Neutralization of Interleukin-18 Ameliorates Ischemia/Reperfusion-induced Myocardial Injury*

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Ischemia/reperfusion (I/R) injury is characterized by the induction of oxidative stress and proinflammatory cytokine expression. Recently demonstrating that oxidative stress and TNF-α each stimulate interleukin (IL)-18 expression in cardiomyocytes, we hypothesized that I/R also induces IL-18 expression and thus exacerbates inflammation and tissue damage. Neutralization of IL-18 signaling should therefore diminish tissue injury following I/R. I/R studies were performed using a chronically instrumented closed chest mouse model. Male C57BL/6 mice underwent 30 min of ischemia by LAD coronary artery ligation followed by various periods of reperfusion. Sham-operated or ischemia-only mice served as controls. A subset of animals was treated with IL-18-neutralizing antibodies 1 h prior to LAD ligation. Ischemic LV tissue was used for analysis. Our results demonstrate that, compared with sham operation and ischemia alone, I/R significantly increased (i) oxidative stress (increased MDA/4-HNE levels), (ii) neutrophil infiltration (increased MPO activity), (iii) NF-κB DNA binding activity (p50, p65), and (iv) increased expression of IL-18Rβ, but not IL-18Rα or IL-18BP transcripts. Administration of IL-18-neutralizing antibodies significantly reduced I/R injury measured by reduced infarct size (versus control IgG). In isolated adult mouse cardiomyocytes, simulated ischemia/reperfusion enhanced oxidative stress and biologically active IL-18 expression via IKK-dependent NF-κB activation. These results indicate that IL-18 plays a critical role in I/R injury and thus represents a promising therapeutic target.

For both men and women, ischemic heart disease is one of the leading causes of death in the United States today, and its pathobiology has been attributed to many factors, including proinflammatory cytokines. Interleukin (IL)-18 is a pleiotropic cytokine belonging to the IL-1 family (1–5), whose expression is up-regulated in numerous immune, infectious, and inflammatory conditions (1–5), which may further amplify the inflammatory cascade by inducing additional cytokines, chemokines, and adhesion molecules (1–5). Elevated plasma IL-18 levels have been detected in patients with acute coronary syndromes (6), and a direct correlation between IL-18 levels and the severity of myocardial dysfunction has been reported. Circulating IL-18 levels have been shown to be independent predictors of coronary events in humans, with increased basal levels of IL-18 observed in individuals who later developed coronary events (7). Increased circulating IL-18 levels have also been measured during heart failure as well as in stroke patients (8, 9).

IL-18 signals via the IL-18 receptor, a heterodimer consisting of a ligand binding α-subunit and a signal-transducing β-subunit (10, 11). Binding of IL-18 to IL-18Rα recruits IL-18Rβ, and this activated complex initiates pleiotropic signal transduction events. Similar to IL-1 receptor antagonist that blocks IL-1 signaling, IL-18-binding protein (IL-18BP) binds IL-18 with high affinity, inhibits IL-18 bioavailability (12), neutralizes IL-18 effects, and thus reduces inflammation. Interestingly, transgenic mice overexpressing human IL-18BP exhibit reduced inflammation and disease severity (13).

Cytotoxic free radicals and proinflammatory cytokines are produced during ischemia/reperfusion (I/R) injury. Using isolated adult rat cardiomyocytes, we have recently described NF-κB-dependent IL-18 and IL-18Rβ expression following hydrogen peroxide and TNF-α treatment in vitro (14). Moreover, although IL-18BP gene expression under basal conditions in these cells is undetectable, TNF-α and H₂O₂ each induce IL-18BP mRNA in a delayed but persistent manner (14), which suggests tight regulation of IL-18 bioavailability. However, the precise role of IL-18 in I/R injury and its regulation in vivo are incompletely understood, and the signal transduction pathway...
ways involved in its induction in myocardial constituent cells in vitro have not been identified.

The purpose of this study was to (i) determine the temporal expression of IL-18, IL-18 receptors, and IL-18BP during I/R in vivo, (ii) define the causal role of IL-18 in myocardial I/R injury in vivo, and (iii) examine the signaling pathways involved in simulated I/R (sI/R)-mediated IL-18 induction in isolated adult mouse cardiomyocytes in vitro. Our results demonstrate that (i) IL-18 is induced in vivo in postischemic myocardium, (ii) its neutralization blunts I/R-induced tissue injury, and (iii) I/R up-regulates IL-18Rβ and IL-18BP but not IL-18Rα expression. Further, sI/R enhances biologically active IL-18 expression in primary adult mouse cardiomyocytes via IKK-dependent IkB degradation and NF-κB activation. Together, these results indicate that IL-18 plays a critical role in ischemia/reperfusion injury and thus is a potential therapeutic target for ischemic heart disease.

EXPERIMENTAL PROCEDURES

Animals—All animal studies conformed to National Institutes of Health guidelines (48), and were approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center (San Antonio, TX). I/R studies were performed using a chronically instrumented closed chest mouse model (15–17). In brief, male C57BL/6 mice weighing ~25–30 g and aged 3–4 months were anesthetized by urethane (1000 mg/kg intraperitoneally) and etomidate (25 mg/kg intraperitoneally) and mechanically ventilated with a rodent ventilator set at 150 breaths/min (100% oxygen). Mice were placed on a heated, temperature-controlled operating table for small animals (Vestavia Scientific). Using microscopic dissection (surgical microscope system; Applied Fiberoptics), the chest was opened along the left side of the sternum by cutting through the ribs to approximately midsternum, and the chest walls were retracted with 6-0 sutures. The pericardium was then gently dissected to allow visualization of coronary artery anatomy. An 8-0 Surgipro monofilament polypropylene suture with the U-shaped tapered needle was passed under the LAD. The needle was then cut from the suture, and the two ends of the 8-0 suture were then threaded through a 0.5-mm piece of PE-10 tubing that was previously soaked in 100% alcohol overnight, forming a loose snare around the LAD coronary artery. Each end of the suture was then exteriorized through each side of the chest wall. The chest was then closed with four interrupted stitches utilizing 6-0 sutures. The ends of the exteriorized 8-0 suture were then tuck under the skin, which was then also closed with 6-0 sutures. At 1 week after instrumentation, the animals were reanesthetized and randomly assigned to sham or I/R groups. The 8-0 suture, which had been previously exteriorized, was cleared of all debris from the skin and chest and carefully taped to heavy metal picks. Ischemia of LAD coronary artery was accomplished by gently pulling the heavy metal picks apart until an S-T segment elevation appeared on the electrocardiogram. The electrocardiogram was constantly monitored throughout the entire ischemic interval to ensure persistent ischemia. After 30 min, reperfusion was accomplished by pushing the metal picks toward the animal and cutting the suture close to the chest wall. Reperfusion was confirmed by resolution of the S-T segment elevation, which usually occurred very quickly. An additional four animals underwent only ischemia (30 min of ischemia and no reperfusion). Sham-operated animals were prepared identically without undergoing the I/R protocol. At the indicated time points (Fig. 1A), the hearts were rapidly excised and rinsed in ice-cold physiological saline. The right ventricle and atria were trimmed away, and the left ventricle was divided into ischemic and nonischemic zones and snap frozen in liquid N2.

