an Eppendorf tube using 1000×*g* for 2 min. The supernatant was aspirated, 20 μl of lysis buffer (10 mM EDTA, 0.5% sarcosyl, 50 mM Tris-HCl, pH 8.0) was added, vortexed and placed at 4°C for 15 min. 1 μl of proteinase K (stock solution 20 mg/ml) was added to each sample. The samples were vortexed, and then incubated for 1 h at 50°C. After incubation for at least 1 h, 1 μl of RNase A (stock solution 10 mg/ml) was added and incubated for an additional hour at 37°C. 5 μl of gel loading buffer was added to the sample, and 10 μl of the DNA sample was electrophoresed on a 1.8% agarose gel with ethidium bromide. DNA laddering was visualized and photographed under ultraviolet transillumination.

3. Results

3.1. Effects of PC and I/R on the consensus AP-1 binding activity

Electrophoretic mobility shift assay indicated increased AP-1 binding activity in the ischemic/reperfused rat heart (Fig. 1, lane B) compared to the control perfused group (lane A). DMTU inhibited this binding activity significantly as shown in Fig. 1C. In ischemically adapted groups (both 1×PC and 4×PC), the binding activity of AP-1 did not exhibit any alteration compared to baseline control (Fig. 1D,E). The binding signal obtained from the image analyzer confirmed that AP-1 has greater binding activity in ischemic/reperfused rat hearts compared to any other groups.

3.2. Effects of PC and I/R on nuclear translocation of NF-κB

NF-κB binding activity was found to be very low in non-ischemic control hearts (Fig. 2A). Reperfusion of ischemic myocardium moderately increased the translocation of NF-κB from cytosol to nucleus (Fig. 2B). Perfusion of the heart with DMTU inhibited NF-κB translocation from cytosol to nucleus as shown in Fig. 2C. NF-κB binding activity was increased significantly (5-fold) for the ischemically adapted hearts when compared to the ischemic/reperfused myocardium. For example, the binding increased slightly for 1×PC hearts (Fig. 2D) and appreciably for 4×PC hearts (Fig. 2E). To confirm the specificity of NF-κB binding activity, we performed supershift assays with polyclonal antibody recognizing NF-κB p65 subunit (results not shown).

3.3. Effects of PC and I/R on Bcl-2 mRNA expression

Northern blot analysis revealed Bcl-2 gene upregulation in ischemically adapted hearts (1×PC, Fig. 3D; 4×PC, Fig. 3E) compared to the control hearts (Fig. 3A). In contrast, prolonged reperfusion (2 h) after acute ischemia (30 min) downregulated Bcl-2 gene significantly as shown in Fig. 3B. Perfusion of the hearts with DMTU increased Bcl-2 mRNA activities (Fig. 3C).

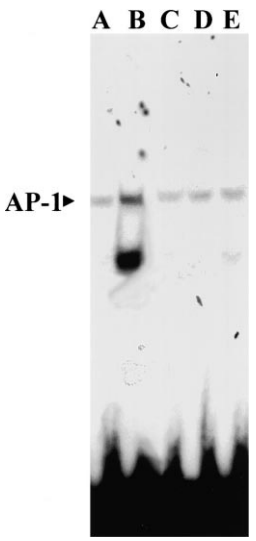

Fig. 1. AP-1 binding activity in rat heart. Ischemia/reperfusion stimulates AP-1 binding activity in rat myocardium. Nuclear extracts were isolated from control and experimental hearts. These extracts were then used for electrophorectic mobility shift assay (EMSA) as described in Section 2. Lane A: control; lane B: ischemia/reperfusion; lane C: DMTU+ischemia/reperfusion; lane D: 1×PC+ischemia/reperfusion; lane E: 4×PC+ischemia/reperfusion.

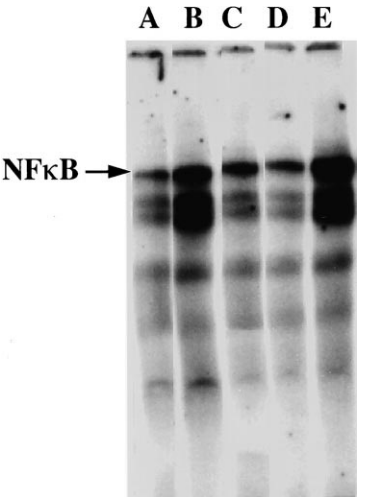

Fig. 2. NF-κB binding activity in rat heart. Ischemia/reperfusion as well as PC increases NF-κB translocation. Nuclear extracts were isolated from control and experimental hearts. These extracts were then used for electrophoretic mobility shift assay as described in Section 2. Lane A: control; lane B: ischemia/reperfusion; lane C: DMTU+ischemia/reperfusion; lane D: 1×PC+ischemia/reperfusion; lane E: 4×PC+ischemia/reperfusion.

