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Redox Regulation of NF-κB and AP-1 in Ischemic Reperfused Heart

HIROAKI SASAKI, NATHANIEL GALANG, and NILANJANA MAULIK

ABSTRACT

Two redox-sensitive transcription factors, AP-1 and NF-κB, have been implicated in the regulation of apoptosis induced by myocardial ischemia and reperfusion. Hearts adapted to ischemic stress by cyclic episodes of short durations of ischemia and reperfusion attenuate apoptotic cell death. This study was designed to examine the pattern of expression of these transcription factors and the redox sensitive transacting molecule, AP-1, NF-κB, and-Bcl-2, during ischemia/ reperfusion and myocardial adaptation to ischemia. NF-κB binding activity was low in nonischemic control heart. Fifteen minutes of ischemia resulted in translocation of NF-κB from cytosol to nucleus followed by activation. The binding activity of NF-κB was further enhanced after 60 min of ischemia. An even higher degree of NF-κB binding was noticed in the ischemically adapted myocardium. In contrast, AP-1 binding activity was highest for the hearts subjected to 15 min of ischemia followed by 2 hr of reperfusion. AP-1 binding was higher in the ischemically adapted heart as compared to the control. The Bcl-2 gene, which was found to be present in the control hearts, had lowered expression after 15 min of ischemia and 2 hr of reperfusion. Significant upregulation of Bcl-2 mRNA was noticed in the ischemically adapted hearts. Apoptotic cardiomyocytes were found only in the hearts that were reperfused for at least 90 min. No apoptosis occurred in hearts subjected up to 1 hr of ischemia or ischemic adaptation. Prolonged reperfusion, and not ischemia up to 1 hr, can induce cardiomyocyte apoptosis. In concert, ischemic/reperfusion increases the nuclear binding of both AP-1 and NF-κB, but downregulates Bcl-2 gene. Ischemic adaptation attenuates apoptotic cell death, further increases NF-κB binding activity and Bcl-2 gene induction, but reduces AP-1 binding activity. These results suggest that AP-1, NF-κB, and Bcl-2 are differentially regulated by ischemia/reperfusion and ischemic adaptation. Antiox. Redox Signal. 1, 317–324.

INTRODUCTION

A SIGNIFICANT NUMBER OF REPORTS exist in the literature to indicate that reactive oxygen species (ROS) are formed during ischemia and reperfusion and play a crucial role in the pathophysiology of ischemic heart disease (Das and Engelman, 1992). Presence of oxygen free radicals in the ischemic reperfused myocardium has been demonstrated directly by using electron spin resonance spectroscopy (ESR) and high-performance liquid chromatography (HPLC) techniques, as well as indirectly from the increased production of lipid peroxidation and protein and DNA damage (Das and Maulik, 1994). To meet the challenge of the potentially destructive reactive oxygen molecules, the heart is well equipped with its own antioxidant defense system (Das *et al.*, 1995). A delicate balance between pro- and anti-oxidants is maintained in the normal heart. This oxidant–antioxidant balance is disturbed in the

Molecular Cardiology Laboratory, Department of Surgery, University of Connecticut School of Medicine, Farmington, Connecticut.

ischemic myocardium because of decreased antioxidant levels and excessive production of free radicals, which leads to modification of the signal transduction system.

Regulation of intracellular redox is a unique control mechanism in signal transduction. Antioxidants steer the intracellular redox status. Major reducing agents inside cells controlling the redox comprise glutaredoxin, thioredoxin, and the glutathione (Wieles *et al.*, 1995). Recent studies demonstrated that all of the aforementioned redox-regulating components are released from the ischemic myocardium leading to a net loss of redox system (Nakamura *et al.*, 1998).

