

Ischemia-Reperfusion of Rat Myocardium Activates Nuclear Factor- κ B and Induces Neutrophil Infiltration Via Lipopolysaccharide-Induced CXC Chemokine

Bysani Chandrasekar, DVM, PhD; Jeffrey B. Smith, MD, PhD; Gregory L. Freeman, MD

Background—Mechanisms by which neutrophils are attracted to the myocardium in ischemia/reperfusion are not fully defined. Lipopolysaccharide-induced CXC chemokine (LIX), cytokine-induced neutrophil chemoattractant (KC), and macrophage inflammatory protein-2 (MIP-2) are rodent chemokines with potent neutrophil-chemotactic activity. The goals of the present study were to evaluate the roles of these chemokines in a rat model of ischemia/reperfusion and to examine the mechanisms of chemokine induction by oxidative stress and cytokines in cultured cardiomyocytes.

Methods and Results—Male Wistar-Kyoto rats underwent 45 minutes of ligation of the left anterior descending coronary artery, followed by reperfusion for various periods. Compared with sham-operated controls, myocardium from reperfused animals had higher levels of free radicals, increased neutrophil infiltration evidenced histologically and by elevated myeloperoxidase activity, and increased nuclear factor (NF)- κ B DNA binding activity. Ischemia-reperfusion also induced the expression of interleukin-1 β , tumor necrosis factor (TNF)- α , LIX, KC, and MIP-2 mRNA and protein. LIX expression was localized to resident myocardial cells, whereas KC and MIP-2 were expressed only in infiltrating inflammatory cells. Neutralization of LIX inhibited 79% of neutrophil infiltration into previously ischemic myocardium. In contrast, neutralization of KC and MIP-2 reduced neutrophil infiltration by only 28% and 37%, respectively. In cultured cardiomyocytes, LIX expression was induced by oxidative stress or TNF- α and was blocked by the NF- κ B inhibitor pyrrolidinedithiocarbamate.

Conclusions—LIX is expressed by resident myocardial cells during ischemia-reperfusion and is induced in cultured cardiomyocytes by oxidative stress or TNF- α via NF- κ B activation. Although KC and MIP-2 are expressed by inflammatory cells infiltrating the myocardium during reperfusion after ischemia, neutrophil recruitment to reperfused rat myocardium is mainly due to cardiomyocyte expression of LIX. (*Circulation*. 2001;103:2296-2302.)

Key Words: ischemia ■ reperfusion ■ chemokines ■ inflammation

Ischemia-reperfusion generates high levels of free radicals composed of both reactive oxygen intermediates and nitric oxide (NO).¹ When generated in sufficient concentrations, free radicals directly injure the myocardium and may even cause cell death. In addition, free radicals activate redox-sensitive transcription factors, including nuclear factor- κ B (NF- κ B), and trigger the expression of interleukin (IL)-1 β , tumor necrosis factor (TNF)- α , and other inflammatory mediators.^{2,3} IL-1 β and TNF- α themselves are potent inducers of NF- κ B activation.²⁻⁶

We have previously shown increased free radical generation, activation of NF- κ B, and induction of the κ B-responsive cytokines IL-1 β , IL-6, and TNF- α in reperfused rat myocardium after 15 minutes of ischemia.⁶⁻⁸ An ischemic episode of 15 minutes does not induce cell death but results in myocardial dysfunction (stunning) that is reversed by prolonged reperfusion.⁹ In contrast, 45 minutes of ischemia is lethal to

the myocardium, and the injury is exacerbated by the recruitment and activation of neutrophils during reperfusion.¹⁰⁻¹³ Although the contribution of neutrophils to ischemia-reperfusion injury is well established, the precise mechanisms responsible for neutrophil recruitment during reperfusion are incompletely understood.

The chemokines are a superfamily of cytokines with diverse biological functions, including the selective recruitment of specific leukocyte classes to sites of inflammation and injury.^{14,15} Members of the CXC branch of the chemokine family have 4 invariant cysteines, the first 2 of which are separated by 1 other amino acid (X). Chemokines containing glutamic acid-leucine-arginine (ELR) immediately preceding the CXC motif are potent neutrophil chemoattractants. In humans, 7 ELR+CXC chemokines are known: IL-8; neutrophil activating peptide-2 (NAP-2); growth-related oncogenes (GRO)- α , - β , and - γ ; epithelial cell-derived neutrophil-

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From the Department of Medicine, University of Texas Health Science Center at San Antonio, South Texas Veterans Healthcare System, Audie Murphy Division, San Antonio, Tex (B.C., G.L.F.), and the Department of Pediatrics, UCLA School of Medicine, Los Angeles, Calif (J.B.S.).