Adult Mouse Cardiomyocytes—Calcium-tolerant adult mouse ventricular myocytes were isolated by modified Langendorff perfusion and collagenase digestion, adapting the methodology described by the Alliance for Cellular Signaling (18) (available on the World Wide Web). In brief, mice were given heparin (1000 units/kg intraperitoneally) and then deeply anesthetized with intramuscular ketamine (43.5 mg/kg), acepromazine (1.5 mg/kg), and xylazine (1.7 mg/kg). Median sternotomy was performed, and the heart was rapidly excised and rinsed with physiologic saline. The aortic lumen was isolated and tied to an 18-gauge cannula, and the heart was perfused with oxygenated (95% O2, 5% CO2), Ca2+-free modified Tyrode’s bicarbonate buffer (126 mM NaCl, 4.4 mM KCl, 1 mM MgCl2, 18 mM NaHCO3, 11 mM glucose, 4 mM HEPES, 10 mM 2,3-butanedi-one monofoxime, 30 mM taurine, pH 7.35) at 37 °C for 5 min. Following this, the heart was perfused with 50 ml of digestion buffer (modified Tyrode’s bicarbonate buffer with 0.25 mg/ml Liberase Blendzyme type 1 (Roche Applied Science), 0.14 mg/ml trypsin (Sigma), and CaCl2 2.5 μM) in a recirculating fashion for 12–15 min. The heart was then removed, and the left ventricle (LV) was separated and dissected with small, blunt forceps in 2–3 ml of digestion buffer. The minced tissue suspension was gently agitated by repeated pipette aspiration and transferred into myocyte stopping buffer 1 (modified Tyrode’s bicarbonate buffer with 10% fetal calf serum and 12.5 μM CaCl2) in a 50-ml conical tube and allowed to sediment for 10 min. The supernatant was transferred to another tube and centrifuged at 90 × g for 2 min. The sediment/pellet from both tubes were combined and resuspended in myocyte stopping buffer 2 (modified Tyrode’s bicarbonate buffer with 5% fetal calf serum and 12.5 μM CaCl2) in a 100-mm culture dish. Small aliquots of CaCl2 were then added in a graded fashion at 4-min intervals to sequentially increase the Ca2+ concentration to 500 μM (five total steps). The suspension was then placed in a 50-ml conical tube and allowed to sediment for 10 min at 22 °C. As above, the supernatant was transferred to another tube and gently centrifuged at 90 × g for 2 min, and myocytes contained in both the sediment and the pellet were combined and resuspended in minimal essential medium (pH 7.35–7.45; catalogue number M1018; Sigma) containing 1.2 mM Ca2+, 12 mM NaHCO3, 2.5% fetal bovine serum, and 1% penicillin/streptomycin. The cells were then plated onto 35-mm cell culture dishes precoated with 20 μg/ml mouse laminin in phosphate-buffered saline with 1% penicillin/streptomycin for 1 h. Cardiomyocytes were maintained under resting conditions in the incubator for at least 16 h before experimentation. Ischemia/reperfusion was simulated by incubating cardiomyocytes 30 min in an “ischemia buffer” containing 118 mM NaCl, 24 mM NaHCO3, 1.0 mM NaH2PO4, 2.5 mM CaCl2, 1.2 mM MgCl2, 20 mM sodium lactate, 16 mM

Interleukin-18 and Reperfusion Injury
KCl, 10 mM 2-deoxyglucose (pH adjusted to 6.2), followed by reoxygenation for 4 h. Reoxygenation was accomplished by replacing the ischemic buffer with normal medium under normoxic conditions. All of the studies were completed within 72 h after isolation.

Cardiomyocytes were treated with PDTC (100 μM in PBS) for 1 h prior to undergoing sl/R. IKK was targeted by SC-514 (10 μM in DMSO for 1 h). In addition, IKKβ and IkB-α were targeted in adenoviral dominant negative IKKβ or IkBα (dnIKKβ or dnIkBα, respectively), as described previously (19). Ad.GFP served as a control. Transfection efficiency of cardiomyocytes with these adenoviral vectors is near 100%. 24 h after infection, cells underwent sl/R.

Splenocyte Isolation—Male C57BL/6 mice weighing ~25–30 g and aged 3–4 months were sacrificed by cervical dislocation. The spleens from three animals were pooled and homogenized in a Dounce homogenizer in RPMI1640 plus 10% fetal calf serum. The cell suspension was then carefully overlaid on Histopaque 1083 (Sigma), centrifuged at 800 × g for 20 min at 22 °C, and the splenic mononuclear cells were carefully collected from the media/Ficoll interface and resuspended in RPMI 1640 plus 10% fetal calf serum. The cell suspension was then carefully overlaid on Histopaque 1083 (Sigma), centrifuged at 800 × g for 20 min at 22 °C, and the splenic mononuclear cells were carefully collected from the media/Ficoll interface and resuspended in RPMI 1640 plus 10% fetal calf serum. Splenocytes (5 × 10⁶/ml) were incubated with and without cardiomyocyte-derived culture supernatants following simulated I/R and LPS (1 μg/ml) for 24 h at 37 °C. After incubation, supernatants were sampled and analyzed for IFN-γ production by ELISA (n = 6).

Adenoviral Transduction—Replication-deficient recombinant adenovirus encoding green fluorescent protein (Ad.GFP), dnIKKβ (Ad.dnIKKβ), and phosphorylation-deficient IkB-α (S32A/S36A; Ad.dnIkB-α) have all been described before (19–21). Cardiomyocytes were infected with adenoviruses at a multiplicity of infection (MOI) of 100 at ambient temperature as described previously (19–21). After 1 h, the adenovirus-containing medium was replaced with medium containing 0.5% bovine serum albumin. 24 h later, cardiomyocytes underwent ischemia/reoxygenation as described above. Adenoviral transduction did not result in cardiomyocyte death (data not shown).

Lipid Peroxidation—Ischemic zones were assessed for lipid peroxidation (22). Left ventricular tissue from sham-operated animals at 2.5 h served as controls. Frozen tissues were homogenized in 250 μl of ice-cold 20 mM Tris-HCl (pH 7.4). After centrifugation, 200 μl of supernatant were analyzed for lipid peroxidation products (malondialdehyde (MDA)/4-hydroxyalkenals (4-HNE)) using a lipid peroxidation assay kit (Calbiochem).

Detection of Intracellular ROS—Intracellular ROS levels were visualized using the cell-permeable, redox-sensitive fluorophore, 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (Molecular Probes, Inc., Eugene, OR). Prior to undergoing simulated ischemia or ischemia/reoxygenation for the indicated time periods, cardiomyocytes were incubated in medium containing 30 μM non-fluorescent DCFH-DA for 1 h to obtain stable intracellular levels of the probe. Similar concentrations were maintained during simulated ischemia or ischemia/reoxygenation. Upon entry into the cell, DCFH-DA is initially converted into non-fluorescent 2′,7′-dichlorofluorescein (DCF) by cellular esterase and then oxidized to fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of ROS (DCFH-DA → DCFH → DCF). DCF fluorescence was visualized by fluorescent microscopy. The intensity of DCF in the cells indicates increased intracellular levels of ROS. Image acquisition and analysis were performed as previously reported (23). In brief, cells were observed through a Plan-Apo ×60 oil immersion objective mounted on an inverted Olympus microscope with an Olympus LSM GB200 confocal imaging system attached. Excitation of dyes was achieved using the 488-nm line of a 15-mWatt argon ion laser. All images were collected at room temperature. Mean fluorescence intensity was calculated by averaging area intensities from a number of outlined cells. For each condition described, six images of different cells were collected, and experiments were repeated at least three times.

MPO Activity—MPO activity in ischemic LV tissue was analyzed as described previously (22). In brief, cardiac tissue samples were first homogenized in 20 mM potassium phosphate (1:10, w/v) and then centrifuged at 20,000 × g for 30 min at 4 °C. The pellets were then frozen (−20 °C) for 12 h. After thawing, the pellet was added to a solution consisting of 0.5% hexadecyltrimethylammonium bromide dissolved in 50 mM potassium phosphate buffer (pH 6.0) containing 30 units/ml aprotinin. Each sample was sonicated for 1 min at 4 °C and then centrifuged at 40,000 × g for 30 min at 4 °C. Aliquots of the supernatant were reacted with a solution of O-dianisidine dihydrochloride (0.167 mg/ml) and 0.0005% H₂O₂, and absorbance at 405 nm was measured over time. MPO activity is defined as the quantity of enzyme degrading 1 μmol of peroxide/min at 37 °C and is expressed as units/g of tissue (wet weight).

