CXC CHEMOKINE LIX (CXCL5) AMPLIFIES A PROINFLAMMATORY CYTOKINE RESPONSE VIA A PHOSPHATIDYLINOSITOL 3-KINASE-NF-κB PATHWAY

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It is well established that cytokines can induce the production of chemokines, but the role of chemokines in the regulation of cytokine expression has not been fully investigated. Exposure of rat cardiac-derived endothelial cells (CDEC) to lipopolysaccharide-induced CXC chemokine (LIX), and to a lesser extent to KC and MIP-2, activated NF-κB and induced κB-driven promoter activity. LIX did not activate Oct-1. LIX-induced interleukin-1β and tumor necrosis factor-α promoter activity, and up-regulated mRNA expression. Increased transcription and mRNA stability both contributed to cytokine expression. LIX-mediated cytokine transcription was inhibited by interleukin-10. Transient overexpression of kinase-deficient NF-κB-inducing kinase (NIK) and IκB kinase (IKK), and dominant negative IκB significantly inhibited LIX-mediated NF-κB activation in rat CDEC. Inhibition of G protein-coupled signal transduction, poly(ADP-ribose) polymerase, phosphatidylinositol 3-kinase, and the 26 S proteasome significantly inhibited NF-κB activating activity in rat CDEC. Various stimuli including cytokines, growth factors, and oxidative stress induce IκB-κB family, including IκB-α, IκB-β, IκB-γ, IκB-ε, all prevent activation and subsequent nuclear translocation of the heterodimer. Various stimuli include cytokines, growth factors, and oxidative stress induce IκB hyperphosphorylation leading to its selective degradation in the cytoplasm by the ubiquitin-26 S proteasome system, resulting in NF-κB activation.

Chemokines are small molecular weight cytokines involved in activation of specific subsets of immune cells and their recruitment to the site of injury and inflammation (1–7). They are classified into C, CC, CXC, and CX3C families (2–4). In the CXC family, the first two conserved cysteines are separated by one nonconserved amino acid (X in CXC). CXC chemokines that have a glutamic acid-leucine-arginine (ELR) sequence immediately preceding the CXC motif are potent neutrophil chemoattractants (ELR+ CXC chemokines) (2, 8, 9). Neutrophil migration is stimulated by a gradient of these chemokines from blood toward the site of inflammation.

We have recently shown up-regulation of the ELR+ CXC chemokines, LIX (lipopolysaccharide-induced CXC chemokine; CXCL5), KC (cytokine-induced neutrophil chemoattractant; CXCL1), and MIP-2 (macrophage inflammatory protein-2; CXCL2) in a rat model of myocardial ischemia/reperfusion injury (10). High levels of myocardial neutrophil infiltration coincided with peak levels of LIX and MIP-2 expression. Neutralization of LIX, KC, and MIP-2 inhibited myeloperoxidase activity, a measure of neutrophil infiltration, by 79, 28, and 37%, respectively, indicating that LIX may be the predominant neutrophil chemoattractant in this model of reperfusion injury (10). Furthermore, the proinflammatory cytokine expression preceded the chemokine expression in this model, suggesting that chemokine expression was a downstream effect of cytokine production (10). This was confirmed in in vitro studies where exposure of cardiomyocytes to TNF-α induced LIX expression via NF-κB activation (10).

NF-κB is a ubiquitous, multisubunit, inducible transcription factor that regulates the expression of various genes involved in the immune and inflammatory processes (11, 12). The p50/p65 heterodimer, which has been most studied, resides in the cytoplasm in an inactive state because of binding of p65 to an inhibitory subunit IκB. The IκB family, including IκB-α, IκB-β, IκB-γ, IκB-ε, all prevent activation and subsequent nuclear translocation of the heterodimer. Various stimuli include cytokines, growth factors, and oxidative stress induce IκB hyperphosphorylation leading to its selective degradation in the cytoplasm by the ubiquitin-26 S proteasome system, resulting in NF-κB activation (11, 12).
A multiprotein complex comprised of IKK (IκB kinase)-α, IKK-β, and a regulatory subunit IKK-γ/NEMO was shown to mediate phosphorylation of IκB by various cytokines (13–17). The cytokine-initiated signal transduction cascade leading to IκB phosphorylation has been shown to converge at activation of the IKK by NF-κB-inducing kinase (NIK). NIK associates with IKK-γ and activates the IKK signalosome. PI 3-kinase, PI-phospholipase C, protein kinase C, and p38 mitogen-activated protein kinase were implicated as upstream regulators of NIK and IKK. Furthermore, poly(ADP-ribose) polymerase 1 (PARP-1), a nuclear protein involved in DNA repair, has been shown to physically and functionally associate with NF-κB (p65-κBα) (20). The NF-κB-driven luciferase reporter plasmid (pNF-κB-Luc) was obtained from Stratagene (La Jolla, CA) and contains five copies of the NF-κB consensus sequence 5′-GGGACTTCC-3′ cloned upstream of a minimal regulatory region of the luciferase reporter gene. pEgFP-Luc was used as a control. The phosphorylation-deficient S32A/S36A mutant of IκB-α (pCMX-IκBα-S32A/S36A) was a gift from Inder Verma (The Salk Institute, La Jolla, CA), and the Myc-tagged phosphorylation-deficient S19A/S32A mutant of IκB-β in pCMV-Tag3B (Stratagene) has been described earlier (22). Kinase-deficient NIK (pRKT7-NIK/IKK-α/β-32A (pcDNA3-IKK-β-Flag), and dominant negative IKK-γ (pDNA3-IKK-γ-HA) were obtained from David V. Goeddel (Tularik Inc., South San Francisco, CA), Tom Maniatis (Harvard University, Cambridge, MA), and Gabriel Nunez (University of Michigan Medical School, Ann Arbor, MI), respectively. Rat CDEC were plated on six-well tissue culture dishes and transfected the following day at ~70–80% confluence using LipofectAMINE 2000™ (Invitrogen, Carlsbad, CA) as described by the manufacturer. pRL Renilla-luciferase reporter gene (100 ng; pRL-TK vector; Promega, Madison, WI) was used as an internal control. The empty vectors pCMX, pCMV-Tag3B, pRKT7, and pDNA3 were used as controls. Data were normalized for transfection efficiency by dividing firefly luciferase activity with that of the corresponding Renilla luciferase, and expressed as mean relative stimulation ± S.E. for a representative experiment from three separate experiments, each performed in triplicate. The amount of DNA transfected was kept constant (2 μg) in all transfection experiments. After transfection, the cells were found to be viable (trypan blue dye exclusion). 24 h after transfection, the media were changed, and the cells were exposed to LIX, KC, or MIP-2 at the indicated concentrations and for the specified time periods. Cell extracts were prepared, and luciferase activity was determined with a TD 20/20 luminometer (Turner Designs, Sunnyvale, CA) using the Promega Biotech™ dual-luciferase reporter assay system.