Correspondence to Bysani Chandrasekar, DVM, PhD, Medicine/Cardiology, The University of Texas Health Science Center, 7703 Floyd Curl Dr, San Antonio, TX 78229-3900. E-mail chandraseka@uthscsa.edu

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activating peptide-78 (ENA-78); and granulocyte chemotactic protein-2 (GCP-2). These chemokines are thought to play a major role in the recruitment of neutrophils to tissue in a wide variety of infectious and inflammatory conditions.^{14,15} IL-8 has been shown to participate in neutrophil recruitment to reperfused myocardium in humans, dogs, and rabbits,^{16–19} but the potential roles of other ELR+CXC chemokines in ischemia-reperfusion have not been evaluated.

Because rats lack an IL-8 homologue, they provide a good model for investigating the roles of other rat ELR+CXC chemokines, including cytokine-induced neutrophil chemoattractant (KC), macrophage inflammatory protein-2 (MIP-2), and lipopolysaccharide (LPS)-induced CXC chemokine (LIX).^{20–22} We found that although myocardial ischemia/reperfusion induced all 3 of these chemokines, neutrophil recruitment to reperfused rat myocardium was dependent primarily on LIX.

Methods

Animal Studies

The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication 85-23, revised 1985). Male Wistar-Kyoto rats (~200 g) were used in these studies. Anesthesia (44 mg/kg ketamine, 1 mg/kg acepromazine, and 8.5 mg/kg xylazine) and surgery were performed as described.^{6–8,23} After 45 minutes of ligation of the left anterior descending coronary artery, the hearts were reperfused for specified times (8 rats per group). Tissue from control animals (8 rats) was evaluated after sham ischemia. The heart was rapidly excised after the experimental period and rinsed in ice-cold physiological saline. The right ventricle and atria were trimmed away. The left ventricle was divided into ischemic and nonischemic zones, which were snap-frozen in liquid N₂ for further analyses.

In a separate experiment, normal rabbit IgG or rabbit anti-LIX, -KC, or -MIP-2 antibodies (500 µg, PeproTech Inc) were injected intraperitoneally 1 hour before sham operation or 45 minutes of left anterior descending coronary artery ligation (4 rats per group). The ischemic zones were collected after 2 hours of reperfusion.

Lipid Peroxidation, NF-κB DNA Binding Activity, and Myeloperoxidase Assays

Measurement of the generation of thiobarbituric acid-reactive substances (TBARS) in myocardial extracts was carried out with a commercially available kit (Lipid peroxidation assay kit, Calbiochem-Novabiochem Corp). Electrophoretic mobility shift assay for NF-κB DNA binding activity and myeloperoxidase (MPO) assay were performed as described.^{8,23}

Northern Blot Analysis

Isolation of total RNA, Northern blotting, autoradiography, and densitometry were performed as described.^{6–8,23} The cDNA probes used for IL-1β and TNF-α were described previously.^{6,23} MIP-2 message (GenBank X65647) was detected with an oligonucleotide probe, AAGGCAAGGCTAACTGACCTGGGAAGGAAGAACA-TGGGCTCCTGTACCTCAACGGGCAGAATC. The probe for KC (GenBank M86536) was a reverse transcription-polymerase chain reaction (RT-PCR) product generated from rat cardiac-derived fibroblasts treated for 12 hours with 1 µg/mL LPS (*Escherichia coli* 055:B5, Sigma) with primers AAGATAGATTGCACCGATGGCG (nt 352 to 373) and CTGAGACGAGAAGGAGCATT (nt 850 to 869). The probe for rat LIX (GenBank U90448) was a 329-bp cDNA cloned in PCR-Script (Stratagene) from an RT-PCR product generated with primers GGTCTGCTCGTCATTCA (nt 41 to 58) and CAGTGCAAGTGCATTCCGCT (nt 350 to 369). A 40-nucleotide

probe for h28S rRNA (Oncogene Science, Inc) was used to control for variations in RNA loading.

Protein Extraction and Western Blot Analysis

Extraction of protein homogenates, Western blotting, autoradiography, and densitometry were performed as described.^{6–8,23} Antibodies to murine IL-1β and TNF-α, which cross-react with their rat counterparts,⁶ were obtained from R&D Systems. Affinity-purified rabbit anti-rat GRO/KC and anti-mouse LIX antibodies were from PeproTech, Inc. Goat anti-mouse MIP-2 antibodies were from Santa Cruz Biotechnology, Inc. The antibodies were used at final concentrations of 2.5 µg/mL (KC and LIX), 3.0 µg/mL (MIP-2), or 3.5 µg/mL (IL-1β, TNF-α).