Transcription Factor Activation-DNA Binding Activity—Nuclear proteins were extracted from the ischemic zones of the myocardium from I/R-treated mice using a nuclear extraction kit (catalog number AY2002; Panomics, Fremont, CA). Formation of NF-κB protein-DNA complexes in postischemic myocardium and isolated cardiomyocytes was analyzed by an electrophoretic mobility shift assay (EMSA), and its subunit composition was analyzed by a supershift assay. In addition, NF-κB activation and its subunit composition were also analyzed by ELISA (TransAM™ TF ELISA kits; catalog number 43296; Active Motif, Carlsbad, CA) (19–21). EMSA was performed using IL18 gene-specific (sense, 5′-CCCTGATAAAGTGTAGATTCGCCTATTATAC-3) and mutant (sense, 5′-CCCTGATAAAATACCTATTCCCTATTATAC-3) oligonucleotides, as described previously (20, 21). Specificity of NF-κB DNA binding activity was determined by competition with unlabeled consensus or mutant NF-κB oligonucleotides or consensus Oct1 (5′-TGT CGA ATGCAA ATC ACT AGA A-3′) oligonucleotides. In addition, labeled mutant NF-κB oligonucleotide also served as a control. In supershift assays, the nuclear protein extracts were incubated with antibodies (1 μg) p50 (sc-7178 X) or p65 (sc-109 X; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 40 min prior to EMSA. Activation of NF-κB in cardiomyocytes was also confirmed by reporter assays using adenoviral transduction with NF-κB (Ad-NF-κB-Luc, 50 MOI; kindly provided by John F. Engelhardt), as described previously (21). Ad-MCS-Luc (Vector Biolabs) served as a control. Ad-β-galactosidase (50 MOI; Vector Biolabs) served as an internal control. β-Galactosidase activity in cell extracts was determined using a luminescent β-galactosid-
Interleukin-18 and Reperfusion Injury

mRNA Analysis—Expression of IL-18, IL-18Rα, IL-18Rβ, and IL-18BP mRNA in the ischemic left ventricular tissue was analyzed by reverse transcription followed by real time quantitative PCR (qPCR) using an ABI GeneAmp 7700 Sequence Detection System (PerkinElmer Life Sciences) according to the manufacturer’s instructions, with SYBR Green fluorescence for amplicon detection. DNA-free total cellular RNA was isolated using the RNAqueous®-4PCR kit (Ambion). RNA quality was assessed by capillary electrophoresis using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). All RNA samples had RNA integrity numbers greater than 8.9 (scale = 1–10), as assigned by default parameters of the Expert 2100 Bioanalyzer software package (version 2.02). Primer pairs were designed to span intron-exon junctions, and thermal melting profiles of amplicons were confirmed for performance. The following primer pairs were used: IL-18, sense (5′-GCCATGCAGAAAGACTCTTGGC-3′) and antisense (5′-GTCAGTGGAAGGTGGGTTGCTC-3′); IL-18Rα, sense (5′-CCCGGCAGAAGAGCCATAGACA-3′) and antisense (5′-TCAGGATGACTCTTGAGCAG-3′); IL-18Rβ, sense (5′-CCAGGGAGTGATGATCG-3′) and antisense (5′-ACCCGCAGAGCCTTTTGAC-3′); and IL-18BP, sense (5′-GTGGCTCAATGAAGGAAACCA-3′) and antisense (5′-ACCAGGCTAGAAGACA-3′). Amplification of β-actin was performed for each sample to control for sample loading. No template controls were performed for each assay, and samples processed without the reverse transcriptase step served as negative controls. Each cDNA sample was run in triplicate, and the amplification efficiencies of all primer pairs were determined by serial dilutions of input template. Using β-actin as the endogenous invariant control gene, all data were normalized and expressed as the -fold difference in gene expression in I/R mice relative to sham-operated mice.

IL-18, IL-18Rα, IL-18Rβ, and IL-18BP mRNA abundance was confirmed by Northern blot analysis, using cDNA amplified from total RNA isolated from mouse myocardium and the following primer pairs: IL-18 (GenBank™ accession number D49949), sense (5′-ACTGTACAACCGCAGTAATAC-3′ (nt 286–308)) and antisense (5′-AGTGACATTCAGTAATCTTCC-3′ (nt 697–719)); IL-18Rα (GenBank™ accession number U43673), sense (5′-CCCGCACAAAGGCAAGCCATAGACA-3′ (nt 1276–1296)) and antisense (5′-TCAGGATGACTCTTGAGCAG-3′); and IL-18Rβ (GenBank™ accession number U4077347), sense (5′-CCCGGCAAGGTCAGGTAAGACA-3′ (nt 1747–1766)) and antisense (5′-ACCCGCAGAAGGCTTTGGC-3′ (nt 2012–2031)). The primers for IL-18BP are specific for both IL-18BPc and IL-18BpD and were derived from sequences for IL-18BPc (GenBank™ accession number AF110802) and IL-18BpD (GenBank™ accession number AF110803): sense, 5′-ACACTTGCTCACCTGAGCAACT-3′; antisense, 5′-TGGGAGGTGCTCAATGAAGGAACCA-3′. The product size was 225 bp. Human 28 S ribosomal RNA (rRNA; 40-mer; Oncogene Science, Inc.) was used as an internal control. The mRNA expression data shown are ratios of the signal obtained for a specific gene to that of corresponding 28 S rRNA.

Protein Analysis—Preparation of protein homogenates, immunoblotting, and densitometry were performed as described previously (19–21, 24). Briefly, myocardial tissue was homogenized in a hypotonic lysis buffer containing 10 mM Tris, pH 7.5, 1 mM EDTA, 1 mM Na3VO4, 10 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 10 mM NaF, and 1% protease inhibitor mixture (Sigma). Protein concentrations were determined using the BCA assay (Pierce) with bovine serum albumin as a standard, and clarified lysates were boiled in SDS sample buffer. Samples were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane and probed with primary antibodies in 2% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TTBS). After incubation overnight at 4 °C, blots were washed in TTBS for 30 min, incubated in horseradish peroxidase-conjugated secondary antibodies in 5% milk in TTBS for 1 h, and detected by chemiluminescence (SuperSignal West Pico; Pierce) supplemented with 5% SuperSignal West Femto (Pierce). Purified rat anti-mouse mature IL-18 antibodies (catalog number D046-3) were obtained from R&D Systems and used at a final concentration of 1.5 μg/ml. β-Actin was used as an internal control. Anti-IkBα (catalog number sc-7977) antibodies were from Santa Cruz Biotechnology. JNK (catalog number 9252), phospho-JNK (catalog number 9251S), phospho-p65 (Ser276; catalog number 3037), and Lamin A/C (catalog number 2032) antibodies were from Cell Signaling Technology, Inc.

IL-18 levels in tissue homogenates and culture supernatants were quantified by a sandwich ELISA (catalog number 7625; R&D Systems). The sensitivity of the assay is 25 pg/ml. IFN-γ levels in splenocyte culture supernatants were quantified by ELISA (Quantikine Colorimetric Sandwich ELISA; sensitivity <10 pg/ml; R&D Systems).

