Early activation of transcription factor NF-κB during ischemia in perfused rat heart

CHUANFU LI, WILLIAM BROWDER, AND RACE L. KAO
Department of Surgery, James H. Quillen College of Medicine, Mountain Home Veterans Affairs Center, East Tennessee State University, Johnson City, Tennessee 37614

LI, Chuanfu, William Browder, and Race L. Kao. Early activation of transcription factor NF-κB during ischemia in perfused rat heart. Am. J. Physiol. 276 (Heart Circ. Physiol. 45): H543–H552, 1999.—The transcription factor nuclear factor κB (NF-κB) regulates multiple immediate-early gene expressions in immune and inflammatory responses and cellular defenses. Ischemia-reperfusion induces many immediate-early gene expressions, but little is known about the NF-κB activation in myocardium during ischemia and reperfusion. This study demonstrated that ischemia alone rapidly induced NF-κB activation in the myocardium of isolated working rat hearts. Electrophoretic mobility shift assay showed that NF-κB binding activity significantly increased in the nucleus after 5 min of ischemia and remained elevated for up to 30 min. Western blot analysis suggested that the levels of inhibitory IκBα protein in the cytoplasm became markedly decreased at 4, 5, 7.5, and 10 min of ischemia but were gradually restored following 10 min of ischemia. Reduction of IκBα protein in the cytoplasm by ischemia resulted in NF-κB translocation to the nucleus. Northern blot hybridization showed that IκBα mRNA levels were not significantly elevated during myocardial ischemia. Pyrroldine dithiocarbamate, an antioxidant, significantly inhibited the loss of IκBα protein from the cytoplasm and prevented NF-κB binding activity in the nucleus. Reperfusion following short periods of ischemia augmented NF-κB binding activity in the nucleus. Western blot analysis suggested that early activation of NF-κB induced by ischemia in the myocardium could be a signal mechanism for controlling and regulating immediate-early gene expression during ischemia-reperfusion.

A NUMBER OF STUDIES HAVE SHOWN that ischemia and reperfusion induce cytokine gene expression including tumor necrosis factor-α (TNF-α), interleukin (IL)-1α, IL-6, IL-8, interferon-γ, and intercellular adhesion molecule-1 (ICAM-1) in myocardium (18, 22–24, 32, 47). These locally overexpressed myocardial cytokines may play a critical role in the progression of myocardial dysfunction, including ischemia-reperfusion injury, vascular wall remodeling, heart failure, and cardiac hypertrophy (5, 7, 21, 22, 32). Recent evidence suggests that locally produced TNF could also contribute to posts ischemic myocardial dysfunction via direct depression of contractility and induction of myocyte apoptosis (30). However, the molecular mechanism for controlling and regulating these immediate-early gene expressions in myocardium during ischemia and reperfusion has not been well studied.

Nuclear factor κB (NF-κB) is an ubiquitous inducible transcription factor that is primarily involved in immune, inflammatory, and stress responses (1, 2). In the majority of cells, NF-κB exists as a latent cytoplasmic complex bound to the inhibitory IκB proteins. In the family of IκB proteins, the most important appear to be IκBα, IκBβ, and the newly discovered IκBε. Treatment of cells with various inducers, including lipopolysaccharide (LPS), mitogens, cytokines, phorbol esters, ultraviolet (UV) radiation, free radicals, and oxidative stress, causes the IκB proteins' phosphorylation, dissociation from NF-κB, and rapid degradation. The released NF-κB translocates to the nucleus, binds to cognate DNA binding sites, and regulates inducible gene expression. Because all known stimuli of NF-κB activity (1, 2) also induce the formation of transient reactive oxygen species (ROS) and the activation of NF-κB can be blocked by antioxidants, the ROS may serve as a common messenger mediating the activation of NF-κB. Recently, increased NF-κB levels in postischemic rat myocardium (8) and a time-dependent increase in NF-κB binding activity induced by hypoxia in cultured cardiac cells (19) have been reported. NF-κB may also be involved in the regulation of ischemic preconditioning of the heart (29). Transfection of NF-κB decoy oligodeoxynucleotides to myocardium significantly reduced the area of infarction (31) and improved tolerance to ischemia-reperfusion injury in association with the inhibition of neutrophil adherence and tissue IL-8 production (36). However, it is unclear when NF-κB is activated and how IκB is regulated in myocardium during ischemia and reperfusion.