Histology and Immunohistochemistry

Cryosections 6 µm thick were stained for chemokines with the Vectastain Elite ABC-peroxidase kit (Vector Laboratories, Inc).⁸ Primary antibodies were used at a final concentration of either 1 µg/mL (KC and LIX) or 2.5 µg/mL (MIP-2).

Cell Culture

Calcium-tolerant myocytes were isolated from rats by the method of Piper et al.²⁴ Cell preparations contained ≥70% rod-shaped, trypan blue-excluding cells. After overnight incubation in M199 medium containing 4% FBS, myocytes were replated at 250 000 cells/T25 flask in fresh M199 medium containing 0.5% BSA and then were incubated for up to 48 hours with 10 ng/mL recombinant rat TNF-α (Biosource International) or with H₂O₂ (100 µmol/L) for up to 8 hours. The TNF-α stock solution contained <0.1 ng endotoxin/µg TNF-α, as determined by the supplier. Pyrrolidinedithiocarbamate (PDTC, 100 µmol/L) was added 1 hour before TNF-α or H₂O₂.

Statistical Analysis

Comparisons between sham-operated controls and each of the 6 reperfusion time periods were performed for measures of MPO activity and NF-κB DNA binding activity by ANOVA with post hoc Dunnett's *t* tests. The antibody neutralization studies were analyzed similarly. *F* tests and Dunnett's *t* tests with values of *P* < 0.05 were considered statistically significant. Because cytokine and chemokine levels of mRNA and protein were undetectable in the controls, the significance of expression in reperfused tissue was evaluated by 1-sample Student's *t* tests. Error bars in figures indicate the SEM.

Results

Ischemia-Reperfusion Generates Free Radicals, Increases NF-κB Binding Activity, and Induces Neutrophil Infiltration

Rat myocardium was rendered ischemic for 45 minutes as described in Methods. Free radical production during subsequent reperfusion was assessed by measurement of TBARS generation (Figure 1A). Compared with tissue from sham-operated controls, TBARS levels in reperfused myocardium were significantly elevated by 30 minutes, peaked at 1 hour (6-fold, *P* < 0.0001), and remained elevated for the remainder of the 6-hour study period. NF-κB binding activity in myocardial nuclear protein extracts was assessed with a gel mobility shift assay (Figure 1B). NF-κB binding activity increased rapidly after ischemia-reperfusion (5-fold at 15 minutes and 6.1-fold at 30 minutes compared with sham-operated controls), then gradually decreased.

The recruitment of neutrophils into previously ischemic myocardium was assessed by measurement of the activity of MPO. Compared with sham-operated controls, MPO activity increased by 30 minutes of reperfusion (2.9-fold, *P* < 0.01), peaked at 1 to 2 hours (17- to 20-fold, *P* < 0.0001), and then