IKK Kinase Activity—IKK kinase activity in cardiomyocytes was determined by an in vitro kinase assay, which evaluates the ability of immunoprecipitated IKKβ to phosphorylate glutathione S-transferase-IκB fusion protein in vitro. The assay was carried out essentially as described by Fan et al. (25). In brief, following 30 min of ischemia and 2 h of reoxygenation, cardiomyocytes were washed in ice-cold PBS and lysed in 1 ml of ice-cold radioimmune precipitation assay buffer (0.15 M NaCl, 50 mM Tris, pH 7.2, 1% deoxycholate, 1% Triton X-100, 0.1% SDS), followed by centrifugation at 10,000 rpm for 10 min at 4 °C. Protein concentrations were determined using the Micro BCA™ protein assay kit. 500 μg of protein was immunoprecipitated with anti-IKKβ antibodies and protein A-agarose beads. 1 μg of glutathione S-transferase-IκB fusion protein was then added to the washed protein A pellets in the presence of 10 μl of kinase buffer (40 mM Hepes, 1 mM β-glycerophosphate, 1 mM nitrophenol phosphate, 1 mM Na3VO4, 10 mM MgCl2, 2 mM dithiothreitol, 0.3 mM cold ATP, and 10 μCi of [γ-32P]ATP) and incubated at 30 °C for 30 min. The reaction was terminated by the addition of SDS loading buffer. Samples were preheated to 98 °C for 5 min and centrifuged to pellet the agarose beads, and the supernatants were separated by 10% SDS-PAGE and exposed to x-ray film.
Infarct Size Measurement—Infarct size was measured at 24 h of reperfusion using the method described by Guo et al. (26). Prior to sacrifice, the LAD coronary artery was reoccluded, and the heart was perfused with a 1% solution of 2,3,5-triphenyltetrazolium chloride in phosphate buffer (pH 7.4, 37 °C). The coronary artery was then tied at the site of the previous occlusion, and the aortic root was perfused with Unisperse blue dye (Ciba-Geigy) through a left ventricular catheter. This procedure delineated the area at risk, which was defined as the portion of the myocardium perfused by the vessel that has been ligated. The animal was then euthanized by an overdose of the anesthetic mixture, and the heart was quickly removed, frozen, cut into five slices parallel to the atrio-ventricular groove, fixed in 10% buffered formaldehyde, weighed, and then photographed on both sides. TTC normally stains tissue dark red; infarcted tissue will be unstained and appear white. The LV area (LV), area at risk (AR), and area of necrosis (AN) for all slices were calculated by planimetry using a digitized tablet and dedicated computer software (Sigma Scan; Jandel Scientific). The AR was expressed as a percentage of LV (percentage of AR = total AR/total LV × 100), and the AN was expressed as a percentage of the AR (infarct size = total AN/total AR × 100). In order to demonstrate the potential role played by IL-18 in I/R injury, mice were treated with IL-18-neutralizing antibodies. Animals received anti-mouse IL-18-neutralizing antibodies (R&D Systems) or normal rat IgG (0.5 mg, intravenously via jugular vein) 1 h prior to LAD coronary artery ligation (n = 4/group). Animals underwent 30 min of ischemia/24 h of reperfusion. Infarct size was measured as described above.

Cell Death Detection ELISA—Cardiomyocytes exposed to either ischemia alone (30 min) or ischemia (30 min), followed by reoxygenation for 4 h, were harvested and analyzed for mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates by ELISA (Cell Death Detection ELISA^PLUS^ kit, Roche Applied Science) (27). Doxorubicin, a potent anti-neoplastic drug, which has previously been shown to induce cardiomyocyte death (28), served as a positive control (1 μM for 24 h; doxorubicin hydrochloride; catalog number D1515; Sigma).

Statistical Analysis—The number of mice required for each study time point was determined by power analysis. Comparisons between experimental groups were made using the two-tailed t test for in vivo analysis, and using two-way analysis of variance for in vitro analyses, with p < 0.05 considered significant. Each experiment was performed at least three times, and group data were expressed as means ± S.E.

RESULTS

I/R Generates Free Radicals and Promotes Neutrophil Infiltration—Reactive oxygen intermediates (e.g. H2O2) are generated during myocardial ischemia/reperfusion and initiate tissue damage through multiple mechanisms, including lipid peroxidation (29). Details of the I/R protocol are shown in Fig. 1A. The levels of MDA/4-HNE levels were significantly elevated by 30 min of ischemia, followed by 2 h of reperfusion (Fig. 1B; 30 min of ischemia/2 h of reperfusion; *, p < 0.001 versus sham-operated and ischemia alone). Circulating neutrophils recruited to the myocardium can exacerbate tissue injury by generating oxygen free radicals via the NADPH oxidase enzyme, releasing proteases and proinflammatory cytokines, and plugging microcapillaries. Furthermore, IL-18 has been shown to directly influence neutrophil function, since IL-18 activates p38 MAP kinase, primes NADPH oxidase, enhances chemotaxis, and increases azurophilic granule release (30). Therefore, we examined neutrophil recruitment during I/R using tissue MPO activity as a biochemical marker. During the reperfusion phase of the I/R injury, there was a significant increase in MPO activity within the ischemic myocardium (*, p < 0.001; Fig. 1C); however, no increase in activity was seen during the ischemic phase. These results demonstrate that I/R results in free radical generation and promotes neutrophil infiltration (Fig. 1).

I/R Activates Nuclear Factor κB DNA Binding Activity—NF-κB is a ubiquitous, multisubunit, inducible transcription factor that is retained in the cytoplasm by the binding of NF-κB to IκB (31). Various stimuli, including cytokines and oxidative stress, induce IκB hyperphosphorylation and degradation, and the subsequent translocation of released NF-κB to the nucleus for κB-dependent gene transcription (31). Because I/R generates free radicals and NF-κB is a redox-sensitive transcription factor, we questioned whether NF-κB DNA binding activity is altered in the postischemic myocardium and determined NF-κB DNA binding activity by two different but complementary methods: EMSA and ELISA (19–21). In the sham-operated animals, minimal NF-κB DNA binding activity was observed (Fig. 2A, lanes 6–9). Similarly, no significant changes were
Interleukin-18 and Reperfusion Injury

FIGURE 2. Ischemia/reperfusion activates NF-κB in postischemic myocardium. A, I/R induced NF-κB DNA-binding activity. Following 30 min of ischemia/reperfusion, nuclear proteins were extracted from postischemic reperfused LV tissue and analyzed for NF-κB DNA binding activity by EMSA as described under “Experimental Procedures.” Sham-operated and ischemia-alone groups served as controls (n = 4/group). Control studies determining the specificity of NF-κB oligonucleotide was shown in the completion experiments (lanes 1–4). Lane 1, protein extract from 30 min ischemia/reperfusion myocardium was preincubated with a 75-fold molar excess of unlabeled double-stranded consensus NF-κB oligonucleotide, followed by the addition of labeled NF-κB-specific probe. Lane 2, competition with cold mutant NF-κB oligonucleotide. Protein extract from 30 min ischemia/reperfusion myocardium was preincubated with a 75-fold molar excess of unlabeled double-stranded consensus NF-κB oligonucleotide, followed by the addition of labeled NF-κB-specific probe. Lane 3, protein extract from 30 min ischemia/reperfusion myocardium was preincubated with a 75-fold molar excess of unlabeled double-stranded consensus Oct1 oligonucleotide, followed by the addition of labeled NF-κB-specific probe. Lane 4, no nuclear protein, but the sample contains [γ-32P]ATP-labeled NF-κB-specific probe. Lane 5, protein extract from 30 min ischemia/reperfusion myocardium was incubated with 32P-labeled mutant NF-κB oligonucleotide. Lanes 6–9, protein extract from LV tissue from Sham-operated animals + NF-κB-specific probe. Lanes 10–13, protein extract from ischemic LV tissue from 30 min of ischemia + NF-κB-specific probe. Lanes 14–17, protein extract from LV tissue from postischemic reperfused LV tissue + NF-κB-specific probe. Solid arrow, NF-κB-X-specific DNA-protein complexes. Solid circle, free probe. B, I/R-induced NF-κB activation was composed of p50 and p65. The nuclear extracts used in lanes 14 in A were preincubated with p50 or p65 antibodies for 40 min prior to incubation with NF-κB-specific probe followed by EMSA (n = 3). Normal IgG served as a control (Control IgG). Solid arrow, NF-κB-X-specific binding region. Open arrow, supershifted bands. Solid circle, free probe. C, I/R had no effect on basal Oct1 DNA binding activity. Nuclear protein extracts described in A were analyzed for Oct1 DNA binding by EMSA (n = 3). Solid arrow, Oct1-specific DNA-protein complexes. Solid circle, free probe. D, I/R-induced NF-κB activation was quantified by ELISA. Nuclear extracts described in A were analyzed for NF-κB activation and subunit composition by ELISA, as described under “Experimental Procedures” (n = 4/group, p < at least 0.001 versus corresponding Sham or ischemia (I) alone).