This redox signaling plays a pivotal role in myocardial ischemic reperfusion injury. We and others have demonstrated that reperfusion of ischemic myocardium results in cardiomyocyte apoptosis (Gottlieb *et al.*, 1994; Kajstura *et al.*, 1996; Maulik *et al.*, 1998a). Reduction of oxidative stress by scavenging free radicals or any means can reduce apoptotic cell death. For example, ebselen, a glutathione peroxidase mimic, was found to reduce cardiomyocyte apoptosis significantly during ischemia and reperfusion (Maulik *et al.*, 1998b). A reduction of oxidative stress by adapting the heart to ischemia resulted in reduction of apoptosis (Maulik *et al.*, 1998c).

There are two well-known redox regulated transcription factors, nuclear factor kappa B (NF-κB) and c-Jun/activator protein-1 (AP-1), which participate in the regulation of apoptosis in many cell types (Sun and Oberley, 1998). The transcription factor NF-κB is a nuclear protein of the Rel oncogene family and involved in the regulation of numerous genes (Ghosh *et al.*, 1990). It exists in the cytoplasm as an inactive form and is stabilized by an inhibitory subunit IκB, which inhibits the DNA binding activity. The transcriptional factors c-Jun and c-Fos form heterodimers or homodimers that binds to DNA, and the complex formed by this proteins is AP-1 (Angel and Karin, 1991). Recent studies have revealed like AP-1, NF-κB stimulates or inhibits oxidative stress-induced apoptosis in a trigger-dependent or a cell-type-specific manner (Lin *et al.*, 1995; Beg and Baltimore, 1996). In this report, we examined the binding pattern of AP-1 and NF-κB in rat heart during ischemia, reperfusion, and ischemic adaptation to determine how these redox-regulated molecules govern apoptosis. To explore further the molecular mechanisms involved in apoptosis, the role of another redox-sensitive transacting molecule, Bcl-2, was also studied.

MATERIALS AND METHODS

Chemicals

p65 antibody was obtained from Santa Cruz Biotechnological Co. (CA). The Gel-Shift Assay kit for NF-κB and AP-1 was purchased from Promega (Madison, WI). The cDNA probe for Bcl-2 was obtained from Oncor (Gaithersburg, MD). DNA labeling kit was purchased from Boehringer Mannheim (Indianapolis, IN). [γ-^{32}P]ATP and [α-^{32}P]dCTP were obtained from Amersham (Arlington Heights, IL). All other chemicals were of high purity and obtained from Sigma Chemical Co. (St. Louis, MO).

Isolated perfused heart preparation

Sprague Dawley rats weighing about 300 grams were anesthetized with pentobarbital (80 mg/kg, i.p.). After intravenous administration of heparin (500 IU/kg), the chests were opened, and the hearts were excised rapidly and mounted on a non-recirculating Langendorff perfusion apparatus (Engelman *et al.*, 1995). The perfusion buffer used in this study consisted of a modified Krebs-Henseleit bicarbonate buffer (KHB) (in m*M*: 118 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 10 glucose, and 1.7 $CaCl_2$, gassed with 95% O_2–5% CO_2, filtered through a 5-mm filter to remove any particulate contaminants, pH 7.4) which was maintained at a constant temperature of 37°C and was gassed continuously for the entire duration of the experiment. Left atrial cannulation was then carried out and, after allowing for a stabilization period of 10 min in the retrograde perfusion mode, the circuit was switched to the antegrade working mode, which allows for the measurement of myocardial contractility as well as aortic and coronary flows, as described in detail in a previous paper (Engelman *et al.*, 1995). Essentially, it is a left-heart preparation in which the heart is per-