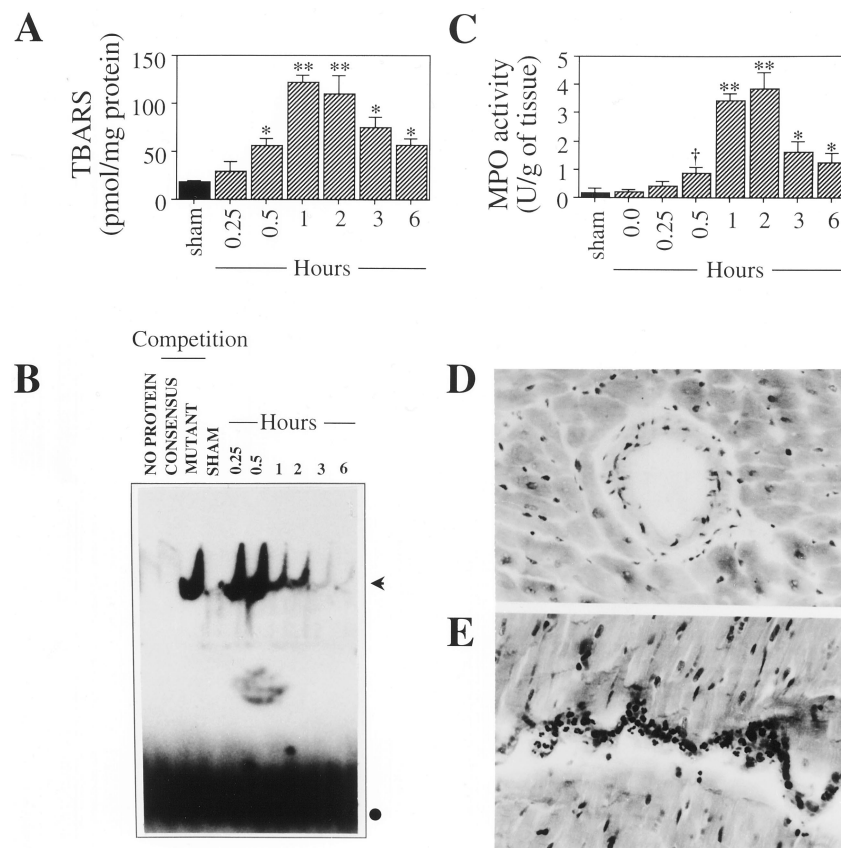


Figure 1. Ischemia-reperfusion induces free radical production, NF- κ B activation, and neutrophil infiltration in myocardium during reperfusion after 45 minutes of ischemia. **A**, Free radical production, as measured by TBARS generation. * P <0.01, ** P <0.0001 vs sham-operated controls. **B**, NF- κ B DNA binding activity in nuclear protein extracts from reperfused and sham-operated myocardium. Each lane contains pooled extracts from 4 rats. Specificity was confirmed by competition with consensus and mutant NF- κ B oligonucleotides. Arrow, NF- κ B complexes; solid circle, unincorporated probe. **C**, MPO activity in reperfused myocardium. † P <0.025, * P <0.01, ** P <0.0001 vs sham-operated controls. **D**, Sham-operated myocardium, with no visible neutrophils. **E**, Previously ischemic myocardium at 2 hours of reperfusion, with many infiltrating neutrophils.

declined. MPO activity remained elevated compared with controls, however, even at 6 hours (6.8-fold, P <0.01). Neutrophil infiltration was also evaluated histologically. Neutrophils were not visible in sham-operated controls (Figure 1D) but were readily detected in previously ischemic myocardium at 2 hours (Figure 1E).

Ischemia-Reperfusion Induces Myocardial Expression of IL-1 β , TNF- α , LIX, KC, and MIP-2

Neither IL-1 β nor TNF- α mRNA was detected in sham-operated animals (Figure 2A). In reperfused tissue, IL-1 β and TNF- α messages were induced within 30 minutes, remained elevated for 3 hours, and then fell to undetectable levels by 6 hours. The protein levels of both cytokines followed a similar time course (Figure 2B).

Ischemia-reperfusion induced the expression of LIX, KC, and MIP-2, but the time courses of induction of these chemokines differed (Figure 3). Message and protein for both LIX and MIP-2 were induced by 1 hour of reperfusion. LIX protein levels then continued to rise, peaking at 2 to 3 hours, and remained detectable at 6 hours. In contrast to LIX, MIP-2 protein decreased after 1 hour and was undetectable by 6 hours. Compared with both LIX and MIP-2, KC induction during reperfusion was delayed. KC message and protein were first detected at 2 hours, and then remained at the same levels through the 6-hour study period. The differences in induction kinetics between LIX, KC, and MIP-2 indicate that they are regulated differently, which suggests that their roles in the biological response to reperfusion might differ.

Localization of LIX Expression in Reperfused Myocardium Differs From That of KC and MIP-2

The differing kinetics of LIX, KC, and MIP-2 expression in reperfused myocardium could be due, in part, to differences in their cellular expression patterns. It is known that LIX is induced by LPS in fibroblasts but not in macrophages, whereas KC and MIP-2 are abundantly expressed by macrophages stimulated with LPS.^{22,25,26} Differential induction of LIX, KC, and MIP-2 in different cell types is one mechanism by which these 3 neutrophil-chemoattractant chemokines could manifest distinct functions in vivo. To localize the sites of LIX, KC, and MIP-2 expression during reperfusion, we evaluated previously ischemic myocardium by immunohistochemistry (Figure 4). We performed the analysis at 2 hours of reperfusion because all 3 chemokines were detectable by Western analysis then, and MPO expression was maximal. In sham-operated controls, no immunoreactivity was detected for LIX (Figure 4A) or for either KC or MIP-2 (not shown). After ischemia-reperfusion, LIX immunoreactivity was readily detected in resident myocardial cells, but not in infiltrating inflammatory cells (Figure 4B). Blood vessel walls also had positive LIX immunoreactivity (Figure 4C). In contrast, KC and MIP-2 immunoreactivity was detected in inflammatory cells but not in resident myocardial cells (Figure 4D and 4E). For each chemokine, no immunoreactivity was detected in controls (not shown) in which (1) primary antibody was omitted, (2) primary antibody was neutralized with the respective recombinant protein, or (3) nonspecific preimmune serum was used in place of the primary antibody.