3.4. Effects of PC and I/R on cardiomyocyte apoptosis

The number of apoptotic cells was significantly higher (23%) in the ischemic/reperfused myocardium (Fig. 4B) compared to the control hearts (Fig. 4A). DMTU significantly reduced the number of apoptotic cells (Fig. 4C) compared to ischemic/reperfused hearts (Fig. 4B). Myocardial adaptation to ischemia reduced the number of apoptotic cells. For 1×PC hearts the extent of apoptotic cell death was reduced to 18% (Fig. 4D) while almost no apoptotic cells were noticed in the 4×PC hearts (Fig. 4E).

3.5. Effects of PC and I/R on cardiomyocyte DNA laddering

DNA fragmentation was clearly visualized in the hearts subjected to 30 min of ischemia followed by 2 h of reperfusion (Fig. 5, lane C). DNA ladders were not apparent in the control hearts (Fig. 5, lane B). DMTU significantly reduced DNA fragmentation when applied to the heart prior to ischemic insult (Fig. 5, lane D). Ischemic adaptation was also associated with a decrease in DNA fragmentation. The extent of inhibition was more in 4×PC (Fig. 5, lane F) than in 1×PC (Fig. 5, lane E) hearts.

3.6. Effects of PC and I/R on MDA formation in heart

The production of MDA is an indicator for the develop-

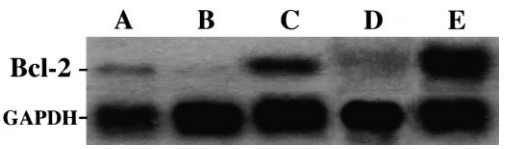

Fig. 3. Northern blot analysis of Bcl-2 mRNA. Northern blot analysis reveals the induction of Bcl-2 in preconditioned rat myocardium. Total RNA was isolated and Northern hybridization was performed as described in Section 2. Lane A: control; lane B: ischemia/reperfusion; lane C: DMTU+ischemia/reperfusion; lane D: 1×PC+ischemia/reperfusion; lane E: 4×PC+ischemia/reperfusion.

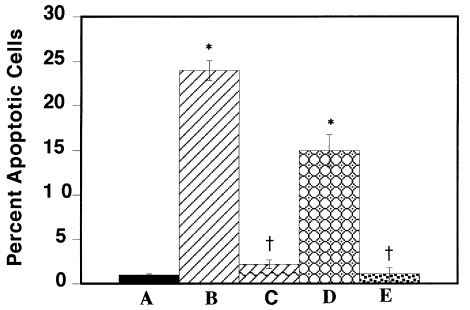

Fig. 4. Evaluation of apoptosis by the TUNEL method. Evaluation of apoptosis reveals an increased number of apoptotic cells in the ischemic/reperfused myocardium. Sections of control and experimental heart tissues were analyzed for apoptosis using APOP TAG kit as described in Section 2. Percent apoptotic cells is shown by bar graphs. Results are expressed as means ± S.E.M of six rats per group. $^{*}P < 0.05$ vs. baseline control, $^{\dagger}P < 0.05$ compared to ischemic/reperfused cntrol. A: control; B: ischemia/reperfusion; C: DMTU+ischemia/reperfusion; D: 1×PC+ischemia/reperfusion; E: 4×PC+ischemia/reperfusion.