fused with a constant preload of 17 cm of H_2O (being maintained by means of a Masterflex variable speed modular pump, Cole Parmer Instrument Company, Vernon Hills, IL) and pumps against an afterload of 100 cm of H_2O. At the end of 10 min, after the attainment of steady-state cardiac function, baseline functional parameters were recorded as usual. The circuit was then switched back to the retrograde mode. The hearts were divided into six groups, with six in each group. After stabilization the hearts were perfused with KHB buffer for 195 min (Group I); Group II hearts were subjected to 15 min of global ischemia; Group III hearts were subjected to 60 min of ischemia only; Group IV hearts were perfused with KHB for 1 hr and then subjected to 15 min of global ischemia followed by 120 min of reperfusion. In Group V, the hearts were subjected to ischemic stress adaptation by repeated ischemia and reperfusion by inducing global ischemia for 5 min followed by 10 min of reperfusion; the process was repeated four times (4×PC) as described previously (Kimura *et al.*, 1992). Group VI hearts were subjected to 4×PC followed by 15 min of ischemia and 120 min of reperfusion. The first 10 min of reperfusion was in the retrograde mode to allow for post-ischemic stabilization and thereafter in the antegrade working mode to allow for assessment of functional parameters. A schematic diagram of the protocol is shown in Fig. 1.

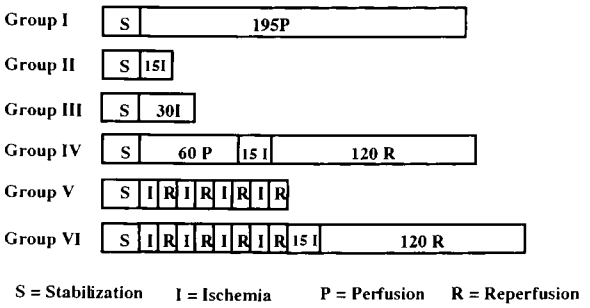

FIG. 1. Experimental protocol.

For NF-κB and AP-1 binding activity, as well as for Bcl-2 gene expression, left ventricles from the control and experimental hearts were kept frozen at the temperature of liquid nitrogen. The extent of myocardial apoptosis was also evaluated in the heart after each experiment.

Electrophoretic mobility assay for NF-κB and AP-1

The electrophoretic mobility shift assay (EMSA) was performed according to the manufacturer's (Promega) protocol with slight modifications.

NF-κB. Nuclear proteins were isolated from the heart to estimate NF-κB translocation according to the method described previously (Maulik *et al.*, 1998d). The nuclear extracts were stored at −70°C. Protein concentration was estimated by using a Pierce protein assay kit (Pierce Chemical Company, Rockford, IL). NF-κB oligonucleotide (AGTTGAGG-GGACTTTCCCAGG) (2.5 μl [20 ng/μl]) was labeled using T4 polynucleotide kinase as previously described. The binding reaction mixture was contained in a total volume of 20.2 μl: 0.2 μl of dithiothreitol (DTT) (0.2 *M*), 1 μl of BSA (20 mg/ml), 4 μl of PdI-dC (0.5 μg/μl), 2 μl of Buffer D^+, 4 μl of Buffer F, 2 μl of ^{32}P-oligo (0.5 ng/μl), and 7 μl of extract containing 10 μg of protein. The composition of Buffer D^+ was 20 m*M* HEPES, pH 7.9, 20% glycerol, 100 m*M* KCl, 0.5 m*M* EDTA, and 0.25% NP 40; Buffer F contained 20% Ficoll 400, 100 m*M* HEPEs, pH 7.9, and 300 m*M* KCl. Incubation was carried out for 20 min at room temperature. Ten microliters 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 up and exposed to Kodak X-ray film at −70°C.

AP-1. The oligonucleotide used for AP-1 consisted of the following sequence: 5′-CGCTTGATGAGTCAGCCGAA-3′. ^{32}P-end-labeled oligonucleotide was incubated in a 10-μl reaction mixture containing 10 mmol/liter Tris-HCl, pH 7.5, 0.5 mmol/liter EDTA, 0.5 mmol/liter DTT, 4% glycerol, 50 mmol/liter NaCl, 1 mmol/liter $MgCl_2$, and 0.5 μg of 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.

Bcl-2. Total RNA was extracted from the heart tissues by the acid-guanidinium thiocyanate-phenol-chloroform method as described previously (Maulik *et al.*, 1996). For northern blot analysis, total RNA was electrophoresed in 1% agarose formaldehyde-formamide gel and transferred to a 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 hr. Each hybridization was repeated at least three times using different membranes. After each hybridization, the residual cDNA was removed and rehybridized with a GAPDH cDNA probe, the results of which served as a loading control. The autoradiograms were quantitatively evaluated by a computerized β-scanner. The results of densitometric scanning were normalized relative to the signal obtained by using GAPDH cDNA.