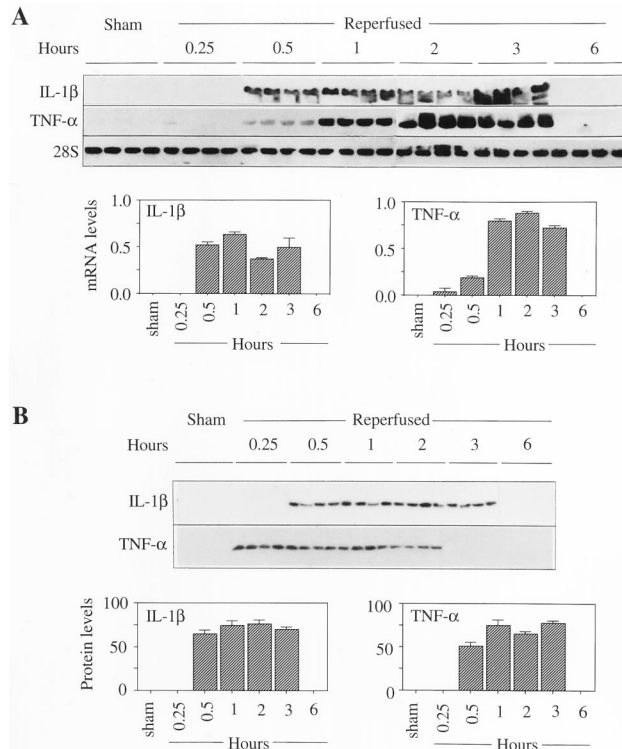


Figure 2. Reperfusion induces expression of IL-1 β and TNF- α in previously ischemic myocardium. A, Cytokine message expression by Northern blot analysis. Each lane contains 30 μ g total RNA from an individual animal. Autoradiographic signal intensity for each probe is expressed in arbitrary units relative to 28S ribosomal RNA. B, Cytokine protein levels by Western blotting. Each lane contains 60 μ g of protein extract from an individual animal. Autoradiographic signal intensity is in arbitrary units for each cytokine.

Neutralization of LIX Markedly Attenuates Neutrophil Accumulation During Reperfusion

The observation that LIX is induced in resident myocardial cells after ischemia-reperfusion, whereas KC and MIP-2 expression is associated with infiltrating inflammatory cells, suggested that LIX may be particularly important for the initiation of neutrophil recruitment during reperfusion. To test this hypothesis, we administered neutralizing anti-LIX, -KC, or -MIP-2 antibodies or control IgG 1 hour before induction of ischemia. Neutrophil accumulation (measured by MPO activity) was determined at 2 hours of reperfusion, which was the time of maximal MPO activity (Figure 1C). The low levels of MPO detected in sham-operated control tissue were not affected by either LIX, KC, MIP-2, or control antibodies (Figure 5). In reperfusion myocardium receiving no antibody, MPO activity increased 21-fold ($P < 0.0001$). Normal rabbit IgG did not affect the reperfusion-induced increase in MPO activity. Anti-LIX antibodies inhibited the increase in MPO activity by 79% ($P < 0.0001$) (Figure 5). In contrast, antibodies to KC and MIP-2 were much less effective, inhibiting the increase in MPO activity by 28% and 37%, respectively ($P < 0.05$). This indicates that although KC and MIP-2 also participate in the process, neutrophil infiltration into reperfusion myocardium in this model is mainly dependent on LIX.