In this report we show, for the first time to our knowledge, that short periods of ischemia alone rapidly diminish IκBα levels in the cytoplasm and induce NF-κB activation in the nucleus of myocardium using an isolated perfused heart preparation, whereas reperfusion augments the NF-κB binding activity. Early activation of NF-κB by ischemia in the myocardium may be responsible for the regulation of immediate-early gene expression during ischemia-reperfusion. Modulation of NF-κB activation could provide a means of reducing myocardial ischemic injury.

MATERIALS AND METHODS

Heart perfusion. Male Sprague-Dawley rats (300–350 g) were purchased from a licensed vendor and maintained in the Animal Care facility of the East Tennessee State University in accordance with the guidelines of the Principles of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals. The research protocol was reviewed by the Institutional Animal Care and Use Committee of East Tennessee State University. Male Sprague-Dawley rats (300–350 g) were used in all experiments.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
and approved by the East Tennessee State University Committee on Animal Care. The rats were heparinized with heparin sodium (2.5 mg/100 g) and anesthetized with chloral hydrate (36 mg/100 g). The hearts were removed and immediately placed into a beaker containing cold (2°C) Krebs-Henseleit bicarbonate buffer (in mM: 119 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, and 25 NaHCO₃; pH 7.4 to 37°C) supplemented with 10 mM glucose and equilibrated with 95% O₂-5% CO₂. Within 1 min (45.6 ± 4 s) of the chest incision, the aorta was cannulated and preliminary retrograde (Langendorff) perfusion at a perfusion pressure of 70 mmHg was begun with 37°C oxygenated Krebs-Henseleit bicarbonate buffer. During the period of preliminary perfusion, the buffer was not recirculated and the cannulation of the left atrium was completed. The hearts were then switched to a working mode. After equilibration (15 min) under stable working conditions, global normothermic ischemia was initiated by clamping the perfusion tubing for 0, 1, 2, 3, 4, 5, 7.5, 10, 15, and 30 min, respectively, with 6 or 7 hearts for each time point. The perfusion apparatus and technique were described previously (20, 46), and the myocardial temperature was maintained at 37°C by keeping the heart within the heart chamber containing the perfusate. Control hearts were perfused for up to 60 min after the preischemia period without interruption of perfusate flow. To examine the effects of antioxidants on NF-κB activation during ischemia, pyrrolidine dithiocarbamate (PDTC), a potent inhibitor of NF-κB activation, was added to the buffer at a concentration of 100 µM from the beginning of perfusion. In other experiments studying the effects of short periods of ischemia followed by reperfusion on NF-κB activation, the rat hearts were subjected to 2, 5, and 15 min of global normothermic ischemia followed by 5, 10, 20, and 30 min of reperfusion for each ischemic duration. Immediately at the completion of the perfusion protocols, ventricles were frozen with Wollenberger clamps precooled in liquid nitrogen and pulverized under liquid nitrogen temperature. The powders of the myocardial samples were extracted for nuclear proteins, cytoplasmic proteins, and total cellular RNA.

Isolation of nuclear and cytoplasmic proteins. Nuclear and cytoplasmic proteins were isolated using a method described previously (23, 35). Briefly, ~0.1 g of pulverized myocardial sample was homogenized in 0.8 ml of ice-cold hypotonic buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT); protease inhibitors: 0.5 mM phenylmethylsulfonyl fluoride, aprotinin, pepstatin, leupeptin (10 µg/ml each); and phosphatase inhibitors: 50 mM NaF, 30 mM β-glycerophosphate, 1 mM Na₂VO₃, and 20 mM p-nitrophenyl phosphate). The homogenates were centrifuged for 30 s at 2,000 rpm at 4°C to eliminate any unbroken tissue. The supernatants were incubated on ice for 20 min, vortexed for 30 s, and then centrifuged for 1 min at 4°C in an Eppendorf centrifuge. Supernatants containing cytoplasmic proteins were collected and stored at −80°C. The pellets, after a single wash with the hypotonic buffer without Nonidet P-40, were suspended in an ice-cold hypertonic salt buffer (20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, protease inhibitors, and phosphatase inhibitors), incubated on ice for 30 min, mixed frequently, and centrifuged for 15 min at 4°C. The supernatants were collected as nuclear extracts and stored at −80°C. The concentration of total proteins in the samples was determined by the Pierce protein assay reagent (Pierce Chemical, Rockford, IL). To estimate possible contamination of the nuclear extracts with the cytoplasmic extracts when preparing the nuclear and cytoplasmic proteins, lactate dehydrogenase (LDH) activity was determined by a commercially available kit for the quantitative kinetic determination of LDH activity (Sigma Chemical, St. Louis, MO). Values were expressed as LDH activity units per milligram of protein. To establish that the nuclear extracts contained mainly nuclear proteins, 40 µg of nuclear protein preparations were subjected to Western blot analysis for histone H3, a nuclear protein, with anti-histone H3 antibody (Upstate Biotechnology, Lake Placid, NY).