FIGURE 3. Ischemia/reperfusion induces IL-18 expression in serum and postischemic myocardium. A, I/R induced IL-18 mRNA expression in a time-dependent manner. IL-18 mRNA in total RNA extracts was quantified by RT-qPCR. β-actin served as an internal control. *p < 0.05; **p < 0.001 versus sham and ischemia (I) alone (n = 4/group). B, I/R-induced IL-18 mRNA expression at 2 h of reperfusion was confirmed by Northern blot analysis. Each lane contains 20 μg of total RNA from an individual animal. 28S rRNA served as an internal control and shows similar levels of RNA loading in each lane. C, I/R induced IL-18 protein expression in a time-dependent manner. IL-18 protein levels were quantified by immunoblotting. *p < 0.01; **p < 0.001 versus sham and ischemia (I) alone (n = 4/group). Representative immunoblotting at 2 h of reperfusion is shown in D. Each lane contains 30 mg of protein extract from an individual animal. β-Actin served as an internal control and shows similar levels of protein loading in each well. E, IL-18 levels in myocardial protein extracts were quantified by ELISA. *p < 0.05; **p < at least 0.01 versus Sham and ischemia (I) alone (n = 4/group). F, I/R enhanced systemic IL-18 levels in a delayed manner. Serum levels of IL-18 at the indicated time periods were quantified by ELISA. *p < 0.05; **p < 0.01 versus sham and ischemia (I) alone (n = 4/group).
Neutralizing Anti-IL-18 Antibodies Attenuate I/R-induced Tissue Injury—We have demonstrated that I/R induces significant and persistent high levels of IL-18 in the postischemic myocardium (Fig. 3). Since IL-18 exerts both proinflammatory and proapoptotic effects, we investigated whether targeting IL-18 expression would blunt I/R-induced tissue injury. IL-18 neutralizing antibodies or normal rat IgG were administered intravenously to mice 1 h prior to LAD coronary artery ligation (Fig. 5A). At 24 h of reperfusion, the heart was harvested, and infarct size was determined by TTC staining and planimetry. In control mice that received neither IL-18-neutralizing antibod-

phase, with nearly maximal and peak up-regulation observed at 1 and 3 h, respectively (Fig. 3B). We also examined the levels of IL-18 mRNA expression at 2 h of reperfusion by Northern blotting and confirmed elevated IL-18 mRNA expression in the I/R group (Fig. 3B, 2.71-fold increase in ratio of IL-18 to 28 S rRNA, as determined by densitometry). We also determined the levels of IL-18 protein in the corresponding samples by immunoblotting. Correlating with the mRNA results (Fig. 3A), low levels of IL-18 protein were present in the sham-operated and ischemia-alone animals, but maximal levels were seen at 3 h I/R (Fig. 3, C and D; 3.9-fold increase, p < 0.001 versus sham-operated; n = 4/group). Induction of IL-18 following I/R was further investigated by ELISA. Significant elevation in IL-18 protein was observed in the clarified tissue homogenates of I/R mice as early as 30 min, which increased to a maximal level by 2 h (Fig. 2E, p < 0.001 versus sham-operated control). Furthermore, a time-dependent increase in the appearance of circulating IL-18 was measured in the I/R mice, with maximal elevation seen at 6 h of reperfusion. Collectively, these results demonstrate that I/R induces IL-18 mRNA and protein expression within the myocardium and a delayed but increased circulating IL-18 (Fig. 3).

I/R Induces IL-18Rβ, but Not IL-18Rα, and Causes a Delayed Induction in IL-18BP Expression—IL-18 exerts its biological effects through by binding to its cognate receptor (IL-18R), a heterodimer composed of α- and β-subunits (10, 11). We have previously demonstrated that both TNF-α and hydrogen peroxide induce IL-18 mRNA and protein expression in isolated adult rat cardiomyocytes and also stimulate IL-18 release into cell culture supernatants (14). However, only TNF-α led to sustained expression. Expression of IL-18Rβ, but not IL-18Rα, was induced by both agonists (14). TNF-α and hydrogen peroxide also induced the delayed expression of IL-18BP, which binds and sequesters IL-18 in the circulation. Since I/R increased IL-18 expression (Fig. 2), we also investigated its effects on IL-18Rα, IL-18Rβ, and IL-18BP. Substantiating our previous observations in vitro (14), I/R failed to significantly alter the expression of IL-18Rα mRNA at any point in our 6-h study period (Fig. 4A), but IL-18Rβ mRNA was significantly induced by 2 h of reperfusion and reached nearly maximal levels by 3 h (Fig. 4B). Northern blot analysis confirmed these observations (Fig. 4C). Furthermore, IL-18BP mRNA was also induced by I/R but in a delayed manner, with significant induction not observed until 6 h of reperfusion (Fig. 4D). Similar results were corroborated by Northern blot analysis (Fig. 4E). Together, these results demonstrate that I/R induces IL-18Rβ expression early during reperfusion and IL-18BP expression in a delayed fashion (Fig. 4).

FIGURE 4. I/R induces robust expression of IL-18Rβ, but not IL-18Rα, and causes delayed expression of IL-18BP mRNA. A, I/R failed to modulate IL-18Rα mRNA expression. IL-18Rα mRNA expression was quantified by RT-qPCR (n = 4). β-ACTIN served as a control. B, I/R induced IL-18Rβ mRNA expression in a time-dependent manner. IL-18Rβ mRNA expression was quantified by RT-qPCR. β-ACTIN served as a control. *, p < 0.05; **, p < 0.01 versus sham at 3 h and 30 min of ischemia (I) alone (n = 4/group). C, I/R-induced IL-18Rα and IL-18Rβ mRNA expressions at 3 h of reperfusion were confirmed by Northern blot analysis. Each lane contains 20 μg of total RNA from an individual animal. 28 S rRNA served as an internal control and shows similar levels of RNA loading in each lane. D, I/R-induced IL-18BP mRNA expression in a delayed manner. IL-18BP mRNA expression was quantified by RT-qPCR. β-ACTIN served as a control. *, p < 0.01 versus Sham at 6 h and 30 min of ischemia alone (n = 4/group). E, I/R-induced IL-18BP expression was confirmed at 6 h of reperfusion by Northern blot analysis. Each lane contains 20 μg of total RNA from an individual animal. 28 S rRNA served as an internal control and shows similar levels of RNA loading in each lane.