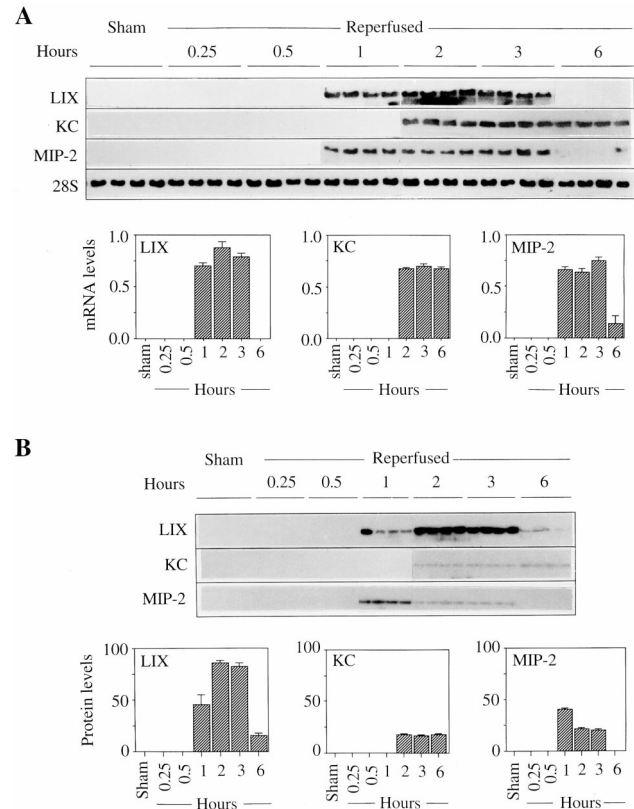


Figure 3. Reperfusion induces expression of LIX, KC, and MIP-2 in previously ischemic myocardium. A, Chemokine message expression by Northern blot analysis. B, Chemokine protein levels by Western blotting. Experiments were performed and analyzed as in Figure 2.

TNF- α and Oxidative Stress Induce LIX Expression Via Activation of NF- κ B

To evaluate the mechanisms of LIX induction that may contribute to its expression in reperfusion myocardium, we investigated the effects of TNF- α and of oxidative stress (H_2O_2) in cultured cardiomyocytes. TNF- α produced a rapid increase in NF- κ B binding activity, which peaked at 30 minutes (2.8-fold) and remained elevated for 48 hours compared with the low levels in unstimulated control cells (Figure 6A). LIX mRNA and protein were induced within 1 hour after TNF- α addition and remained elevated for 48 hours (Figure 6B and 6C). A 1-hour pretreatment with PDTC, a specific inhibitor of NF- κ B activation, strongly inhibited both TNF- α -stimulated NF- κ B DNA binding activity (Figure 6D) and LIX message and protein induction (Figure 6E and 6F).

Compared with TNF- α , the effects of H_2O_2 were delayed and transient (Figure 7). NF- κ B activity was induced 2 hours after H_2O_2 addition, peaked at 4 hours, and then fell to basal levels by 8 hours (Figure 7A). H_2O_2 -induced LIX mRNA and protein expression was maximal at 4 hours (Figure 7B and 7C). PDTC pretreatment markedly attenuated NF- κ B activity (Figure 7D) and abrogated LIX mRNA and protein expression (Figure 7E and 7F). The ability of PDTC to block LIX induction by either TNF- α or H_2O_2 indicates that NF- κ B activation is required for cardiomyocyte induction of LIX by these agents.

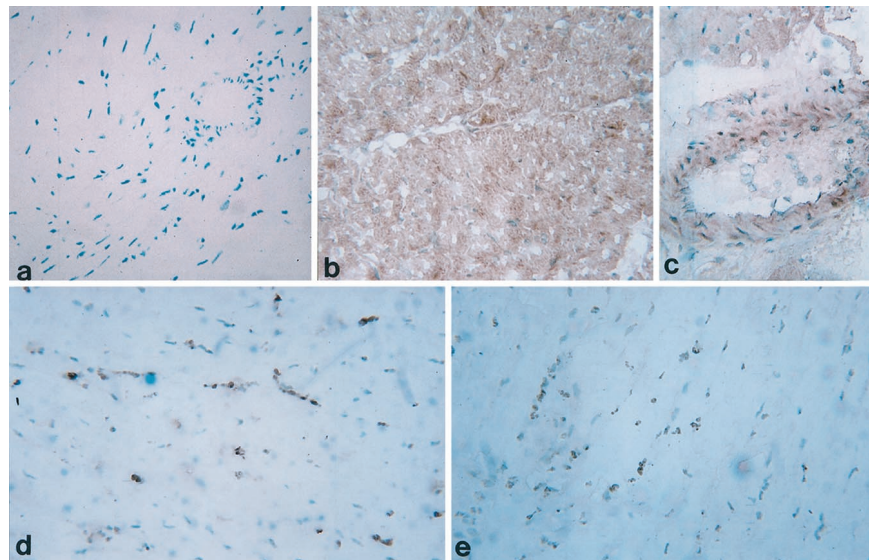


Figure 4. Immunohistochemical localization of LIX, KC, and MIP-2 in myocardium at 2 hours of reperfusion. A, Sham-operated control showing absence of LIX immunoreactivity. B, Reperfused myocardium with LIX immunoreactivity (brown) widely expressed in myocardial cells. C, Reperfused myocardium with LIX immunoreactivity in blood vessels. D, KC and E, MIP-2 immunoreactivity in reperfused myocardium, localized mainly to infiltrating inflammatory cells. Magnification $\times 250$.