Electrophoretic mobility shift assay. NF-κB binding activity was performed as described previously (3, 14) in a 15-µl binding reaction mixture containing 1× binding buffer [50 µg/ml of double-stranded poly(dl-dC), 10 mM Tris·HCl (pH 7.5), 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 1 mM MgCl₂, and 10% glycerol], 15 µg of nuclear proteins, and 35 fmol (~50,000 cpm, Cherenkov counting) of double-stranded NF-κB consensus oligonucleotide (5′-AGT TGA GGG GAC TTT CCC AGG C-3′), which was end-labeled with [γ-32P]ATP (3,000 Ci/mmol at 10 µCi/ml; Amersham Life Sciences, Arlington Heights, IL) using T4 polynucleotide kinase (Promega, Madison, WI). The binding reaction mixture was incubated at room temperature for 20 min and analyzed by electrophoresis on 5% nondenaturing polyacrylamide gels. After electrophoresis, the gels were dried by Gel-Drier (Bio-Rad Laboratories, Hercules, CA) and exposed to Kodak X-ray films at −70 °C. The binding bands were quantified by scanning densitometry of a Bio-Image Analysis System (Millipore Imaging System, Ann Arbor, MI). The results for each time point from each group were expressed as relative integrated intensity compared with the normal heart group measured in the same batch because the integrated intensity of group samples from different electrophoretic mobility shift assay (EMSA) batches would be affected by the half-life of the isotope, exposure time, and background levels.

Competition experiments were performed to assess the specificity of NF-κB binding activity determined by EMSA. Nuclear extracts from ischemic myocardium were incubated with unlabeled double-stranded NF-κB or AP-1 oligonucleotides for 5 min (14). After addition of [γ-32P]ATP end-labeled NF-κB oligonucleotides into the binding reaction for 20 min, the reaction mixtures were analyzed by 5% nondenaturing polyacrylamide gel electrophoresis. Antibody supershift assays were carried out to confirm that NF-κB binding activity in the myocardium contains subunits p50 and p65. Nuclear extracts from ischemic myocardium were incubated with [γ-32P]ATP end-labeled, double-stranded NF-κB oligonucleotides for 20 min followed by addition of 2 µl of the appropriate antibody (Santa Cruz Biotechnology, Santa Cruz, CA) specific to NF-κB subunits p50 and p65 into the reaction mixtures. After incubation for 2 h at 4°C, the protein DNA complexes were resolved by electrophoresis on 5% nondenaturing polyacrylamide gels. Alternatively, nuclear extracts from ischemic myocardium were incubated with the appropriate antibodies for 1 h, and the [γ-32P]ATP end-labeled NF-κB oligonucleotide probes were then added into the reaction mixtures. After incubation for 20 min, the reaction mixtures were run on 5% nondenaturing polyacrylamide gels.

Western blot analysis of IκBα. Western blot analysis of cytoplasmic protein lysates and NF-κB binding activity were carried out to confirm that NF-κB binding activity in the myocardium contains subunits p50 and p65. Nuclear extracts from ischemic myocardium were incubated with the appropriate antibodies for 1 h, and the [γ-32P]ATP end-labeled NF-κB oligonucleotide probes were then added into the reaction mixtures. After incubation for 20 min, the reaction mixtures were run on 5% nondenaturing polyacrylamide gels.
three times for 10 min each in TBS-0.05% Tween 20, and incubated with a primary IκBα antibody (Santa Cruz Biotechnology) in TBS-0.05% Tween 20 containing 5% nonfat dry milk for 1–2 h at room temperature. After being washed three times for 10 min each in TBS-0.05% Tween 20, the membranes were incubated with a second antibody of peroxidase-conjugated goat anti-rabbit immunoglobulin G (Sigma) for 1 h at room temperature. After washing, the membranes were analyzed by the enhanced chemiluminescence system according to the manufacturer’s protocol (Amersham). The IκBα protein signal was quantified by scanning densitometry of a Bio-Image Analysis System (Millipore). The results from each experimental group were expressed as relative integrated intensity compared with normal hearts measured with the same batch.