Transfection efficiency was determined by transfecting rat CDEC with pEgFP-N1 vector (Clontech, Palo Alto, CA) that constitutively expresses the enhanced green fluorescent protein (EGFP) under the regulation of CMV promoter and enhancer. Once the cells reached ~70% confluency, the media was replaced with M199 + 0.5% BSA. After overnight culture, cells were transfected with pEgFP-N1 and LipofectAMINE 2000. 24 h later, the cells were trypsinized, seeded onto Lab-Tek II chamber slide (Nunc™), and cultured for an additional 48 h. Cells were then washed in PBS (pH 7.4), fixed in 4% paraformaldehyde in PBS for 30 min at room temperature. After washing in PBS, coverslips were mounted using ProLong™ Antifade kit ( Molecular Probes, Eugene, OR). After the mounting media was dried, the coverslips were sealed with black nail polish, and stored at 4 °C in the dark. A nicked DNA visualization assay was performed by fluorescent microscopy (Nikon Inc., Melville, MA), and 1,000 cells were counted under ×20 objective, and bright to very-bright green fluorescent cells were considered positive for the expression of EGFP, and the others as nontransfected (controls). The transfection efficiency varied between 37 and 46% with an average of 38.4 ± 5.9%. To determine the role of CXC receptors in NF-κB activation, the cells were exposed to LIX, KC, or MIP-2 at the indicated concentrations and for the specified time periods.

Electrophoretic Mobility Shift Assay

NF-κB DNA binding activity was measured in the nuclear protein extracts by electrophoretic mobility shift assay (EMSA) as described in Materials and Reagents.

EXPERIMENTAL PROCEDURES

Cell Culture

Nontransformed rat cardiac-derived endothelial cells (rat CDEC; a generous gift of G. C. Digilio, Ref. 20) and nontransformed mouse cardiac-derived endothelial cells, described previously (21), were cultured in medium 199 with 10% fetal calf serum, endothelium growth supplement (30 mg/liter), heparin (100 mg/liter), penicillin (100,000 units/liter), and streptomycin (100 μg/ml) at 37 °C in a humidified atmosphere of 95% air, 5% CO2. At ~70–80% confluency, the media was replaced with serum-free medium 199 containing 0.5% BSA. After overnight culture, LIX, KC, MIP-2, or PBS was added and incubated for the indicated time periods. To inhibit NF-κB DNA binding activity, the cells were pretreated for 1 h with 3-aminobenzamide (10 μM in ethanol), wartmanin (50 nM), LY 294002 (20 μM), chelerythrine chloride (60 μM), and MG-132 (5 μM) in MeSO4, pertussis toxin (100 ng/ml) in PBS or for 4 h with IL-10 (10 ng/ml) or corresponding vehicle before the addition of LIX.
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earlier (10, 26). In the gel supershift assay, the protein extract (10 μg) was preincubated for 40 min on ice with either anti-p50 or p65 subunit-specific polyclonal antibodies (1 μg) or control IgG (1 μg) prior to the addition of 32P-labeled double stranded NF-κB consensus oligonucleotide (5′-AGTTGAGGGGACCTTCCGACG-3′). Absence of protein extract, competition with 100-fold molar excess unlabeled consensus NF-κB, and mutant NF-κB oligonucleotide (5′-AGTTGAGGGGACCTTCCGACG-3′) served as controls. Incubation of Oct-1, a constitutively expressed transcription factor, were also measured by EMSA using Oct-1 consensus sequence (5′-TGCTGGATGCAAATCTAGAA-3′; Santa Cruz Biotechnology, Inc.).

Northern Blot Analysis

Total cellular RNA was isolated using the TRIzol reagent (Invitrogen). 20 μg of total RNA were resolved on a 0.8% agarose-formaldehyde gels and transferred onto nylon membranes. After prehybridization for 4 h, hybridizations were carried out at 42 °C for 16 h, followed by high stringency washing at 68 °C in 0.1× SSC, 0.1% SDS. The CDNAs were amplified using total RNA isolated from rat CDEC and gene-specific primers (rat IL-1β, GenBank™ accession number NM_031512, 324 bp product, sense, 5′-CTCTGCTGACTGCGGATG-3′ and antisense, 5′-CTCTCTCTATTTGTTGTTG-3′ (bases 684-707); TNF-α, GenBank™ accession number AF329985, 295 bp product, sense, 5′-TACTAAGTCTGCTGGTAGTTGCTGC-3′ (bases 955-979) and antisense, 5′-CAGCGGCTGGCTTCAAGAAGAACC-3′ (bases 2161-2138). The PCR products were cloned into pCR™2.1-TOPO™ vector (Invitrogen) and sequenced on both strands for confirmation. The probe for rat IL-1 (GenBank™ accession number U90448) was a 329-bp cDNA cloned into pCR™2.1-TOPO™ vector (Invitrogen). cDNAs were labeled with [γ-32P]ATP using T4 polynucleotide kinase (26).

NF-κB-labeled double stranded NF-κB consensus oligonucleotide (5′-AGTTGAGGGGACCTTCCGACG-3′) served as controls. Incubation of Oct-1, a constitutively expressed transcription factor, were also measured by EMSA using Oct-1 consensus sequence (5′-TGCTGGATGCAAATCTAGAA-3′; Santa Cruz Biotechnology, Inc.).