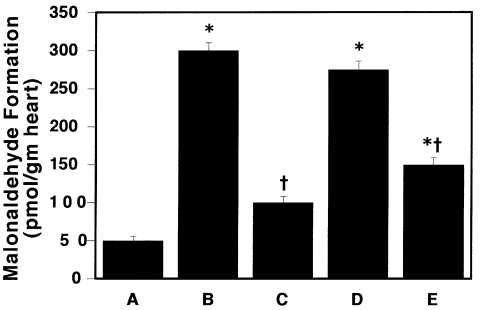

Fig. 6. Estimation of MDA production by HPLC. Control and experimental hearts were processed, MDA extracted and derivatized with DNPH as described in Section 2. MDA formation is shown by bar graphs. Results are expressed as means ± S.E.M of six rats per group. $^{*}P < 0.05$ vs. baseline control, $^{\dagger}P < 0.05$ compared to ischemic/reperfused control. A: control; B: ischemia/reperfusion; C: DMTU+ischemia/reperfusion; D: 1×PC+ischemia/reperfusion; E: 4×PC+ischemia/reperfusion.

ment of oxidative stress. We have estimated the level of MDA formation to monitor the extent of lipid peroxidation. As shown in Fig. 6B, after 30 min of ischemia followed by 120 min of reperfusion, MDA content was 300 ± 10.5 pmol/g compared to only 50 ± 6 pmol/g for the perfused hearts (Fig. 6A). At the end of 2 h reperfusion, MDA content in the DMTU-treated hearts was only 100 ± 8 pmol/g demonstrating that DMTU lowered the oxidative stress in the heart. In the 1×PC group (Fig. 6D), MDA content at the end of the experiment was 275 ± 11 pmol/g compared to 150 ± 9 pmol/g MDA observed for the 4×PC group (Fig. 6E).

4. Discussion

The heart possesses a remarkable ability to adapt itself to any stressful situation by increasing resistance to the adverse consequences. Creating a stress reaction by repeated ischemia and reperfusion or subjecting the hearts to heat or oxidative stress enables the heart to meet the future stress challenge by upregulating its cellular defense through direct accumulation of intracellular mediators which constitute the material basis of increased adaptation to stress. Thus, the powerful cardioprotective effect of adaptation is likely to originate at the cellular and molecular levels. In this report, we demonstrate that repeated cyclic episodes of ischemia and reperfusion reduced cardiomyocyte apoptosis and DNA fragmentation. This was associated with the induction of the expression of Bcl-2 mRNA and translocation and activation of NF-κB. Another transcription factor, AP-1, remained unaffected by repeated ischemia and reperfusion, but exhibited significant upregulation by a single episode of 30 min ischemia followed by 2 h of reperfusion. This activation of AP-1 was inhibited by a scavenger of oxygen free radicals, DMTU, when administered prior to ischemia. Thirty minutes ischemia and 120 min reperfusion inhibited the induction of the expression of Bcl-2, but activated NF-κB binding activity to some extent.

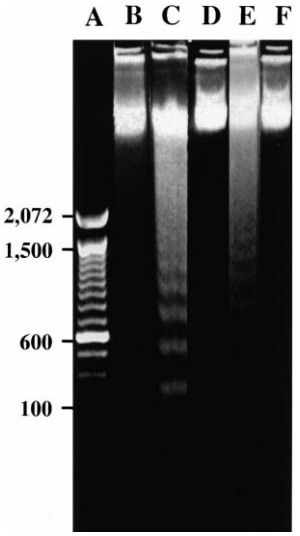

Fig. 5. DNA fragmentation in ischemic/reperfused heart. DNA fragmentation was increased in the ischemic/reperfused myocardium. Cardiomyocytes were isolated and DNA was extracted from control and experimental rat myocardium as described in Section 2. Lane A: DNA marker; lane B: control; lane C: ischemia/reperfusion; lane D: DMTU+ischemia/reperfusion; lane E: 1×PC+ischemia/reperfusion; lane F: 4×PC+ischemia/reperfusion.

Ischemic preconditioning is the manifestation of the earlier stress response that occurs during repeated episodes of brief ischemia and reperfusion, and can render the myocardium more tolerant to a subsequent potential lethal ischemic injury [22,23]. This transient adaptive response has been demonstrated to be associated with decreased reperfusion-induced arrhythmias [24], increased recovery of postischemic contractile functions [25,26], and reduction of infarct size [27]. The adaptive protection is believed to be mediated by gene expression and their transcriptional regulation [28–30]. Recent findings indicate that multiple kinases including MAP kinases and MAPKAP kinase 2 are likely to be involved in the adaptive signaling process [31–33]. The acutely developing adaptive effect is short-lived, lasting for only up to 2–3 h. Hearts can subsequently undergo a secondary and delayed adaptation to stress presumably through the induction of the expression of new genes and their subsequent translation into proteins. A number of genes and proteins have been identified as possibly involved in the development of delayed preconditioning including heat shock proteins, superoxide dismutase, catalase, nitric oxide synthase as well as ATPase 6 and cytochrome *b* subunits [31]. Such an adaptive response becomes evident only after approximately 24 h of stress treatment and may include

stress induced by heat shock, oxidant or other stress-inducible agents. MAPKAP kinase 2 appears to link the early preconditioning effect to the delayed adaptive response [34].