RNA isolation and Northern blot analysis. Total cellular RNA was isolated from rat myocardium using an Ultraspec-II RNA isolation System (Biotecx Laboratories, Houston, TX). Briefly, pulverized heart samples were homogenized with 2 ml of the Ultraspec RNA solution, and one-fifth of the homogenate volume of chloroform was added. After incubation on ice for 5 min, the lysate was centrifuged at 12,000 g for 15 min at 4°C. Total RNA in the aqueous phase was transferred to a clean RNase-free tube. Water by vortex. After centrifugation, the supernatant containing purified RNA was transferred to a clean RNase-free tube. The concentration of total RNA isolated was quantified by UV spectrophotometry at 260/280 nm (16).

For Northern blot hybridization, 10 µg of total RNA was denatured for 2 min at 95°C and fractionated by electrophoresis on 1% agarose gels (SeaKem LE Agarose; FMC Products, Rockland, ME) containing formaldehyde in 1× MOPS buffer (20 mM MOPS, 5 mM sodium acetate, and 10 mM EDTA). The gels were washed twice for 20 min each in 1× SSC (1.5 M NaCl, 0.15 M citrate), and the total RNA on the agarose gels was transferred onto nylon membranes (Schleicher & Schuell, Keene, NH) by capillary blotting and subsequently cross-linked under UV light. The membranes were incubated with a primary IgG antibody (Santa Cruz Biotech- nology or loading of the total RNA samples (16). The autoradiograms were quantified by scanning densitometry of a Bio-Image Analysis System (Millipore). The results for each time point from each group were expressed as relative integrated intensity compared with the normal heart group measured under identical conditions.

Statistical analysis. Results are expressed as means ± SE. For tests of significance between the different time points and normal hearts, one-way ANOVA was performed, with P < 0.05 considered to be significant.

**RESULTS**

Activation of NF-κB by ischemia. NF-κB binding activity in the nuclear extracts of myocardium was determined by EMSA after rat hearts were subjected to various periods of ischemia. NF-κB binding activity was present at very low levels in normal and control (nonischemic) rat myocardium but significantly increased after the hearts were subjected to ischemia. NF-κB binding activity rapidly increased (42% at 4 min of ischemia, 66% at 5 min of ischemia), and the increment persisted to 30 min of ischemia (Fig. 1A). NF-κB binding activity did not significantly increase when the rat hearts were subjected to continuous perfusion for 5, 10, and 15 min but became markedly elevated after 30 and 60 min (Fig. 1B). The data suggest that both early ischemia and prolonged oxygenated perfusion for more than 30 min induce NF-κB activation in rat myocardium in an isolated working heart preparation.

To assess specific binding of NF-κB in ischemic myocardium, 100-fold excesses of unlabeled NF-κB or AP-1 oligonucleotides were added to the EMSA reaction. Unlabeled NF-κB oligonucleotides competed for the binding proteins in nuclear extracts prepared from ischemic myocardium, whereas the unrelated AP-1 oligonucleotides did not (Fig. 2). To confirm that the predominant protein complex of NF-κB in the ischemic myocardium is composed of p65 and p50 subunits, antibody supershift assays were performed with polyclonal antibodies recognizing NF-κB p65 and p50 subunits. Both antibodies considerably shifted the major ischemia-induced NF-κB binding complex (Fig. 2). These results confirm that the activated NF-κB in ischemic rat myocardium contains subunits p65 and p50.

The possibility that the cytoplasmic extracts contaminated the nuclear extracts was assessed by detecting LDH activity as a marker. The levels of LDH activity in the nuclear extracts were almost 20-fold less than that in the cytoplasmic extracts (3.67 ± 0.12 U/mg nuclear protein vs. 152 ± 42 U/mg cytoplasmic protein, n = 14). To confirm that the nuclear extracts contain primarily nuclear proteins, immunoblot analysis of the nuclear extracts was performed with an antibody specific to histone H3, a nuclear protein. Histone H3 was primarily found to be in the nuclear extracts (n = 14).