LIX-mediated IL-1β and TNF-α Transcription (Nuclear Run-on)

After treating rat CDEC with LIX (100 ng/ml) for 4 h in M199 medium containing 0.5% BSA, nuclei were isolated, counted, and resuspended in a homocytometer, and resuspended (2 × 10⁶/ml) in a storage buffer (50 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 1.0 mM EDTA, 2 mM dithiothreitol, 40% glycerol) as described in detail previously (29). The nuclei were allowed to snap freeze in liquid nitrogen ice bath and stored in liquid nitrogen until use. For labeling RNA, nuclei were thawed (100 μl), mixed with equal volumes of labeling mixture (200 mM KCl, 8 mM MgCl₂, 1.0 mM each of ATP, UTP, and CTP, 100 μM GTP) and 10 μl of [α-32P]UTP (800 Ci/mmol). The mixture was incubated at 30 °C for 30 min and 20 μl (4 μg) of RQ1 RNase-free DNase I (Fermentas Corp.) was added and incubated for an additional 10 min. After digestion with proteinase K (60 μg in 20 μl) in a buffer containing 1% SDS, 50 mM Tris-HCl (pH 7.0), 50 mM EDTA for 30 min at 42 °C, it was subjected to phenol/chloroform/isoamyl alcohol and chloroform extractions. The aqueous phase was ethanol-precipitated in the presence of 20 μg of carrier RNA (Escherichia coli transfer RNA, RNase-free, Roche Molecular Biochemicals). The pellet was dissolved in 20 μl of TE buffer (pH 8.0) and subjected to autoradiography using a slot-blot apparatus (HYBRIDIZATION MANIFOLD, Invitrogen). After fixing the DNA to the membranes by UV cross-linking, prehybridization was performed at 52 °C overnight, followed by hybridization for 3 days with ~10⁶ cpm/ml of labeled RNA. The filters were then washed three times for 10 min each in 2× SSC plus 0.1% SDS, two times in 0.1× SSC plus 0.1% SDS at 65 °C for 15 min each, and then treated with RNase A (10 ng/ml) for 30 min at 37 °C in 2× SSC. Finally the membranes were washed with 2× SSC at 37 °C for 30 min, and subjected to autoradiography, and the visualized bands were semiquantitated by densitometry.

Marine IL-1β Promoter—The murine IL-1β promoter (−4093 to +45) construct in pBluescript vector was a kind gift from Clifford J. Bellione (St. Louis University School of Medicine, St. Louis, MO; Ref. 27). This construct contains 4,093 bp of the 5′-flanking sequence that includes the first exon, first intron, and untranslated region of the second exon. This promoter construct has been demonstrated to confer a strong responsiveness to lipopolysaccharide (27). Rat CDEC were transfected with 3 μg of either the IL-1β−4093 to +45-CHL (chloramphenicol acetyl transferase) vector or a mock plasmid that contains CAT reporter gene alone (pFR-CAT; Stratagene). To compensate for variations in transfection, cells were cotransfected with a β-galactosidase reporter construct (pSV-β-galactosidase control vector, Promega) in which the SV40 early promoter and enhancer drives transcription of the lacZ gene, which encodes the β-galactosidase enzyme. 24 h later, the media was changed, and the cells were treated with LIX (100 ng/ml), neutralized LIX, or vehicle. Seven hours later the cells were processed for CAT levels using a CAT enzyme-linked immunosorbent assay kit (Roche Molecular Biochemicals) and β-galactosidase levels by using the β-galactosidase assay kit (Invitrogen) essentially as described by the manufacturers.

Marine TNF-α Promoter—Rat CDEC were transfected with pTNF−1080/+138, pTNF−85/+135, or empty vector (pGL3 basic). These murine TNF-α promoter reporter constructs were described earlier (28). The 1.1-kb TNF-α promoter construct (−1080/+138 nucleotides; relative to the transcription start site) contains 4 NF-κB response elements, and has been demonstrated to confer responsiveness to a variety of stimuli including lipopolysaccharide. Its deletion mutant construct (−85/+135) lacks all four NF-κB response elements but contains only TATA box and Sp1 site, and responds poorly to lipopolysaccharide (28). The cells were co-transfected with pRK-Renilla to compensate for transfection efficiency. 24 h after transfection, the media was changed, and the cells were treated with LIX, neutralized LIX, or vehicle. Seven hours later the cells were processed for luciferase activity by the dual luciferase assay kit.

mRNA Stability-Actinomycin D Pulse—Rat CDEC were cultured in M199 medium containing 10% fetal calf serum. At 70–80% confluency, the complete medium was replaced with M199 + 0.5% BSA, and cultured for an additional 18 h. The cells were then treated with either LIX (100 ng/ml) or vehicle (control) for 4 h. Actinomycin D (0.5 μg/ml; Sigma), a potent inhibitor of RNA polymerase II-dependent transcription, was then added. At the indicated time periods 1.5, 3, 4.5, and 6 h), cells were harvested for total RNA isolation. RNA was isolated using TRIzol reagent, and analyzed by Northern blot hybridization to quantitate IL-1β and TNF-α mRNA levels as described above.

Enzyme-linked Immunosorbent Assay

NF-κB (sensitivity 0.7 pg/ml) and IL-1β (sensitivity <3.0 pg/ml) levels in culture supernatants were measured by enzyme-linked immunosorbent assay using commercially available kits (BIOSOURCE International; Ref. 30). Studies were performed as per the manufacturer’s instructions.

Measurement of PI3K Activity

P13K lipid kinase assays were performed essentially as described by Foukas et al. (31). After overnight incubation in 0.5% BSA, M199 media, rat CDEC were treated with RLIIX (10 ng/ml) for 5 min with and
without LY 294002. Cleared cell lysates were prepared by centrifugation at 10,000 × g for 30 min at 4 °C, and protein concentration was determined.