Neutralizing Anti-IL-18 Antibodies Attenuate I/R-induced Tissue Injury—We have demonstrated that I/R induces significant and persistent high levels of IL-18 in the postischemic myocardium (Fig. 3). Since IL-18 exerts both proinflammatory and proapoptotic effects, we investigated whether targeting IL-18 expression would blunt I/R-induced tissue injury. IL-18 neutralizing antibodies or normal rat IgG were administered intravenously to mice 1 h prior to LAD coronary artery ligation (Fig. 5A). At 24 h of reperfusion, the heart was harvested, and infarct size was determined by TTC staining and planimetry. In control mice that received neither IL-18-neutralizing antibod-

FIGURE 5. Administration of IL-18-neutralizing antibodies attenuates I/R-induced tissue injury. A, male C57Bl/6 mice underwent 30 min of LAD coronary artery ligation followed by reperfusion for 24 h. Mice were administered with anti-IL-18-neutralizing antibodies 1 h prior to I/R. Details of the I/R protocol and that of intervention are shown. B, IL-18-neutralizing antibodies attenuated I/R-induced tissue injury. IL-18-neutralizing antibodies (500 μg/mouse, intravenously) were administered 1 h prior to LAD coronary artery ligation. Normal rat IgG served as a control. After 30 min of ischemia/24 h of reperfusion, animals were sacrificed, and infarct size was measured (n = 12/group). A representative TTC-stained tissue is shown in the inset. *, p < 0.01 versus control (untreated) or normal rat IgG-treated.
Interleukin-18 and Reperfusion Injury

ies nor normal rat IgG, the mean I/R-induced infarct size was 47.2%, and pretreatment with normal rat IgG failed to influence I/R-induced tissue injury (Fig. 5B). In contrast, administration of IL-18-neutralizing antibodies attenuated I/R-induced tissue injury, as evidenced by a significant reduction in infarct size (Fig. 5B; 32% reduction versus control and normal rabbit IgG, $p < 0.01, n = 12$). A representative TTC-stained myocardial tissue section is shown in the inset in Fig. 5B. These important in vivo results demonstrate that IL-18 plays a causal role in I/R-induced myocardial injury (Fig. 5).

sI/R Induces Oxidative Stress and Activates NF-κB in an IKK-dependent Manner—Having demonstrated in vivo that I/R induces oxidative stress (Fig. 1B), NF-κB activation (Fig. 2), and IL-18 expression (Fig. 3) in the postischemic myocardium, we next determined whether these responses could be modeled by simulated I/R in vitro. Isolated adult mouse cardiomyocytes underwent 30 min of ischemia followed by reoxygenation for up to 4 h (schematic in Fig. 6A). Under these conditions, no cell death, as measured by an ELISA technique, could be detected. Doxorubicin (Dox) was used as a positive control (Fig. 6B). Thirty minutes of simulated ischemia followed by reoxygenation for 4 h resulted in increased oxidative stress, as evidenced by a significant increase in MDA levels. To confirm further sI/R-induced oxidative stress, cardiomyocytes were loaded with the redox-sensitive fluorophore DCF prior to undergoing sI/R for up to 4 h. Rapid generation of reactive oxygen species, as evidenced by an increase in the fluorescence intensity, was seen as early as 15 min of sI/R (versus simulated ischemia alone) and remained at these levels throughout the 4-h study period (Fig. 6D). Further, sI/R increased the oxidative stress-responsive transcription factor NF-κB in a time-dependent manner. Although low levels of NF-κB DNA binding activity were detected in cardiomyocytes at basal levels (data not shown), ischemia alone failed to modulate its activity (control (C)). However, sI/R significantly increased NF-κB DNA binding activity at 15 min ($p < 0.05$ versus control, $n = 3$/group). This activity was increased at 30 min, peaked at 1 h, and was maintained at these high, but comparable, levels at 2 h (Fig. 6E). The EMSA results were confirmed by ELISA, which showed similar time-dependent increases in NF-κB p65 levels in the nuclear protein extracts (Fig. 6F). Importantly, our results demonstrate that ROS accumulation (DCF fluorescence) precedes NF-κB activation in cardiomyocytes following sI/R (Fig. 6G). In addition to EMSA and ELISA, activation of sI/R-mediated NF-κB activation was investigated by reporter gene activity and immunoblotting. sI/R induced a significant increase in NF-κB-dependent reporter gene activity (Fig. 6H) and enhanced phospho-p65 levels in the nuclear protein extracts (Fig. 6I; a representative of three independent experiments is shown; $n = 3$/group; densitometric analysis, 3.56-fold, $p < 0.005$ versus normoxia). Together, these results demonstrate that sI/R induces oxidative stress in isolated adult cardiomyocytes and activates oxidative stress-responsive NF-κB and that oxidative stress precedes NF-κB activation (Fig. 6).

sI/R Induces NF-κB Activation via IKK-mediated IkB Degradation—We have now demonstrated that sI/R induces oxidative stress and activates the oxidative stress-responsive transcription factor NF-κB in adult cardiomyocytes (Fig. 6). Activation of NF-κB is tightly regulated by the IKK complex composed of catalytic subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ/NEMO (nuclear factor κB essential modulator) (31). Classical stimulatory signals activate NF-κB via IKK-β-mediated site-specific phosphorylation and subsequent degradation of IkB by the 26 S proteasome, which results in NF-κB translocation and the subsequent induction of κB-dependent genes regulating inflammation, immunity, cell death, and cell survival (31). We observed that sI/R significantly increases IKKβ activity, an effect that was blunted by the adenosoreal scavenger of dnIKKB (Fig. 7A; $n = 3$, densitometry, 2.31-fold, $p < 0.01$ versus normoxia). Further, dnIKKB inhibited sI/R-induced IkB-α degradation (Fig. 7B) and NF-κB activation (Fig. 7C). sI/R-induced NF-κB activation was also inhibited by the free radical scavenger PDTC (Fig. 7C), by adenosoreal overexpression of phosphorylation-deficient IkB-α (Fig. 7C), and by the proteasomal inhibitor MG-132 (Fig. 7D). Collectively, these results demonstrate that sI/R induces NF-κB activation in adult cardiomyocytes via IKKβ-mediated IkB-α degradation (Fig. 7).