Discussion

Reperfusion after ischemia triggers a cascade of responses, including the activation of NF- κ B and the production of the inflammatory cytokines TNF- α and IL-1 β . We found that ischemia-reperfusion also induces the expression of the neutrophil chemoattractant chemokines LIX, KC, and MIP-2. LIX was expressed by resident myocardial cells during reperfusion and was induced in isolated cardiomyocytes by oxidative stress or TNF- α . KC and MIP-2 were also expressed in reperfused heart tissue but were localized to infiltrating inflammatory cells. Neutralization of LIX blocked nearly 80% of neutrophil recruitment to rat myocardium during reperfusion. Neutralization of KC or MIP-2 had much smaller effects. Taken together, our results show for the first time that neutrophil recruitment during myocardial ischemia-reperfusion in rats is mediated primarily by myocardial cell expression of LIX.

LIX was initially cloned as an LPS-induced, glucocorticoid-attenuated response gene in fibroblasts.²² LIX

was subsequently shown to be a potent neutrophil chemoattractant^{27,28} and to be abundantly induced in multiple organs during endotoxemia.²⁹ Interestingly, the organ expressing LIX message most abundantly (per microgram of RNA) during endotoxemia was the heart.²⁹ LIX is also induced in the lung during pneumonia.³⁰ The human chemokines most similar to LIX are ENA-78 and GCP-2.^{31,32} Although LIX has been called murine GCP-2,^{27,28} this designation is potentially misleading. The human ENA-78 and GCP-2 peptides are much more closely related to each other than they are to LIX, and phylogenetic analysis confirms that human GCP-2 is no more closely related to LIX than is human ENA-78.^{31,32} Moreover, the ENA-78 and GCP-2 genes have very high nucleotide similarity in noncoding as well as coding regions, suggesting that they are the result of an evolutionarily recent gene duplication.³² Because of these and other differences between the rodent and human ELR+CXC chemokine systems, including the lack of an IL-8 homologue in rodents, results for LIX in rats cannot be directly extrapolated to either ENA-78 or GCP-2 in humans. Our findings strongly suggest, however, that the roles of ENA-78 and GCP-2 in human myocardial ischemia-reperfusion should be investigated.

A key observation in the present study is the differential localization of the expression of LIX, KC, and MIP-2 in previously ischemic myocardium. LIX immunoreactivity was localized in cells resident to myocardium, whereas KC and MIP-2 were found in infiltrating inflammatory cells but not in myocardial cells (Figure 4). It has previously been reported that adult rat cardiomyocytes do secrete MIP-2 after prolonged stimulation with IL-1 β , TNF- α , or LPS.³³ We evaluated only short-term expression, however, so these results are compatible with our observations. The immunohistochemical localization of LIX to resident myocardial cells in reperfused ischemic myocardium is supported by our *in vitro* studies showing that LIX message and protein are rapidly inducible in isolated cardiomyocytes (Figures 6 and 7). Induction of LIX by TNF- α or H₂O₂ was abrogated by pretreatment with PDTC, indicating that NF- κ B activation is required for LIX induction by either agent. LIX induction by TNF- α was rapid

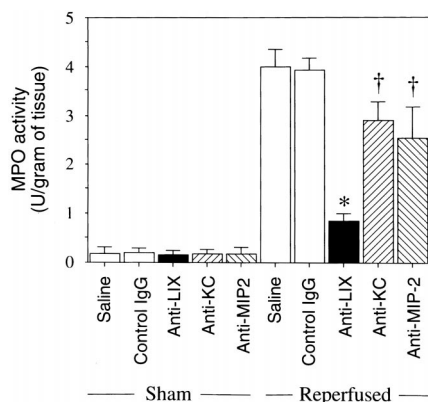


Figure 5. Neutralization of LIX markedly attenuates neutrophil infiltration into previously ischemic myocardium. MPO activity was determined after 2 hours of reperfusion in rats preinjected with saline, control IgG, or rabbit anti-LIX, -KC, or -MIP-2 antibodies. * $P < 0.0001$, † $P < 0.05$ vs reperfused animals given control IgG.