Ischemia causes rapid, transient loss of IκBα protein in cytoplasm. Because ischemia rapidly induces NF-κB binding activity in the myocardium, the effects of ischemia on the dynamics of IκBα protein in the cytoplasm were investigated. Hearts were subjected to various periods of ischemia, and the cytoplasmic extracts were assayed by Western blot analysis with an antibody specific to IκBα. Figure 3A shows that IκBα levels in the cytoplasm began to decrease after 3 min of ischemia and were reduced 75.5% at 4 min and 74.4%
or longer can cause consistent loss of IκBα protein from
the cytoplasm, only 4 min of ischemia results in signifi-
cant, rapid, but transient loss of IκBα protein from
the cytoplasm. Because the kinetics of NF-κB binding
activity in the nuclear extracts parallels the kinetics of
IκBα decrement in the cytoplasm, the data suggest that
the transient ischemia induced the loss of IκBα protein
from the cytoplasm and translocation of activated
NF-κB to the nucleus.

IκBα mRNA expression during ischemia. Because
IκBα protein in the cytoplasm diminished following 4
min of ischemia but began to increase after 10 min of
ischemia, IκBα mRNA levels in rat myocardium were
determined. Total cellular RNA was isolated from each
heart, separated by electrophoresis on 1% agarose gels,
and subjected to Northern blot analysis for both IκBα
and GAPDH mRNA levels. As shown in Fig. 4A, IκBα
mRNA levels increased 40 and 64% after 7.5 and 10 min
of ischemia, respectively, and peaked (74%) at 15 min of
ischemia compared with that of the normal hearts.
Although these findings did not achieve statistical
significance, IκBα mRNA levels did not change in
continuously perfused hearts during the entire perfu-
sion period (Fig. 4B).

Antioxidant prevents ischemic effects. To investigate
the effects of the antioxidant PDTC on ischemia-
induced loss of IκBα protein from the cytoplasm and
increased NF-κB activity in the nucleus, PDTC was
added to the buffer at a concentration of 100 µM from
the beginning of perfusion. The EMSA results showed

Fig. 1. Electrophoretic mobility shift assay (EMSA) of nuclear factor (NF)-κB binding activity in rat hearts. A: ischemia rapidly induces NF-κB binding activity. Nuclear extracts were prepared from normal rat hearts (N) and hearts undergoing ischemia for 0, 1, 2, 3, 4, 5, 7.5, 10, 15, and 30 min, respectively. B: effects of continuous perfusion without ischemia on NF-κB binding activity. Nuclear extracts were prepared from normal rat hearts and hearts subjected to perfusion for 0, 5, 10, 20, 30, and 60 min, respectively. EMSA was performed with 15 µg of the nuclear proteins from each sample. Representative EMSA picture is shown at top of each panel, and NF-κB (2 bands) and the nonspecific (ns) band are labeled at left. Free probe is not shown. Results are expressed as means ± SE of 6 or 7 hearts for each time point. *P < 0.05 compared with normal hearts.

at 5 min of ischemia compared with that of normal
hearts. After that, the IκBα levels in the cytoplasm
gradually increased. As shown in Fig. 3A, IκBα levels at
5 min of ischemia rose from 26.6% to 52, 75, and 89%
of the normal levels after ischemia for 10, 15, and 30 min,
respectively. In control (nonischemic) hearts, however,
perfusion for 5 min did not reduce IκBα levels in the
cytoplasm, but the levels dropped ~15% following 10
and 15 min of perfusion without ischemia. Significant
decrement of IκBα levels in the cytoplasm was only
found when the nonischemic hearts were continuously
perfused for 30 and 60 min (Fig. 3B). The results
showed that although continuous perfusion for 30 min

Fig. 2. Specific NF-κB binding activity was analyzed by addition of unlabelled oligonucleotides and by an antibody supershift gel assay. Lanes in descending order are nuclear extract (NE; from ischemic heart), NE plus unlabeled NF-κB oligonucleotides, NE plus unlabeled AP II oligonucleotides, NE plus antibody against p50, NE plus antibody against p65, and NE plus antibodies against both p50 and p65. NF-κB and nonspecific band are labeled at left, and raised bands supershifted by antibodies are indicated by arrow at right. Free probe is not shown. Results are representative of 3 experiments.
that PDTC suppressed the ischemic-induced activation of NF-κB in the nuclear extracts (Fig. 5A). Western blot analysis demonstrated that PDTC prevented the loss of IκBa protein from the cytoplasm of ischemic myocardium (Fig. 5B). PDTC, however, did not alter IκBa mRNA levels as shown by Northern blot hybridization (Fig. 5C). The results demonstrate that both IκBa decrement in the cytoplasm and NF-κB activation in the nucleus, induced by ischemia, can be prevented by this antioxidant.