sI/R Enhances Biologically Active IL-18 Expression via IKK-dependent NF-κB Activation—We and others have previously demonstrated that IL-18 is an NF-κB-responsive cytokine (32, 33). Since sI/R affects NF-κB activation via oxidative stress and IKK-dependent IkB degradation (Figs. 6 and 7), we next questioned whether sI/R induces IL-18 expression in cardiomyocytes via IKK-NF-κB signaling. sI/R potently induced IL-18 mRNA expression in cultured cardiomyocytes as determined by Northern blotting (a representative of three independent experiments is shown in Fig. 8A), and this induction was significantly inhibited by the free radical scavenger PDTC (Fig. 8A). Similarly, adenoviral transduction of dnIKKB or dnIkB-α blunted sI/R-induced IL-18 mRNA expression (Fig. 8B). Similar to sI/R, TNF-α also enhanced IL-18 mRNA expression. Since TNF-α is induced during I/R and is a potent inducer of NF-κB activation, we also investigated whether sI/R-mediated IL-18 expression is TNF-α-dependent. Results in Fig. 8C demonstrate that TNF-α-neutralizing antibodies fail to modulate sI/R-mediated IL-18 expression. Similarly, normal IgG failed to modulate sI/R-mediated IL-18 expression. However, TNF-α-neutralizing antibodies significantly blunted TNF-α-mediated IL-18 expression (Fig. 8D), demonstrating the specificity of these antibodies. Similar to its effects on IL-18 mRNA expression (Fig. 8A), PDTC blocked sI/R-mediated IL-18 protein levels (Fig. 8E). Similarly, adenoviral transduction of dnIKKB and dnIkB-α inhibited sI/R-mediated IL-18 protein expression (Fig. 8F). Further, pretreatment with SC-514, a pharmacological IKKβ inhibitor, and the proteasomal inhibitor MG-132 blunted sI/R-mediated IL-18 protein expression (Fig. 8G). To demonstrate the efficacy and specificity of dnIKKB and dnIkB-α on inhibition of NF-κB activity, we next examined whether adenosoreal transduction of these mutant constructs block TNF-α-mediated NF-κB activation. NF-κBp65 levels in the nuclear protein extracts were quantified by ELISA. Results in Fig. 8H show that indeed adenosoreal transduction of dnIKKB and dnIkB-α significantly inhibit TNF-α-mediated NF-κB activation. In contrast, dnIKKB and dnIkB-α failed to modulate sI/R-mediated JNK phosphorylation (Fig. 8I). Our results also show...
FIGURE 6. si/R activates NF-κB in isolated adult mouse cardiomyocytes. A, adult mouse cardiomyocytes underwent 30 min of ischemia followed by reoxygenation for up to 4 h. Details of the si/R and of intervention are shown. B, si/R did not induce cell death during the 4-h study period. Cardiomyocytes were exposed to normoxia, ischemia for 30 min, or si/R for 4 h. Cell death was analyzed by ELISA as described under “Experimental Procedures.” Doxorubicin hydrochloride (Dox.; 1 μM for 24 h) was used as a positive control. *, p < 0.001 versus control (normoxia) and ischemia alone. C, si/R-induced oxidative stress. Oxidative stress was quantified at 4 h after reoxygenation by quantitating the lipid peroxidation products MDA and 4-HNE, as described under “Experimental Procedures.” *, p < 0.001 versus normoxia; n = 6). D, si/R-mediated oxidative stress was confirmed by DCF fluorescence. Cardiomyocytes loaded with the ROS-sensitive fluorophore dichlorofluorescein diacetate were exposed to ischemia alone or si/R. Representative fluorescent micrographs at the indicated time periods are shown (n = 3). E, si/R-activated NF-κB DNA binding activity. Cardiomyocytes were exposed to si/R for up to 2 h, and nuclear extracts were assessed for NF-κB DNA binding activity by EMSA, as described under “Experimental Procedures” (n = 3/group). A representative of three independent experiments is shown. Arrow, NF-κB-specific DNA-protein complexes. F, si/R-mediated NF-κB p65 translocation into the nucleus. Cardiomyocytes underwent si/R for up to 2 h, and nuclear protein extracts were analyzed for p65 levels by ELISA (n = 3; *, p < 0.05; †, p < 0.01 versus normoxia). G, ROS were generated prior to NF-κB activation. Results from D and E are compared to demonstrate that ROS (oxidative stress) are generated prior to activation of NF-κB in cardiomyocytes following si/R. *, p < 0.01 versus normoxia; †, p < 0.05; ††, p < 0.001 versus normoxia (n = 3). H, si/R-stimulated NF-κB-driven reporter gene activity. Cardiomyocytes were transduced with adenoviral NF-κB-Luc vector and 24 h later were exposed to si/R (n = 12). Cotransfection with a β-galactosidase vector (Ad.β-galactosidase) was used to control for transfection efficiency. Ad.MCS-Luc served as a control. Firefly luciferase and β-galactosidase levels were assayed after 12 h. *, p < 0.01 versus normoxia. I, si/R induces p65 phosphorylation. Cardiomyocytes were exposed to si/R for 2 h, and nuclear extracts were analyzed for phospho-p65 levels by immunoblotting. A representative of three independent experiments is shown. Lamin A/C levels demonstrate the purity and equal loading of the nuclear extracts in each lane.
that treatment with PDTC and adenoviral transduction of dnIKKβ or IkB-α blunt sI/R-induced IL-18 secretion (Fig. 8). Importantly, the secreted IL-18 is biologically active, as evidenced by the significant dose-dependent production of IFN-γ by mouse splenocytes incubated with culture supernatants following sI/R (Fig. 8A). The specificity of IL-18 action was verified by preincubating culture supernatants with IL-18-neutralizing antibodies prior to its addition to splenocytes. Our results show that IL-18-neutralizing antibodies, but not control IgG, blunt IL-18 + LPS-mediated IFN-γ production (Fig. 8K). Together, these results demonstrate that sI/R stimulates the production and secretion of biologically active IL-18 in adult cardiomyocytes via IKK-dependent NF-κB activation (Fig. 8).

**DISCUSSION**

Ischemia/reperfusion is characterized by oxidative stress and by the induction of proinflammatory cytokines, both of which induce IL-18 expression. In isolated adult rat cardiomyocytes, we have previously shown that hydrogen peroxide induces IL-18 expression (14). Concordant with those results, the present study demonstrates that I/R in vivo and sI/R in vitro induce oxidative stress and IL-18 expression and that sI/R-mediated IL-18 expression is significantly attenuated by the free radical scavenger PDTC.

We have also shown that I/R induces activation of NF-κB both in vivo (Fig. 2) and in vitro (Fig. 6). NF-κB is an inducible, oxidative stress-responsive multisubunit transcription factor involved in the induction of diverse κB-responsive genes including cytokines, chemokines, and adhesion molecules (31, 34). Both I/R in vivo and sI/R in vitro induced the activation of NF-κB, comprising predominantly p50 and p65 subunits. Simulated I/R induced IkB-α degradation in an IKKβ-dependent manner and stimulated the phosphorylation of p65, suggesting that I/R induces NF-κB activation via several mechanisms. Activation of NF-κB may lead to the inflammation, inflammatory cell recruitment, and cell death characteristic of ischemic heart disease (31, 34).

Since the activation of NF-κB induces IL-18 expression (32, 33) and since IL-18 potently stimulates NF-κB activation (19), targeting NF-κB activation may be a viable strategy to attenuate I/R-mediated tissue injury. Importantly, we have demonstrated previously that inhibition of NF-κB by diethyldithiocarbamate can significantly attenuate proinflammatory cytokine expression in the postschismic myocardium (35). Similarly, Liu et al. (36) reported that inhibition of NF-κB with PDTC attenuates cytokine, chemokine, and adhesion molecule expression in the hearts of mice treated with LPS. More recently, Brown et al. (37) have demonstrated a significant reduction in I/R-induced tissue injury in transgenic mice overexpressing the phosphorylation-deficient mutant of IkBα (S32A, S36A, Y42F) in a cardiac specific manner. Targeting NF-κB activation may not only inhibit IL-18 action and its further induction but also the expression of other cytokines, chemokines, and adhesion molecules, thereby limiting neutrophil infiltration. In contrast to these reports, using IκBAN transgenic mice to block NF-κB activation, Misra et al. (38) have demonstrated, however, that permanent occlusion of the LAD coronary artery (without reperfusion) results in increased tissue injury. Similarly, Xuan et al. (39) demonstrated that activation of NF-κB plays a protective role in preconditioning, suggesting that activation of NF-κB may also play a protective role. These important but conflicting studies indicate that activation of NF-κB plays either a protective or deleterious role, depending on the type of insult (ischemia alone versus ischemia/reperfusion). In the present study, we report that targeting NF-κB-responsive IL-18 expression significantly reduces I/R-mediated tissue injury. Whether neutralization of IL-18 exerts such protective effects in vivo following permanent ligation is not known but is currently under active investigation.