Equal amounts of protein were immunoprecipitated with affinity purified antibodies against the p85 regulatory subunit of PI3K (Santa Cruz Biotechnology, Inc., number sc-423) for 2 h followed by washing the immunoprecipitates (IP) in Tris-HCl (100 mM, pH 7.4) containing 0.5 M LiCl and kinase assay buffer (2 mM MgCl₂, 100 mM NaCl, 2 mM dithiothreitol), the immunoprecipitates were resuspended in 50 μL of 1× kinase assay buffer containing 5 mM MgCl₂, 100 μM ATP (plus 0.1 μCi of [γ-³²P]ATP/assay), and 200 μg/ml phosphatidylinositol as a substrate. The reaction was incubated at 25 °C for 20 min. The reaction was stopped by the addition of 100 μL of 0.1 M HCl and 200 μL of chloroform/methanol (1:1). The lower organic phase containing phospholipids was recovered and spotted on silica gel thin-layer chromatography plates (Gel-60, Merck), impregnated with 1% (v/v) potassium oxalate, 1 mM EDTA in water/methanol (6:4), and developed in a mixture of chloroform, methanol, 4 mM NH₄ (9:2:4). The radioactivity on the dried plate was visualized and quantified by autoradiography and densitometry.

**Measurement of Intracellular Calcium**

Intracellular calcium measurements were made in rat CDEC using the calcium-sensitive probe Fura-2/AM (Molecular Probes). The cells were loaded with Fura-2/AM pentapotassium salt (5 μM) in M199 medium supplemented with 10% fetal calf serum. After incubation for 45 min at 37 °C, the cells were washed and resuspended at 2 × 10⁶ cells/ml in 137 mM NaCl, 4.5 mM KCl, 1.2 mM MgCl₂, 7H₂O, 4.9 mM KCl, 1.2 mM NaH₂PO₄, 20 mM HEPES, 15 mM d-glucose, 1.8 mM CaCl₂ (pH 7.4). The cell suspension was placed in a fluorimetry cuvette and stirred continuously at 37 °C. After equilibrating at 37 °C for 10 min, rLIX (100 ng/ml), neutralized LIX, or PBS were added, and fluorescence was monitored at excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm on a Hitachi F-2000 fluorescence spectrophotometer; the results were calculated as the ratio of emission following excitation at 340 nm with that produced by excitation at 380 nm.

**Results**

The Proinflammatory Cytokines IL-1β and TNF-α Induce LIX Expression—Both proinflammatory cytokines and chemokines are induced during inflammation and endotoxemia. We have previously demonstrated induction of the neutrophil chemotaxtrants LIX, KC, and MIP-2, members of the ELR⁺/CXC chemokines, during ischemia/reperfusion injury (26). In isolated adult rat cardiomyocytes, TNF-α-induced LIX expression in a NF-κB-dependent manner (26). In the present study, we investigated whether IL-1β and TNF-α induce LIX expression in rat cardiac-derived endothelial cells. EMSA showed rapid and sustained induction of NF-κB DNA-binding activity by
either cytokine in rat CDEC (Fig. 1, A and B). The induction was observed at 5 min after addition of IL-1/α (100 ng/ml) or TNF-α (100 ng/ml) and persisted up to 48 h. However, no synergy was observed when the cells were treated with IL-1/α and TNF-α together (Fig. 1C). Furthermore, IL-1/α and TNF-α induced LIX mRNA expression in a sustained manner (Fig. 1, D and E). These results indicate that cytokines induce chemokine expression, probably through activation of NF-κB.

**ELR** CXC Chemokines Activate NF-κB—To determine whether cytokine-chemokine cross-talk amplifies the proinflammatory cytokine cascade, we investigated whether ELR** CXC chemokines induce cytokine expression, and the role of NF-κB in chemokine-mediated cytokine expression. EMSA showed that unstimulated rat CDEC contained low levels of NF-κB in the nuclear protein extracts. Treatment with all three ELR** CXC chemokines increased NF-κB activity in a dose-dependent manner (Fig. 2A). LIX-induced NF-κB activity increased to near peak levels at 100 ng/ml, with a slight further increase when the concentration was increased to 1000 ng/ml. Even at 1000 ng/ml, KC- and MIP-2-induced NF-κB activity in rat CDEC did not reach levels comparable with that induced by LIX. To confirm the EMSA results we performed transient transfections with a pNF-κB luciferase reporter vector. LIX, KC, and MIP-2 induced NF-κB-driven luciferase activity (Fig. 2B), and LIX was the most potent inducer of NF-κB activation. Neutralization of LIX with anti-LIX antibodies completely blocked LIX-induced NF-κB-activation (Fig. 2B). LIX-induced NF-κB degradation was associated with transient increase in the phosphorylated form of IκB-α (P-IκB-α; E). However, β-actin levels showed no variations between samples. Furthermore, LIX-induced NF-κB contained both p50 and p65 complexes as assessed by gel supershift assay (F).
FIG. 3. LIX treatment up-regulated the NF-κB-responsive proinflammatory cytokines IL-1β and TNF-α, increased transcription rate and mRNA half-life, and induced cytokine promoter activity in rat CDEC. After rat CDEC reached 70–80% confluency, the complete media was replaced with M199 containing 0.5% BSA. After overnight culture, LIX (100 ng/ml) was added and the incubation continued up to 48 h. At the end of experimental period, media was separated and the cells were rinsed with ice-cold PBS. Cells were then processed for mRNA expression by Northern blotting. Culture supernatants were assayed for cytokine protein levels by enzyme-linked immunosorbent assay. Our results indicate that LIX up-regulated both IL-1β and TNF-α mRNA expression in a time-dependent manner with significant increases detected around 4 h post-treatment (A). Similarly, treatment with LIX, but not LIX after neutralization, significantly increased cytokine protein levels in culture supernatants at 4 h (B; *, p < 0.001 (versus control); †, p < 0.001 (versus LIX)). Because increased transcription and/or mRNA stability contribute to mRNA expression, we then studied the effects of LIX on cytokine transcription by nuclear run-on assay and mRNA stability by actinomycin D pulse. Our results indicate that LIX treatment significantly increased transcripts for IL-1β (C) and TNF-α (D) in nuclei isolated from LIX-treated rat CDEC. Actinomycin D pulse following LIX treatment showed increased stability of IL-1β mRNA as compared with control (E). However, mRNA half-life for TNF-α and controls was similar (F). To demonstrate the effects of LIX on cytokine promoter activity, rat CDEC were transiently transfected with 3 μg of IL-1β promoter construct (IL-1β-4093 to +45-CAT) or a mock plasmid (pFR-CAT). Cells were cotransfected with pSV-β-galactosidase vector to compensate for variability in transfection efficiency. CAT and β-galactosidase levels were measured, and CAT expression was normalized to that of β-galactosidase, and represented as normalized CAT expression. The results indicate that LIX significantly induced IL-1β promoter activity (G), and neutralizing LIX with anti-LIX antibodies prevented its stimulatory effects on the promoter activity. To demonstrate LIX effects on TNF-α promoter activity, rat CDEC were transfected with a 1.1-kb TNF-α promoter that contained 4 NF-κB response elements (TNF-α-1080/-138), its deletion mutant that lacks all NF-κB response elements (TNF-α-855/-138), or the empty vector (pGL3 basic).
LIX treatment had no effect on the expression of Oct1, a constitutively expressed transcription factor (Fig. 2D).