Reperfusion augments NF-κB binding activity induced by ischemia. To investigate the effects of reperfusion following short periods of ischemia on NF-κB binding activity, rat hearts were subjected to 2, 5, and 15 min of ischemia followed by reperfusion for 5, 10, 20, and 30 min for each ischemic duration. As shown in Fig. 6A, IκBa levels in the cytoplasm did not significantly change in myocardium subjected to 2 min of ischemia followed by reperfusion for 5 min. When the ischemic hearts were subjected to reperfusion for 10, 20, and 30 min, the IκBa levels in the cytoplasm were reduced ~32–37%, whereas NF-κB binding activity in the nucleus was increased 18.8–38.8% compared with those from normal hearts (Fig. 6B). After 5 min of reperfusion, the NF-κB binding activity was increased by 10.2 ± 0.3% compared with normal hearts.
ischemia and reperfusion for 5, 10, 20, and 30 min, cytoplasmic I\(\kappa\)B\(\alpha\) remained at the same low levels as after 5 min of ischemia alone, whereas NF-\(\kappa\)B binding activity in the nucleus was increased 65–82.7% compared with those from normal hearts as shown in Fig. 7. Figure 8A shows that I\(\kappa\)B\(\alpha\) levels in the cytoplasm were restored to normal levels after ischemia for 15 min followed by reperfusion for 10, 20, and 30 min, but NF-\(\kappa\)B binding activity in the nucleus was increased from 158 to 212% after reperfusion for 20 and 30 min compared with that of normal hearts (Fig. 8B).

**DISCUSSION**

A significant finding of this study is that ischemia alone rapidly reduces I\(\kappa\)B\(\alpha\) levels in the cytoplasm, which results in the activation and translocation of NF-\(\kappa\)B to the nucleus in isolated perfused heart. Inflammatory gene expression, including IL-1, TNF-\(\alpha\), inducible nitric oxide synthase, ICAM-1, granulocyte macrophage colony-stimulating factor, IL-6, and IL-8 are controlled by NF-\(\kappa\)B in most types of cells. Recent studies have shown that these cytokine gene expressions are also induced by ischemia and reperfusion in myocardium (12, 18, 21, 24, 28, 32, 39, 47) and are suggested to be important factors involved in the depression of cardiac functions, mediation of remodeling, and cardiac hypertrophy (5, 7, 21, 22, 28, 32). Specific blocking of NF-\(\kappa\)B activation with NF-\(\kappa\)B decoy oligodeoxynucleotides has been shown to improve the recovery of cardiac function after ischemia-reperfusion insult (31, 36). The present study suggests that early activation of NF-\(\kappa\)B by ischemia could be a potential acute molecular mechanism for regulating inflammatory cytokine gene expression in the heart. The other important observation is that continuous perfusion without ischemia for more than 30 min also induced NF-\(\kappa\)B activation in isolated perfused rat hearts, which may account for the suppressed myocardial performance during prolonged perfusion observed in our earlier studies (20, 46). In addition, reperfusion after ischemia only augmented NF-\(\kappa\)B binding activity in the nucleus, which was previously induced by short periods of ischemia, suggesting that induction of increased NF-\(\kappa\)B activity in the postischemic myocardium (8) may have been initiated during the periods of ischemia. PDTC, a well-known antioxidant, prevented the decrement of I\(\kappa\)B\(\alpha\) levels in the cytoplasm and the activation of NF-\(\kappa\)B in the nucleus induced by ischemia.