Our laboratory demonstrated that ischemic preconditioning triggers a signaling pathway by potentiating tyrosine kinase phosphorylation [34]. The signal transduction involves phospholipase D which subsequently transmits the signal via the activation of MAP kinases. Our results clearly indicated a role of tyrosine kinase, because inhibition of tyrosine kinase phosphorylation by genistein almost completely blocked the activation of protein kinase C, MAP kinases and MAPKAP kinase 2. Subsequently, we were able to identify p38 MAP kinase as one of the potential targets for tyrosine kinase phosphorylation. A role of tyrosine kinase and p38 MAP kinase in ischemic adaptation has been supported by other investigators [35,36]. For example, a correlation was found between p38 MAPK phosphorylation and protection by preconditioning, suggesting that its activation might represent a crucial step in the signal transduction cascade of ischemic preconditioning. In this study, the investigators showed that simulated preconditioning in isolated rabbit cardiomyocytes was prevented by SB203580 and was mimicked by the p38-MAPK/JNK activator, anisomycin [35]. An inhibitor of tyrosine kinase, genistein, blocked the myocardial adaptation to ischemia [36].

Studies from different laboratories including our own demonstrated that reperfusion of ischemic myocardium results in cardiomyocyte apoptosis in addition to necrosis. We have shown that a hallmark of apoptosis, translocation of phosphatidyl serine and phosphatidyl ethanolamine, occurs during ischemia, but execution of apoptosis does not occur until the late phase of reperfusion [5]. Oxidative stress developed in the ischemic/reperfused myocardium was found to be instrumental for apoptotic cell death [9]. Another related study showed that myocardial adaptation to ischemia provided cardioprotection by blocking apoptotic cell death [6]. Prolonged reperfusion after ischemia caused downregulation of the antioxidant gene, Bcl-2, in concert with enhanced DNA fragmentation [17].

Evidence is rapidly accumulating to support the notion that oxygen-derived free radicals are involved in transmembrane signaling processes. In a recent study, a tyrosine kinase inhibitor, herbimycin A, and a free radical scavenger, *N*-acetylcysteine, were found to inhibit free radical-induced activation of NF-κB indicating that activation triggered by reactive oxygen species is dependent on tyrosine kinase activity [37]. A large number of studies from different laboratories including our own showed the induction of the expression of antioxidant genes during preconditioning [28]. Our recent study on the role of NF-κB in tyrosine kinase signaling of ischemic adaptation and MAPKAP kinase 2 phosphorylation supports these earlier findings [11]. Existing evidence that oxidative stress/free radicals lead to the activation of NF-κB thereby inducing the expression of genes also supports the role of reactive oxygen species as messenger molecules [37,38].

A significant amount of evidence exists in the literature indicating a crucial role of oxygen free radicals in myocardial ischemia/reperfusion injury. The presence of reactive oxygen species, especially hydroxyl radical ($OH^{\bullet}$), in the ischemic/reperfused heart has been demonstrated both directly [39] and indirectly [40]. It is possible that $OH^{\bullet}$ radical formed by transient metal-catalyzed Fenton reaction during the reperfusion of ischemic heart [41] induced NF-κB activation. Inhibition of NF-κB translocation and AP-1 induction by DMTU, a $OH^{\bullet}$ scavenger, further supports a role of free radicals as a signaling molecule.

The most interesting finding of this study is that both AP-1 and NF-κB are activated after 30 min ischemia followed by 2 h of reperfusion. Such activation of AP-1 and NF-κB was blocked by scavenging the reactive oxygen species suggesting that the activation of these transcription factors is mediated by oxidative stress. Myocardial adaptation induced by cyclic episodes of short-term ischemia and reperfusion results in the activation of NF-κB by 5-fold, while AP-1 remained unaffected. Interestingly, the Bcl-2 gene is downregulated after 30 min ischemia and 2 h reperfusion, but induced significantly after 5 min ischemia/10 min reperfusion and further enhanced after four cyclic episodes of ischemia and reperfusion. In concert, MDA formation, a presumptive marker for oxidative stress, which is increased after the initial phase of ischemia/reperfusion, decreased after ischemic adaptation. Cardiomyocyte apoptosis and DNA fragmentation also increased after prolonged reperfusion, but decreased after ischemic adaptation.