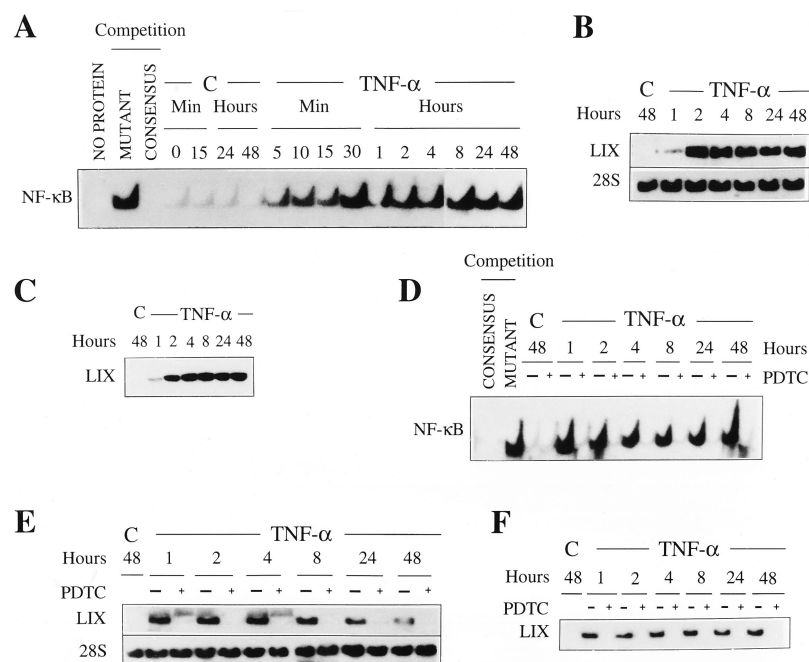


Figure 6. TNF- α induces LIX expression in rat cardiomyocytes via activation of NF- κ B. A, NF- κ B binding activity. B, LIX mRNA expression. C, LIX protein levels. D, Preincubation with PDTC blocks TNF- α induction of NF- κ B activation and abrogates induction of (E) LIX mRNA and (F) LIX protein. Results are representative of 2 independent experiments.

and persistent, however, whereas LIX induction by H₂O₂ was delayed and transient. These differences are consistent with other studies indicating that TNF- α and H₂O₂ activate NF- κ B through different mechanisms.³⁴ The relative importance of cytokine- and free radical-mediated pathways for LIX induction during reperfusion and their roles in LIX-dependent neutrophil recruitment and reperfusion injury are subjects for future study.

Selective targeting of neutrophil chemoattractant chemokines (or their receptors) is an attractive strategy for reducing neutrophil-mediated myocardial injury. We have found that a single chemokine, LIX, is the principal neutrophil chemoattractant in a model of reperfusion after lethal ischemia in rats.

This suggests that in addition to IL-8, the LIX homologues GCP-2 and ENA-78 might have important roles in human myocardial ischemia-reperfusion. It has previously been observed that many cytokines, chemokines, and adhesion molecules expressed after reperfusion are dependent on NF- κ B activation.^{2,3} Because NF- κ B activation was required for induction of LIX by oxidative stress or TNF- α in cardiomyocytes, our data provide further support for the idea that targeted blunting of NF- κ B activation could also be an effective strategy for limiting myocardial injury after ischemia.

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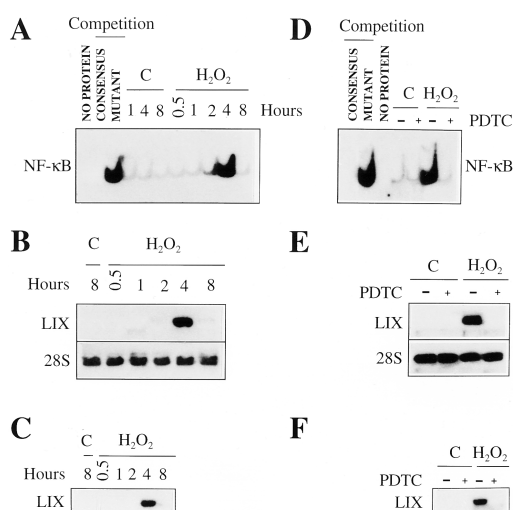


Figure 7. H₂O₂ induces LIX expression in rat cardiomyocytes via activation of NF- κ B. A, NF- κ B binding activity. B, LIX mRNA expression. C, LIX protein levels. D, Preincubation with PDTC blocks H₂O₂ induction of NF- κ B activation and abrogates induction of (E) LIX mRNA and (F) LIX protein. Results are representative of 2 independent experiments.

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