---

**Fig. 5.** Prevention of the loss of I\(\kappa\)B\(\alpha\) protein in the cytoplasm and the activation of NF-\(\kappa\)B in the nucleus in the presence of antioxidant pyrrolidine dithiocarbamate (PDTC). Samples were normal rat hearts and hearts treated with the antioxidant PDTC (100 µM) and subjected to ischemia for 0, 3, 4, 5, 7.5, 10, 15, and 30 min, respectively. The cytoplasmic and nuclear proteins and total RNA were isolated. A: I\(\kappa\)B\(\alpha\) protein levels were determined by Western blot analysis with specific antibody to I\(\kappa\)B\(\alpha\) with 40 µg of cytoplasmic proteins from each sample. Representative Western blot result is shown at top, and I\(\kappa\)B\(\alpha\) protein is labeled at left. B: NF-\(\kappa\)B binding activity was analyzed by EMSA in 15 µg of nuclear extract from each sample. Representative EMSA of NF-\(\kappa\)B binding activity is shown at top, and NF-\(\kappa\)B (2 bands) and nonspecific band are labeled at left. C: I\(\kappa\)B\(\alpha\) mRNA levels were measured by Northern blot hybridization with [\(^{32}\)P]dCTP-labeled I\(\kappa\)B\(\alpha\) cDNA probe. Total RNA (10 µg) from each sample was subjected to hybridization with [\(^{32}\)P]dCTP-labeled I\(\kappa\)B\(\alpha\) and GAPDH cDNA probes, respectively. Representative results of Northern blot hybridization of both I\(\kappa\)B\(\alpha\) and GAPDH mRNA are shown at top and labeled at left. Results are expressed as means ± SE of 4 hearts for each time point.
inhibition of NF-κB activation by PDTC could be due to
the prevention of the loss of IκBα protein from the
cytoplasm during ischemia, by suppression of IκBα release from the latent cytoplasmic form of NF-κB (38)
and inhibition of IκBα degradation (40). Because the
inhibitory effects of PDTC on NF-κB activation have
been attributed to its antioxidant and metal-chelating
properties, the prevention of the activation of NF-κB in ischemic rat hearts by the antioxidant suggests the involvement of ROS in the activation of NF-κB. It has been
demonstrated that the activation of NF-κB by different stimuli can be blocked by antioxidants (1, 2, 38, 40). Antioxidants and metal chelators prevent NF-κB
activation induced not only by oxidizing agents, like hydrogen peroxide, but also by inducers unrelated to oxidizing agents, such as inflammatory cytokines, mitogens, protein synthesis inhibitors, and certain drugs (38). Overexpression of the antioxidative enzyme thioredoxin can also prevent the activation of NF-κB (37).

These data suggest that the activation of NF-κB is controlled by ROS and the intracellular redox state (34) and that multiple signaling pathways could be involved in the release of ROS as a common signaling moiety.

Both hypoxia-reoxygenation and ischemia-reperfusion can induce ROS formation and oxidative stress (11), and ROS are hypothesized to be responsible for myocardial ischemic and reperfusion injury. Our finding that activation of NF-κB by ischemia is involved in ROS production during ischemia is consistent with a num-

![Fig. 6.](http://ajpheart.physiology.org/)
NF-κB activation during myocardial ischemia

commonly observed in myeloid, epithelial, and fibroblast cells stimulated with cytokine, phorbol myristate acetate (PMA), and LPS (4, 6, 17). The rate of IκBα degradation in cytoplasm varied among different types of cells, but translocation of the released NF-κB to the nucleus paralleled the loss of IκBα protein from the cytoplasm. In U937 cells, the restoration of IκBα occurred within 20-40 min, but replacement required 1-2 h in HeLa, J urkat, and THP-1 monocyteic cells (4, 6, 10, 41). In the present study, ischemia caused rapid loss of IκBα protein from the cytoplasm within 4-7.5 min, whereas restoration began after 10 min of ischemia. Restoration of IκBα in myocardium responding to ischemia was more rapid than that of other cell types. Because IκBα may be involved in the regulation of the multiple NF-κB-dependent gene expressions (35, 40), degradation of IκBα coupled with the activation of NF-κB in the myocardium during ischemia and consequent restoration of IκBα could play important roles in the regulation of inflammatory cytokine gene expression and/or the mediation of immediate-early gene expression in the ischemic-reperfused heart (11).

IκBα mRNA levels were increased ~64% after 10 min of ischemia and peaked at 74% after 30 min of ischemia. Although the increased IκBα mRNA levels did not achieve statistical significance because of variance among the animals and limited experimental numbers, the increased mRNA levels could account for the restoration of IκBα protein in the cytoplasm after 10 min of ischemia. It has been demonstrated that activation of NF-κB by various inducers can subsequently upregulate IκBα mRNA expression (9, 25), and newly synthesized IκBα protein replaces the depleted pool of IκBα protein in the cytoplasm. Upregulation of IκBα mRNA levels by activated NF-κB has been confirmed by the presence of NF-κB binding sites in the IκBα gene promoter (4, 5, 10, 17, 41). Recent studies have suggested the existence of an autoregulatory feedback of NF-κB/IκBα activation. In this model, IκBα controls the NF-κB activation, while activated NF-κB also in turn regulates the expression of the IκBα gene, thus facilitating an autoregulatory feedback mechanism that serves to temporarily restrict NF-κB activation (6, 41).