In the majority of cells, NF-κB exists as a cytoplasmic complex by binding with its inhibitory protein IκB. Phosphorylation of IκB by oxidative stress induced by ischemia/reperfusion can cause dissociation from NF-κB. We have shown moderate translocation of NF-κB in the ischemic/reperfused heart and significant translocation in the preconditioned heart from the cytosol to the nucleus by EMSA assay. We have also investigated the loss of IκB protein after dissociation in the cytoplasm caused by ischemia/reperfusion and preconditioning. The kinetics of NF-κB binding activity in the nuclear extracts correlate with the kinetics for IκBα protein disappearance in the cytoplasm. The data (not shown) suggest that the disappearance of IκBα protein from cytoplasm resulted in the translocation of the NF-κB complex to the nucleus as an active form.

Preconditioning of the ischemic myocardium increased the extent of NF-κB translocation (Fig. 2, lane E) to a greater extent than ischemia and reperfusion (Fig. 2, lane B). In concert, Bcl-2 gene expression in the ischemic reperfused myocardium was significantly reduced (Fig. 3, lane B) when compared to the preconditioned heart (Fig. 3, lane E). Therefore, when the extent of NF-κB translocation is significantly elevated in the preconditioned heart, significant upregulation of Bcl-2 also occurs.

The results of our study indicate a direct role of AP-1 on cardiomyocyte apoptosis. Reperfusion of ischemic myocardium results in the activation of AP-1 simultaneously causing apoptotic cell death. Conversely, ischemic stress adaptation downregulates AP-1 in concert with the attenuation of apoptosis. These results receive support from the previous observations that increased expression of components of AP-1 is linked to apoptosis [42–44]. A recent study demonstrated that c-Jun/AP-1, but not NF-κB, is a mediator for oxidant-initiated apoptosis in glomerular mesangial cells [45]. In this study, using Northern blot analysis and transient transfection assays with reporter plasmids, the authors showed that H_2O_2 activated both AP-1 and NF-κB. Downregulation of c-Jun/AP-1 using a transdominant negative mutant of c-Jun inhibited apoptotic cell death while use of a transdominant negative mutant of p50 NF-κB subunits did not affect H_2O_2-medi-

ated apoptosis. Our study showed activation of both AP-1, but the anti-apoptotic signal induced by ischemic adaptation did not alter AP-1. NF-κB, on the other hand, was slightly activated after ischemia/reperfusion, but significantly activated during ischemic stress adaptation. A previous study demonstrated that activation of NF-κB is a necessary step for myocardial adaptation to ischemic stress. Taken together, the present results suggest that activation of NF-κB during ischemic adaptation is associated with a decrease in cardiomyocyte apoptosis when AP-1 remains downregulated at the baseline level. In contrast, an increase in AP-1 during ischemia/reperfusion is associated with cardiomyocyte apoptosis when NF-κB remains significantly downregulated compared to that in the preconditioned heart.

In summary, to the best of our knowledge, the present findings demonstrated for the first time differential regulation of AP-1, NF-κB and the Bcl-2 gene in myocardial adaptation to ischemia. Ischemic adaptation resulted in reduced oxidative stress, decreased apoptosis and unchanged AP-1 binding activity, but increased NF-κB and upregulation of the Bcl-2 gene. Analysis of these seemingly conflicting data suggests that while both AP-1 and NF-κB are induced through the oxidative stress developed in the ischemic/reperfused myocardium, cardiomyocyte apoptosis occurs when AP-1 remains high and NF-κB remains low as in the case of ischemia/reperfusion. Cardiomyocyte death due to apoptosis remains low when NF-κB binding activity is high and AP-1 is low as in the case of ischemic preconditioning. The Bcl-2 gene is decreased after prolonged reperfusion (2 h) following an acute ischemic insult (30 min), but it becomes upregulated by ischemic adaptation. The results of this study also suggest that oxygen free radicals play a role in the signal transduction process triggered by ischemic adaptation.

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