Activation of NF-κB results from phosphorylation and dissociation of IκB-α (61), and also by its nondegradability after LIX treatment (A). Expression of IκB-α mutant (pCMX-IκB-α(S32A/S36A)) was confirmed in an immunoblotting by its slower mobility as compared with the wild type IκB-α (61), and also by its nondegradability after LIX treatment (A). Expression of dnIκB-β (pCMV-Tag3B-IκB-β(S19A/S23A)), kinase-deficient IKK-β (pRK5-IKK-β-Flag), kdNIK (pRK7-NIK(KK429-430AA)-Flag), and dnIKK-γ (pcDNA3-IKK-γ-HA) was confirmed by immunoblotting using anti-Myc, -FLAG, and -hemagglutinin antibodies. B, β-actin levels demonstrated similar amounts of protein loading. Cells transfected with empty vectors (pCMX, pCMV-Tag 3B, pRK5, pRK-7, and pcDNA3) were used as controls. 24 h after transfection, the media was changed, and the cells were treated with LIX (100 ng/ml) for either 1 (C) or 4 h (D and E). The results indicate that pretreatment with the anti-inflammatory cytokine IL-10 (10 ng/ml for 4 h) and overexpression of dnIκB-α or dnIκB-β inhibited LIX-induced NF-κB activation (C), and cytokine mRNA (D) and protein levels (E). Similar results were obtained by the overexpression of kdNIK, kdIKK-β, and dnIKK-γ (*, p < 0.001 versus empty vector transfected cells).

Cells were cotransfected with pRK-Renilla to compensate for variability in transfection efficiency. The results are represented as a ratio of firefly luciferase to Renilla luciferase. The results indicate that LIX significantly increased TNF-α promoter activity (H), and lack of κB-response elements abrogated LIX-mediated luciferase activity. *, p < 0.01 (versus LIX-treated empty vector transfected cells).

Fig. 4. LIX-induced NF-κB activation involves NIK, IKK, and IκB. Rat CDEC were transiently transfected with either empty vectors or dominant negative IκB-α, IκB-β, IKK-γ, kinase deficient NIK or IKK-β. Expression of IκB-α mutant (pCMX-IκB-α(S32A/S36A)) was confirmed in an immunoblotting by its slower mobility as compared with the wild type IκB-α (61), and also by its nondegradability after LIX treatment (A). Expression of dnIκB-β (pCMV-Tag3B-IκB-β(S19A/S23A)), kinase-deficient IKK-β (pRK5-IKK-β-Flag), kdNIK (pRK7-NIK(KK429-430AA)-Flag), and dnIKK-γ (pcDNA3-IKK-γ-HA) was confirmed by immunoblotting using anti-Myc, -FLAG, and -hemagglutinin antibodies. B, β-actin levels demonstrated similar amounts of protein loading. Cells transfected with empty vectors (pCMX, pCMV-Tag 3B, pRK5, pRK-7, and pcDNA3) were used as controls. 24 h after transfection, the media was changed, and the cells were treated with LIX (100 ng/ml) for either 1 (C) or 4 h (D and E). The results indicate that pretreatment with the anti-inflammatory cytokine IL-10 (10 ng/ml for 4 h) and overexpression of dnIκB-α or dnIκB-β inhibited LIX-induced NF-κB activation (C), and cytokine mRNA (D) and protein levels (E). Similar results were obtained by the overexpression of kdNIK, kdIKK-β, and dnIKK-γ (*, p < 0.001 versus empty vector transfected cells).

Likewise, LIX treatment had no effect on the expression of Oct1, a constitutively expressed translation factor (Fig. 2D).
CDEC treated with LIX (Fig. 2F). Because LIX was the most potent inducer of NF-κB activation, in all subsequent experiments we used LIX at a concentration of 100 ng/ml.

**LIX Induces Proinflammatory Cytokine Expression**—The
NF-κB in Chemokine-Cytokine Cross-talk

Increased mRNA levels reflect enhanced transcription and/or increased mRNA half-life. To determine whether increase in cytokine mRNA expression is because of increased gene transcription, we performed nuclear run-on analyses. As illustrated in Fig. 3, C and D, low levels of IL-1β and TNF-α transcripts were detected in control rat CDEC, but were significantly increased at 4 h after LIX treatment (IL-1β, 4.8-fold; TNF-α, 11.3-fold; p < 0.001). These results indicate that LIX-induced cytokine expression is regulated at the transcriptional level.

To determine mRNA half-life, cells were treated with LIX for 4 h, followed by actinomycin D pulse for up to 6 h. Fig. 3, E and F, shows that the half-life of LIX-induced IL-1β mRNA was twice that of untreated controls (t1⁄2 control, 2.5 h, IL-1β, −4.25 h). However, the half-life of TNF-α mRNA was similar in untreated and LIX-treated cells (t1⁄2 control −2.75 h). These results indicate that while increased transcription and mRNA stability contributed to LIX-induced IL-1β induction, increased transcription contributed to LIX-induced TNF-α expression.