Detection of apoptotic cells. Apoptotic cells were detected by labeling the 3'OH ends of DNA using digoxigenin incorporation by TDT enzymes (Maulik *et al.*, 1998a). In brief, paraffinized sections were deparaffinized with xylene and washed in succession with different concentrations of ethanol. Sections were treated with Proteinase K followed by TDT at 37°C for 1 hr. After using stop/wash solution to the sections, anti-digoxigenin-fluorescein was added to the slides and incubated for 30 min, washed, and counterstained with PI/antifade directly on the slides. Apoptotic cells were detected by direct fluorescence detection of digoxigenin-labeled genomic DNA by epifluorescence using standard fluorescein excitation and emission filters with an Axiovert 100 TV microscope.

Statistical analysis

For statistical analysis, a two-way analysis of variance (ANOVA) followed by Scheffe's test was first carried out using Primer Computer Program (McGraw-Hill, 1988) to test for any differences between groups. If differences were established, the values were compared using Student's *t*-test for paired data. The values were expressed as mean ± SEM. The results were considered significant if p was less than 0.05.

RESULTS

The NF-κB binding activity during different duration of ischemia, ischemia reperfusion, and ischemic preconditioning

NF-κB binding activity was found to be very low in nonischemic control hearts (Fig. 2A). Global ischemia for 15 min, as well as for 60 min, significantly increased the translocation of NF-κB from cytosol to nucleus (Fig. 2B,C). NF-κB binding activity was also increased significantly (five-fold, Fig. 2F) for the ischemically adapted hearts when compared to the ischemic reperfused myocardium (Fig. 2D). To confirm the specificity of NF-κB binding activity, we performed supershift assays with a polyclonal antibody recognizing the NF-κB p65 subunit (results not shown).

The consensus AP-1 binding activity during different duration of ischemia, ischemia/reperfusion, and ischemic preconditioning

EMSA indicated increased AP-1 binding activity in the ischemic (15 min as well as 60 min) rat heart (Fig. 3, lanes B and C) compared to

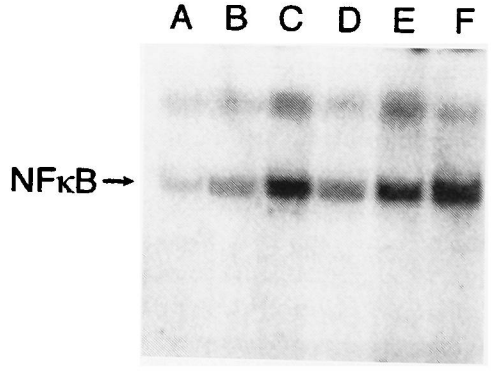

FIG. 2. Effect of ischemia, ischemia/reperfusion, and ischemic preconditioning on the binding activity of NF-κB. Nuclear protein extracts were isolated from control and experimental hearts. The extracts were then used for EMSA as described in the Materials and Methods section. Lane A, Perfused control heart; lane B, 15 min ischemia; lane C, 60 min ischemia; lane D, 15 min ischemia and 120 min reperfusion; lane E, 4×PC; lane F, 4×PC and 120 min reperfusion.