It has been reported that NF-κB activation has been detected within 15 min of LPS or PMA stimulation of pre-B cells, whereas the levels of IκBα mRNA expression increased after 15 min of stimulation and reached maximum levels by 60 min (9). Stimulation of U937 monocytes by PMA or TNF-α for 20 min significantly increased IκBα mRNA expression (6), and the new IκBα protein synthesis was associated with a massive increase in IκBα mRNA. A recent study (27) has shown that the IκBα levels were markedly diminished within 5 min of monocyte adhesion and rapidly replaced within 20 min thereafter. The accumulation of IκBα mRNA was not due to mRNA stabilization, and the IκBα gene transcription rate was unchanged. Increased IκBα mRNA levels were found in the nuclei, but not in the cytoplasm, suggesting that a translation-dependent degradation mechanism may maintain the
low levels of IκBα mRNA in the cytoplasm. Because immediate-early gene expression has been observed in myocardium subjected to hypoxia, ischemia, reperfusion, hyperthermia, and oxidative stress [11], it is possible that the autoregulatory feedback of NF-κB/IκBα activation in the myocardium during short periods of ischemia may be involved in the modulation of the expression of multiple NF-κB-dependent immediate-early genes.

A full understanding of the contribution of reperfusion to myocardial injury after ischemia is still lacking. Whether reperfusion causes further injury to ischemic tissue or simply unmasks irreversible myocardial damage previously induced by ischemia has not been resolved. To investigate the effects of reperfusion following short periods of ischemia on NF-κB activation, 2, 5, and 15 min were chosen as ischemic time points followed by 5, 10, 20, and 30 min of reperfusion for each ischemic duration. The three ischemic time points were chosen because IκBα levels in the cytoplasm did not change during 2 min of ischemia but dropped to the lowest levels at 5 min of ischemia and were restored to 75% of normal levels after 15 min of ischemia, whereas NF-κB binding activity was not induced at 2 min of ischemia but significantly increased after 5 min of ischemia, and the increment remained at 15 min of ischemia. Patterns of IκBα levels in the cytoplasm and NF-κB binding activity in the nucleus generated by 2 min of ischemia followed by reperfusion were similar to those of the perfusion group. Interestingly, IκBα levels in the cytoplasm after reperfusion following 5 min of ischemia were continually maintained at the same low levels as 5 min of ischemia alone but restored to normal levels after reperfusion following 15 min of ischemia. However, NF-κB binding activity in the nucleus was further increased even if IκBα levels were restored in the cytoplasm. Recent studies have proposed that inducers such as LPS or differentiation signals cause persistent NF-κB activation by affecting IκBβ complexes, which act as chaperones to protect NF-κB from IκBα, allowing NF-κB/β to translocate to the nucleus [42, 43]. It is possible that the increased ROS generation during reperfusion (33) and the IκBβ complexes may be involved in the persistent activation of NF-κB during ischemia and reperfusion.

In conclusion, the data presented suggest that short periods of ischemia rapidly reduce IκBα levels in the cytoplasm and increase NF-κB binding activity in the nucleus of isolated perfused rat hearts. Early activation of NF-κB in ischemic myocardium could be a potential acute molecular mechanism for regulating cytokine gene expression. PDTC, an antioxidant, significantly prevents the loss of IκBα protein from the cytoplasm and inhibits NF-κB binding activity in the nucleus. Reperfusion after short periods of ischemia, however, only augments NF-κB binding activity in the nucleus that was previously induced by ischemia. Because NF-κB activation is induced during the early stages of ischemia, modulation of NF-κB activation might prove to be beneficial for preventing myocardial ischemia and reperfusion injury.

The authors gratefully acknowledge Dr. Tuanzhu Ha, Dr. Liping Liu, and J anet Davis for technical assistance; Margaret Hatch for secretarial support; and Dr. Donald A. Ferguson, J r., for critical reading of the manuscript.

This work was supported in part by National Heart, Lung, and Blood Institute Grant HL-54286, a Veterans Affairs Merit Review Grant, and a Cardiovascular Research Institute Grant of East Tennessee State University.

Address for reprint requests: R. L. Kao, Dept. of Surgery, James H. Quillen College of Medicine, East Tennessee State University, PO Box 70575, Johnson City, TN 37614-0575.

Received 27 May 1998; accepted in final form 13 October 1998.

REFERENCES