Because LIX up-regulated cytokine mRNA expression, we next determined whether LIX regulates cytokine promoter activity. Rat CDEC were transiently transfected with IL-1β or TNF-α promoter-reporter constructs. Fig. 3G shows that LIX, but not neutralized LIX, significantly increased IL-1β promoter activity, as seen by increased CAT expression (p < 0.01). To determine TNF-α promoter activity, we used a 1.1-kb TNF-α promoter (TNF-α (−1080/+135)) that contains 4 NF-κB sequence and a deletion construct (TNF-α (−85/+135)) that lacks all four NF-κB response elements (Fig. 3H). Treatment with LIX, but not neutralized LIX, induced a 3.5-fold increase in luciferase activity as compared with control (untreated and untransfected cells) and LIX-treated empty vector-transfected rat CDEC (p < 0.01). In contrast, cells transfected with the TNF-α promoter construct that lacks all four NF-κB sites failed to respond to LIX (Fig. 3H).

Fig. 7. CXCR2-specific blockade inhibits LIX-mediated NF-κB activation and sB-driven luciferase activity. Reverse transcriptase-PCR was performed to demonstrate expression of CXCR1 and CXCR2 in rat CDEC using primers designed based on published sequences for CXCR1 and CXCR2 in rats. Our results show that rat CDEC expresses both CXCR1 and CXCR2 at basal conditions (A). B. NF-κB DNA binding activity by EMSA; C. sB-driven luciferase activity. Treatment with a specific CXCR2 antagonist (SB447232, 10 nM, 10 min) followed by LIX treatment for 1 h attenuated NF-κB activation in rat CDEC that express both CXCR1 and -R2, and abrogated LIX-mediated NF-κB activation in mouse CDEC that express only R2, indicating that LIX signals via both CXCR1 and -R2.

Promoter/enhancer regions of proinflammatory cytokines contain binding elements for various stress-responsive transcription factors that are regulated by oxidative stress and proinflammatory stimuli. Therefore, we assessed the effects of LIX on IL-1β and TNF-α expression in rat CDEC. The results are shown in Fig. 3. IL-1β and TNF-α mRNA were detected at low levels under basal conditions, and were up-regulated by LIX, with peak levels detected around 4 h (Fig. 3A). Whereas IL-1β expression returned to near basal level by 48 h, LIX-induced TNF-α expression remained high. In addition to IL-1β and TNF-α gene transcription, LIX treatment also increased cytokine protein levels in the culture supernatants (Fig. 3B). Both cytokines were induced at high levels by LIX at 4 h, and their expression was blocked when rat CDEC were treated with LIX after antibody neutralization. Thus, LIX induces both transcription and translation of IL-1β and TNF-α.

Because LIX up-regulated cytokine mRNA expression, we next determined whether LIX regulates cytokine promoter activity. Rat CDEC were transiently transfected with IL-1β or TNF-α promoter-reporter constructs. Fig. 3G shows that LIX, but not neutralized LIX, significantly increased IL-1β promoter activity, as seen by increased CAT expression (p < 0.01). To determine TNF-α promoter activity, we used a 1.1-kb TNF-α promoter (TNF-α (−1080/+135)) that contains 4 NF-κB sequence and a deletion construct (TNF-α (−85/+135)) that lacks all four NF-κB response elements (Fig. 3H). Treatment with LIX, but not neutralized LIX, induced a 3.5-fold increase in luciferase activity as compared with control (untreated and untransfected cells) and LIX-treated empty vector-transfected rat CDEC (p < 0.01). In contrast, cells transfected with the TNF-α promoter construct that lacks all four NF-κB sites failed to respond to LIX (Fig. 3H).

LIX-induced Cytokine Expression Is Dependent on Activation of NF-κB, and Involves NIK, IKK, and IκB—The signaling cascade initiated by free radicals and proinflammatory cytokines resulting in NF-κB activation has previously been shown to converge at IKK. The NIK associates with IKK- and IκB- kinases (Fig. 4A and B). Because LIX activated NF-κB (Fig. 1, A and B) and induced proinflammatory cytokines (Fig. 3, A and B) in rat CDEC, we next determined if LIX-mediated cytokine expression was dependent on activation of NF-κB, and whether LIX-mediated NF-κB activation proceeds via NIK, IKK, and IκB. We used a series of vectors that expressed dominant negative IκB-α, IκB-β, or IκK-γ or kinase-deficient NIK or IKK-β. We confirmed that the dominant negative or kinase-deficient vectors expressed the appropriate protein (Fig. 4, A and B). We then studied the effects of LIX on rat CDEC that had been transiently transfected with the above expression vectors. The results are shown in Fig. 4. LIX-mediated NF-κB activation was significantly inhibited by overexpression of dnIKK-α, dnIKK-β, and dnIKKK-α, but not by the corresponding empty vectors (Fig. 4C). LIX-mediated cytokine mRNA (Fig. 4D) and protein (Fig. 4E) was also similarly inhibited by these dominant negative and kinase-deficient expression vectors as compared with empty vector-transfected cells.

In addition, we have also tested the effects of IL-10, an
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Comparison of SB 447232 with SB 225002 as selective CXCR2 antagonists. CXCR1 and CXCR2 binding was done using membranes from Chinese hamster ovary cells stably expressing the individual receptors and 125I-labeled IL-8. Calcium mobilization was done with RBL-2H3 cells stably expressing the individual receptors with IL-8 being used as the agonist. Human (hPMN) and rat (rPMN) peripheral blood neutrophils were evaluated as suspensions in calcium mobilization using ligands defined in the table.

| Assay                              | SB 447232 | SB 225002 |
|------------------------------------|-----------|-----------|
| CXCR1 binding (Kᵦ, nM)             | 5,610 ± 1,900 (n = 6) | >3,300*   |
| CXCR2 binding (Kᵦ, nM)             | 26.8 ± 3.5 (n = 6)     | 22*       |
| CXCR1 Ca²⁺ (IC5₀, nM)              | 2,200 (n = 2)          | >1,000*   |
| CXCR2 Ca²⁺ (IC5₀, nM)              | 12 (n = 2)             | 20 (n = 2) |
| hPMN IL-8 Ca²⁺ (IC5₀, nM)          | 590 ± 220 (n = 4)      | 1,433 nM  |
| hPMN GROα Ca²⁺ (IC5₀, nM)          | 4.5 ± 1.6 (n = 5)      | 30*       |
| rPMN rGROβ Ca²⁺ (IC5₀, nM)         | 16 (n = 2)             | ND*       |

* SB 225002 data taken from Ref 31 and cloned receptor calcium data is from the present study.