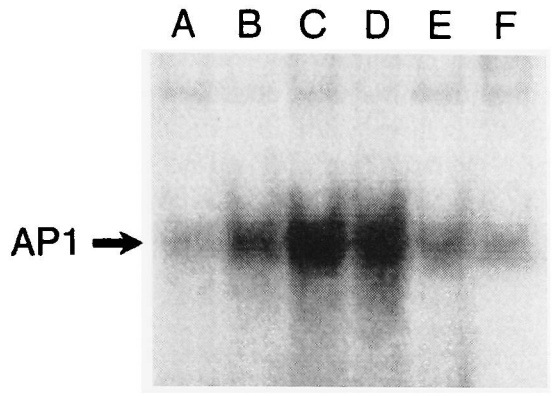

FIG. 3. Effect of ischemia, ischemia/reperfusion, and ischemic preconditioning on AP-1 activity. Nuclear extracts were isolated from control and experimental hearts. These extracts were then used for EMSA as described in the Materials and Methods section. Lane A, Perfused control heart; lane B, 15 min ischemia; lane C, 60 min ischemia; lane D, 15 min ischemia and 120 min reperfusion; lane E, 4×PC; lane F, 4×PC and 120 min reperfusion.

the control perfused group (lane A). Reperfusion of 15-min ischemic hearts for 120 min also demonstrated significantly higher AP-1 binding activity compared to the control. In ischemically adapted groups [both 4 × PC and 4 × PC 2h reperfusion], the binding activity of AP-1 was significantly affected. The densitometry scanning by image analyzer showed a significant decrease in its binding activity in the ischemic preconditioned group compared to the ischemic as well as ischemic/reperfused group; the value is almost close to the control group.

The expression of Bcl-2 during ischemia, ischemia/reperfusion, and ischemic preconditioning

Northern blot analysis revealed Bcl-2 gene upregulation in ischemically adapted hearts [4 × PC and 4 × PC 2 hr reperfusion; Fig. 4] compared to the control baseline hearts. To the contrary, prolonged reperfusion (2 hr) after acute ischemia (15 min) downregulated Bcl-2 gene significantly, as shown in Fig. 4. Ischemia alone has no influence on Bcl-2 gene expression.

Evaluation of apoptotic cells during ischemia, ischemia/reperfusion and preconditioning

We were unable to detect apoptotic cells in the control and in the ischemic hearts that were not subjected to reperfusion. Even 60 min of global ischemia could not induce apoptosis, and there was no sign of fragmented nuclear DNA in these biopsies. Apoptotic cells were identified only in the reperfused hearts. The extent of apoptosis increased with the progression of reperfusion time. Apoptotic cells were first evidenced after 90 min of reperfusion. The number of apoptotic cells increased after 2 hr (Fig. 5) reperfusion, as evidenced from the immunohistochemical staining of the extended DNA in these hearts. Ischemic preconditioning reduces the number of apoptotic cells in the reperfused myocardium significantly.

DISCUSSION

The ability of the myocardium to adapt itself successfully against any stress by increasing resistance to an adverse consequence, ultimately determines whether the heart will fail or maintain its function. 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. Such adaptation occurs through upregulation of the heart's own cellular defense through accumulation of intracellular mediators and reprogramming of gene expression, which

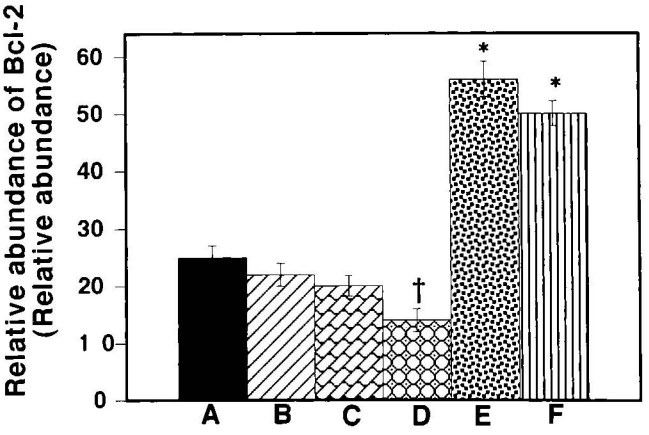

FIG. 4. Effect of ischemia, ischemia/reperfusion, and ischemic preconditioning on the relative abundance of Bcl-2 mRNA in heart tissue. The results of densitimetric scanning (mean ±SEM) for six different experiments for each time point are shown by bar graph. A, Perfused control heart; B, 15 min ischemia; C, 60 min ischemia; D, 15 min ischemia and 120 min reperfusion; E, 4×PC; F, 4×PC and 120 min reperfusion. †$p < 0.05$ decreased compared to control; *$p < 0.05$ increased compared to control.