I/R is also characterized by neutrophil infiltration, and IL-18 is a potent chemoattractant and inducer of ELR + CXC chemokines. In addition, neutrophils are able to proteolytically activate both recombinant and native pro-IL-18, similar to that mediated by ICE (interleukin-1-converting) or caspase-1 (40), suggesting that neutrophils may amplify IL-18 processing and signaling at the site of injury/inflammation. Further, IL-18 primes neutrophils, and IL-18-primed neutrophils are more responsive to subsequent stimuli (30). In anti-neutrophil cytoplasm autoantibody-associated systemic vasculitis, autoantibody-induced neutrophil activation was shown to cause organ damage (41). Priming of neutrophils by IL-18 enhanced production of superoxide and other free radicals in response to anti-neutrophil cytoplasm autoantibody (41). It is therefore possible that in addition to attracting neutrophils to the site of injury, I/R-induced IL-18 may sensitize neutrophils to other induced cytokines, such as TNF-α. Further, IL-18 has also been shown to stimulate CD11b expression, NADPH oxidase-catalyzed oxyradical generation (superoxide anion, hydrogen per-
Simulated ischemia/reperfusion induces biologically active IL-18 via IKK-NF-κB signaling. A, PDTC inhibited sI/R-mediated IL-18 mRNA expression. Cardiomyocytes pretreated with PDTC (100 μM in PBS for 1 h) were exposed to sI/R for 4 h, and IL-18 mRNA expression was analyzed by Northern blotting. 28S rRNA served as a control. A representative of three independent experiments is shown. B, sI/R induced IL-18 mRNA expression via IKK and IκB-α degradation. Cardiomyocytes transfected with adenoviral dnIKKβ, dnIKK-α, or GFP for 24 h were exposed to sI/R for 4 h. IL-18 mRNA expression was analyzed as in A (n = 3). TNF-α (10 ng/ml) served as a positive control. C, sI/R-mediated IL-18 expression was independent of TNF-α. Cardiomyocytes were incubated with TNF-α-neutralizing antibodies or control IgG (10 μg/ml for 1 h) prior to sI/R. IL-18 protein levels were analyzed by immunoblotting (n = 3). Actin served as a loading control. D, TNF-α-neutralizing antibodies attenuated TNF-α-induced IL-18 expression. To demonstrate the efficacy of TNF-α-neutralizing antibodies, cardiomyocytes were incubated with αTNF-α antibodies or control IgG (10 μg/ml for 1 h) prior to TNF-α addition (10 ng/ml for 4 h). IL-18 protein levels were quantified by immunoblotting (n = 3). Actin served as a loading control. E, PDTC inhibited sI/R-mediated IL-18 protein expression. Cardiomyocytes treated as in A were analyzed for IL-18 protein expression by immunoblotting (n = 3). Actin served as a control. F, sI/R induced IL-18 protein expression via IκBβ and IκB-α degradation. Cardiomyocytes treated as in B were analyzed for IL-18 protein levels by immunoblotting. TNF-α served as a positive control. G, the proteasome inhibitor MG-132 and the IKK inhibitor SC-514 inhibited sI/R-mediated IL-18 protein expression. Cardiomyocytes pretreated with MG-132 (5 μM in DMSO for 1 h) or SC-514 (10 μM in DMSO for 1 h) were exposed to sI/R for 4 h. IL-18 protein expression was analyzed by immunoblotting (n = 3). Actin served as a control. H, dnIKK and dnIKK-α blunted TNF-α-induced NF-κB activation. Cardiomyocytes transfected as in B were treated with TNF-α (10 ng/ml for 1 h) and were analyzed for nuclear NF-κB p65 levels by ELISA (n = 3). *, p < 0.001 versus untreated; †, p < 0.001 versus TNF-α, ‡, dnIKK and dnIKK-α failed to modulate sI/R-mediated JNK phosphorylation. Cardiomyocytes transfected as in B were exposed to sI/R for 2 h. Total and phospho-JNK levels in cleared cell lysates were analyzed by immunoblotting (n = 3). I, sI/R-stimulated IL-18 secretion via IKK and IκB-α degradation. Cardiomyocytes were treated as in A and B, and IL-18 levels in culture supernatants were analyzed for IL-18 levels by ELISA (n = 6). TNF-α served as a positive control (10 ng/ml for 4 h). J, IL-18 levels in culture supernatants were analyzed for IL-18 levels by ELISA (n = 6). TNF-α served as a positive control (10 ng/ml for 4 h). K, IL-18 levels in culture supernatants were analyzed for IL-18 levels by ELISA (n = 6). TNF-α served as a positive control (10 ng/ml for 4 h).

The IL-18BP promoter has been cloned and characterized, identifying one IRF-1 (IFN regulatory factor-1) and two C/EBPβ binding sites that are important for both basal and inducible activity (44). Following IFN-γ exposure, it was reported that IRF-1 forms a complex with C/EBPβ and that this complex is critical for the induction of IL-18BP gene expression (44); however, it is not known whether a similar mechanism modulates IL-18BP expres-

oxide, hydroxyl radicals, etc.), p38 mitogen-activated protein kinase activation, and cytokine and inflammatory molecule production in neutrophils (30). In addition, activated neutrophils secrete specific enzymes, including proteases, collagenases, lipooxygenases, phospholipases, and myeloperoxidase, that degrade components of the extracellular matrix (42, 43). The neutrophil thus plays several critical roles in mediating the postischemic events, which culminate in myocardial damage, and the induction of IL-18 may therefore be essential for this process.

I/R induced a rapid and persistent expression of IL-18Rβ, the signal-transducing subunit of the functional IL-18 receptor, but failed however to modulate the basal expression of IL-18Rα, the ligand-binding receptor subunit. Since the expression of IL-18 and IL-18Rβ is increased in postischemic myocardium, it is possible that I/R amplifies IL-18 signaling in vivo. In contrast to the rapid and persistent induction of IL-18 and IL-18Rβ, I/R induced IL-18BP expression in vivo in a delayed fashion; significant I/R-induced IL-18BP expression was only observed at 6 h of reperfusion. Similarly, we reported previously that both hydrogen peroxide and TNF-α also induce IL-18BP expression in cardiomyocytes in a similarly delayed manner (14) but did not determine the signaling mechanisms involved.

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Interleukin-18 and Reperfusion Injury

Reperfusion following a lethal period of ischemia is characterized by an early stage of cardiomyocyte death, which is followed by hypertrophy of the surviving cardiomyocytes in the nonischemic myocardium. Interestingly, our results illustrate that neutralization of IL-18 significantly attenuates I/R-induced tissue injury in vivo, suggesting that IL-18 plays an active role in cardiomyocyte death. In fact, we and others have demonstrated that IL-18 can paradoxically exert both proapoptotic and prohypertrophic effects in various cell types. Although IL-18 induces cell death in cardiac endothelial cells (27, 46), IL-18 potently induces prohypertrophic effects in primary neonatal rat ventricular myocytes and in the HL-1 atrial cardiomyocyte cell line (47). However, when used in combination with either TNF-α or hydrogen peroxide, IL-18 induces significant cardiomyocyte death (data not shown). Moreover, IL-18 in combination with TNF-α significantly inhibits the expression of prosurvival Bcl-2 in cardiomyocytes. Thus, when used alone, IL-18 displays prohypertrophic properties (47), whereas when combined with other proinflammatory cytokines, it becomes proapoptotic. Consequently, IL-18 may exert both proapoptotic and prohypertrophic effects in vivo within the postischemic myocardium, and studies to characterize IL-18-mediated cardiomyocyte death are in progress.

In summary, the present studies demonstrate that I/R induces IL-18 expression in the postischemic myocardium in vivo and that targeting IL-18 expression significantly attenuates I/R-induced tissue injury. Further, our studies in vitro demonstrate that simulated I/R enhances biologically active IL-18 expression in isolated adult mouse cardiomyocytes, which is dependent on IKK-NF-κB signaling. Collectively, these results indicate that IL-18 plays a critical role in I/R-induced tissue injury and thus represents a promising therapeutic target for ischemic heart disease.

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