** ND, not determined.

anti-inflammatory cytokine, on LIX-mediated NF-κB activation. Our results indicate that pretreatment with IL-10 for 4 h significantly inhibited LIX-induced NF-κB activation and cytokine expression (Fig. 4, B-D).

LIX-mediated NF-κB Activation Involves PI3K, PKC, PARP-1, and Proteasome—The signal transduction pathway(s) initiated by ELR⁺ CXC chemokines in the activation of NF-κB are not fully known. To determine the role of PI3K, PKC, PARP-1, and proteasome in LIX-mediated activation of NF-κB, rat CDEC were pretreated with selective inhibitors. Inhibition of PI3K with the selective inhibitor LY 294002 significantly inhibited LIX-mediated b activation and cytokine expression (Fig. 5A). Similar results were obtained with wortmannin. Furthermore, 3-amino-benzamide, a specific PARP-1 inhibitor, and MG-132, a proteasome inhibitor, also inhibited LIX-mediated b activation and cytokine expression (Fig. 5, B and C). On the other hand, inhibition of PKC by chelerythrine chloride partially, but significantly, attenuated b activation and cytokine expression.

To confirm activation of PI3K by LIX, we performed PI3K lipid kinase assays. Fig. 5D shows that LIX, but not neutralized LIX, activates PI3K (2.4-fold increase, p < 0.01). Whereas MeSO and LY 294002 had no effect on basal levels of PI3K, pretreatment with LY 294002 significantly inhibited LIX-induced activation of PI3K (p < 0.01; Fig. 5D).

LIX-mediated NF-κB Activation Is G Protein-dependent—The ELR⁺ CXC chemokines signal through the 7 transmembrane domain G protein-coupled receptors CXCR1 (R1) and CXCR2 (R2). Damaj et al. (34) have shown that addition of IL-8 to cell lines that specifically express either R1 or R2, or to neutrophils that express both R1 and R2 resulted in the formation of immunoprecipitatable complexes containing the receptors and the α subunits of G proteins. In addition, IL-8-mediated increase in cytosolic-free calcium was inhibited by pertussis toxin indicating a G protein-coupled signal transduction (35). Therefore, we determined the role of G proteins in LIX-mediated cellular and NF-κB activation. Rat CDEC were pretreated with pertussis toxin for 1 h followed by LIX stimulation. G-protein function was determined by measuring intracellular calcium levels in Fura-2/AM-loaded rat CDEC. Fig. 6 illustrates that treatment with LIX (100 ng/ml), but not PBS, increased intracellular calcium levels rapidly but transiently (compare Fig. 6, A and B). Pretreatment with pertussis toxin significantly attenuated LIX-induced calcium transient (Fig. 6C). Furthermore, LIX-mediated NF-κB activation and cytokine expression were also blocked by pertussis toxin (Fig. 6, D and E) indicating the role of inhibitory G proteins in LIX-mediated NF-κB activation.

LIX-mediated NF-κB Activation Involves Both CXCR1 and CXCR2—CXCR1 and -2 have a 78% sequence homology within the transmembrane domains, but differ in the extracellular, intracellular, and NH₂- and COOH-terminals, leading to distinct ligand specificity and signaling (36–39). Therefore, we determined the role of CXCR1 and -2 in LIX-mediated NF-κB activation. Rat CDEC that express both R1 and R2 (Fig. 7A), and mouse CDEC that express only R2 were pretreated with SB447232, a CXCR2-specific antagonist (32), and LIX-induced NF-κB activation was determined. SB 447232 is a potent CXCR2 antagonist with similar selectivity to SB 225002 (32) and SB 265610 (33), i.e. >100-fold higher affinity for human CXCR2 versus CXCR1 (binding IC₅₀ values of 26.8 ± 3.5 (n = 6) and 5,610 ± 1,433 nM, respectively (Table I). In addition, SB 447232 was a potent inhibitor of CXCR2 (0.1 μM rat GROβ-inducible calcium mobilization) on rat neutrophils with an IC₅₀ of 16 nM (Table I). The advantage of SB 447232 over SB 225002 and SB 265610 is that the former compound has much better bioavailability in rodents and will be useful for in vivo studies in the future. In mouse CDEC, CXCR2 blockade completely abrogated NF-κB activation and b-driven luciferase activity. Blockade of R2 in rat CDEC attenuated LIX-induced NF-κB activation and b-driven luciferase activity by 50% (Fig. 7, B and C) indicating that LIX signals through CXCR2, and presumably CXCR1.

DISCUSSION

Our results indicate for the first time that the ELR⁺ CXC chemokines LIX, KC, and MIP-2 up-regulate the proinflammatory cytokines IL-1β and TNF-α in cardiac-derived endothelial cells via activation of NF-κB. LIX-mediated NF-κB activation and b-responsive gene transcription involves IkB hyperphosphorylation leading to its selective degradation in the cytoplasm by the proteasome system. The LIX signaling was inhibited by IL-10 and NF-κB pathway-specific mutant expression vectors. LIX signals via both CXCR1 and -2 in inducing NF-κB activation. Specific blocking of CXCR2 attenuated NF-κB activation in rat cardiac-derived endothelial cells that express both R1 and R2, and completely abrogated LIX-induced NF-κB activation and b-driven luciferase activity in mouse cardiac-derived endothelial cells that express only R2.

The ELR⁺ CXC chemokines primarily attract and activate neutrophils to the site of injury/inflammation (1–7). We have previously shown that LIX, KC, and MIP-2 are expressed in the post-ischemic myocardium, and most notably LIX is expressed by all myocardial constituent cells (10). Although activated neutrophils at the site of myocardial ischemic injury play a role in scavenging damaged tissue and subsequent remodeling, at least initially they exacerbate tissue injury through generation of free radicals, and secretion of various proteolytic enzymes and proinflammatory cytokines (40–48). The results presented here demonstrate that chemokines (LIX) in addition to the recruitment of neutrophils to the site of myocardial ischemic injury may contribute to myocardial inflammation by the direct
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Induction of cytokine expression.