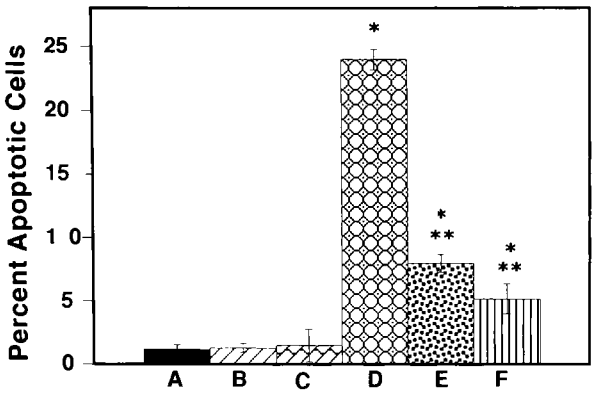

FIG. 5. Evaluation of apoptosis by tunnel method. Evaluation of apoptosis reveals an increased number of apoptotic cells in the ischemic/reperfused myocardium. Sections of control and experimental heart tissues were analyzed for apoptosis using the APOPTAG® kit, as described in Materials and Methods. The percent of apoptotic cells is shown by bar graph. A, Perfused control heart; B, 15 min ischemia; C, 60 min ischemia; D, 15 min ischemia and 120 min reperfusion; E, 4×PC; F, 4×PC and 120 min reperfusion. Results are expressed as means ± SEM of six different rats per group. $^{*}p < 0.05$ compared to control; $^{**}p < 0.05$ compared to the ischemic/reperfused group.

constitute the material basis of increased adaptation to stress. Recent studies suggest that the cytoprotection resulting from ischemic preconditioning could be applied to the human heart and may constitute anti-ischemic therapy to cure ischemic heart disease. Altered gene expression in the cardiovascular tissues of diseased heart has been reported (Nishio *et al.*, 1998). The possible cause of the altered gene regulation is the abnormal binding activities of some of the transcription factors like NF-κB and AP-1. Thus, the powerful cardioprotective effect of adaptation is likely to originate at the cellular and molecular levels.

In this report, we demonstrate the binding activities of NF-κB and AP-1 during different durations of ischemia, reperfusion, and ischemic adaptation. Myocardial adaptation to ischemia was achieved by four cyclic episodes of 5 min of ischemia each followed by 10 min of reperfusion. Such adaptation, also known as ischemic preconditioning, was previously shown to exert powerful cardioprotective effects for ischemic hearts (Das *et al.*, 1995). The results of the present study demonstrated that the binding activities NF-κB and AP-1 increased progressively and steadily as a function of the duration of ischemia. During subsequent reperfusion, NF-κB binding activity goes down while AP-1 activity contributes to increase further with reperfusion time. In adapted myocardium, NF-κB activity remains high while AP-1 binding activity comes down to the baseline level. In majority of cells, NF-κB exists as a cytoplasmic complex by binding with its inhibitory protein 1κBα. Phosphorylation of 1κBα by oxidative stress resulting from ischemia/reperfusion can cause dissociation IκB from NF-κB. We have shown in Fig. 2 early activation of NF-κB by ischemia in the myocardium; this may be a signaling mechanism for the induction of immediate-early gene expression during subsequent stress adaptation. In the preconditioned myocardium, significantly higher binding activity of NF-κB prevails while AP-1 binding becomes downregulated.

We have also investigated the loss of 1κBα protein after dissociation in the cytoplasm caused by ischemia/reperfusion and preconditioning. The kinetics of NF-κB binding activity in the nuclear extracts correlated with the kinetics for 1kBα protein disappearance in the cytoplasm. The data (not shown) suggest that the disappearance of 1κBα protein from the cytoplasm resulted in the translocation of NF-κB complex to the nucleus as an active form.

The results of our study indicate a direct correlation of cardiomyocyte apoptosis with AP-1 and indirect or minimal correlation with NF-κB. For example, ischemia, as well as reperfusion of ischemic myocardium, resulted in the activation of AP-1 simultaneously, causing apoptotic cell death (Figs. 3 and 5). Conversely, ischemic stress adaptation downregulated AP-1 (close to baseline control) and attenuated apoptosis. These results are consistent with previous observations that increased expression of components of AP-1 is linked to apoptosis (Marti *et al.*, 1994; Sawai *et al.*, 1995; Watabe *et al.*, 1998). A recent study demonstrated that c-Jun/AP-1, but not NF-κB, is a mediator for oxidant-initiated apoptosis in glomerular mesangial cells (Ishikawa *et al.*, 1997). 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. Downregula-

tion of c-Jun/AP-1 using a transdominant negative mutant of c-Jun inhibited apoptotic cell death whereas use of a transdominant negative mutant of p50 NF-κB subunits did not affect H_2O_2-mediated apoptosis. NF-κB, on the other hand, was activated during ischemia is Chemia/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 (Maulik *et al.*, 1998d).

Bcl-2 proved to be a unique gene by blocking programmed cell death. This protein has been localized to mitochondria, endoplasmic reticulum, and nuclear membrane and also it is found to be localized at the sites of ROS. A recent study demonstrated that Bcl-2 activates the transcription factor NF-κB by regulating IκBα (Moissac *et al.*, 1998), suggesting a link between Bcl-2 and the NF-κB signaling pathway in the suppression of apoptosis. This study prompted us to determine the relationship between Bcl-2 and NF-κB during ischemia and reperfusion. Previously, we demonstrated that prolonged reperfusion after ischemia caused downregulation of Bcl-2 in consert with enhanced DNA fragmentation (Maulik *et al.*, 1997). The present study showed upregulation of Bcl-2 by myocardial preconditioning (Fig. 4).

In summary, the results of our study demonstrated differential regulation of NF-κB, AP-1, and Bcl-2 during ischemia/reperfusion and ischemic adaptation. It appears that when AP-1 binding to the heart remains low and the extent of NFκB translocation is significantly elevated, upregulation of Bcl-2 also occurs. This is correlated with the decrease in cardiomyocyte apoptosis. However, our study does not demonstrate that Bcl-2 is regulating NF-κB. Several reports suggest that the activation of NF-κB by different types of stress can be prevented by antioxidants as well as thioredoxin, indicating that ROS and the intracellular redox state may serve as a common intracellular signaling pathway for the activation of NF-κB (Baeuerle and Henkel, 1994; Pinkus *et al.*, 1996). It is tempting to speculate that increased NF-κB binding prevents apoptosis through the upregulation of the Bcl-2 gene. Another striking feature of this study is that NF-κB remains higher compared to the baseline in the reperfused myocardium when cardiomyocyte apoptosis is prevalent, and it becomes further activated in the adapted myocardium when apoptosis is significantly attenuated. Thus, it seems likely that NF-κB operates both as a pro and anti-apoptotic factor depending on the redox state of the cell.

ACKNOWLEDGMENTS

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ABBREVIATIONS

ANOVA, analysis of variance; AP-1, c-Jun/activator protein-1; BSA, bovine serum albumin; DTT, DL-dithiothreitol; EMSA, electrophoretic mobility shift assay; ESR, electron spin resonance spectroscopy; HPLC, high-performance liquid chromatography; KHB, Krebs Hensleit bicarbonate buffer; NF-κB, nuclear factor kappa B; ROS, reactive oxygen species; TDT, terminal deoxynucleotidyl transferase.

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Address reprint requests to:
Dr. Nilanjana Maulik
Molecular Cardiology Laboratory
Cardiovascular Div, Dept. of Surgery
University of Connecticut School of Medicine
Farmington, CT 06030-1110

E-mail: nmaulik@panda.uchc.edu

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