Interleukin-1β and TNF-α are κB-responsive proinflammatory cytokines with known negative myocardial inotropic effects. In isolated cardiomyocytes, papillary muscles, myocardial segments, Langendorff preparations, and in whole animals addition/infusion of TNF-α has been shown to depresses contractile function via induction of the inducible form of nitric oxide synthase and sustained generation of high levels of nitric oxide (49–54). High levels of TNF-α expression have also been shown to induce cell death by apoptosis (49). Both TNF-α and IL-1β are known free radical generators and NF-κB activators (11). We have previously shown that TNF-α induces LIX via activation of NF-κB in isolated cardiomyocytes (10). Furthermore, we demonstrated activation of NF-κB and induction of LIX by IL-1β and TNF-α in rat CDEC (Fig. 1). In the present study, we describe the converse, that is, the induction of proinflammatory cytokines by chemokines via NF-κB activation. Together, these observations indicate that NF-κB activation plays a central role in regulating cross-talk between chemokines and cytokines in myocardial cells.

The ELR⁺ CXC chemokines bind and exert their biological effects via the seven-transmembrane heterotrimeric G protein-coupled receptors CXCR1 and CXCR2. The sequences of CXCR1 and -2 within the seven-transmembrane domains and the connecting loops are homologous, but differ in the N and C-terminal domains, leading to overlapping as well as distinct ligand-binding and selective signal transduction pathways (36–39). Whereas all ELR⁺ CXC chemokines bind with high affinity to CXCR2, IL-8, because of the presence of Tyr13 and Lys15 in the N terminus has been shown to also bind R1 with high affinity (55). Recently, granulocyte chemotactic protein-2 has also been shown to bind R1 with high affinity because of the presence of Arg20 (56) indicating that other ELR⁺ CXC chemokines may bind to both R1 and R2 with high affinity. In the present study, we demonstrated that LIX-induced NF-κB activation is mediated in part through R2 in rat CDEC that express both R1 and R2, and fully through R2 in mouse CDEC that express only this receptor. Presumably, in the rat CDEC that express both receptors, the R2-dependent signaling occurs through R1.

Pretreatment of endothelial cells with pertussis toxin, which specifically blocks the coupling of CXC receptor to G proteins, attenuated the LIX-induced increase in intracellular calcium levels and completely inhibited LIX-induced NF-κB activation. Similarly, treatment with LY294002, a specific PI3K inhibitor, inhibited LIX-induced activation of PI3K activity and completely blocked NF-κB activation and κB-responsive cytokine gene transcription. In contrast, chelerythrine chloride, a PKC inhibitor, partially inhibited LIX-mediated NF-κB activation and cytokine expression. Collectively, these data indicate that LIX signals via inhibitory G proteins and PI3K, and partially via PKC. Although other G proteins may be involved in chemokine-mediated cell signaling (57, 58), our results exclude this possibility because LIX-mediated NF-κB activation was completely blocked by pertussis toxin.

Interleukin-10 is an anti-inflammatory cytokine, and has been shown to block expression of various proinflammatory cytokines via inhibition of NF-κB activation (59). In the present study we demonstrate that IL-10 blocked LIX-induced NF-κB activation and κB-responsive gene transcription. It has been previously shown that IL-10 could block NF-κB activation by inhibiting IKK-mediated IκB phosphorylation and degradation (55). Because the inhibitory effects of IL-10 are not cell-specific, and can inhibit activation of NF-κB in response to various proinflammatory stimuli, IL-10 may have a therapeutic potential in ischemia/reperfusion injury by blocking induction of proinflammatory cytokines and chemokines (60).

In the present study, we demonstrate for the first time that inhibition of PARP-1 activation prevents LIX-mediated NF-κB activation and IL-1β and TNF-α expression. It has been demonstrated recently that PARP-1, a nuclear protein involved in repairing DNA strand breaks, has also been shown to activate NF-κB (18). PARP-1 activation has been described during endotoxin and inflammation (55–60). Administration of lipopolysaccharide to mice activated PARP-1, and resulted in PARP-1-dependent κB-responsive IL-1, IL-6, TNF-α, iNOS gene expression, and iNOS-mediated NO generation (63). Further, in the murine system PARP-1 gene disruption or pharmacological inhibition of PARP-1 activation has been shown to reduce free radical generation, attenuate κB-responsive gene transcription, and reduce neutrophil infiltration in the lungs (64). Whether PARP-1 may be a logical target for inhibition to attenuate post-ischemic myocardial injury will require further study.

Taken together, our results indicate that the ELR⁺ CXC chemokines, besides being potent neutrophil chemoattractants, also induce proinflammatory cytokine expression via activation of NF-κB. Blunting the activation of NF-κB or other components of the signaling cascade, rather than targeting inhibition of individual cytokines, chemokines, or adhesion molecules, may be a valid strategy to attenuate myocardial tissue injury during various inflammatory conditions.

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REFERENCES

1. Oppenheim, J. J., Zachariae, C. O., Mukaida, N., and Matsushima, K. (1991) Annu. Rev. Immunol. 9, 617–648
2. Baggiolini, M., Dewald, B., and Moser, B. (1994) Adv. Immunol. 55, 97–179
3. Rollins, B. J. (1997) Blood 90, 905–908
4. Schall, T. J., and Bacon, K. B. (1994) Curr. Opin. Immunol. 6, 865–873
5. Rovai, L. E., Herschman, H. R., and Smith, J. B. (1998) J. Leukocyte Biol. 64, 494–502
6. Maekawa, T., and Ishii, T. (2000) Intern. Med. 39, 90–100
7. Simonini, A., Moscucci, M., Muller, D. W., Bates, E. R., Pagani, F. D., Burdick, M. D., and Strieeter, R. M. (2000) Circulation 101, 1519–1526
8. Lukacs, N. W., Hogaboam, C., Campbell, E., and Konkel, S. L. (1999) Chem. Immunol. 72, 102–120
9. Kukielka, G. L., Smith, C. W., LaRosa, G. L., Manning, A. M., Mendoza, L. H., Daly, T. J., Hughes, B., Youkey, K. A., Hawkins, L. H., Rot, A., and Entman, G. M. (1995) J. Clin. Invest. 95, 89–103
10. Chandrasekar, B., Smith, J. B., and Freeman, G. L. (2001) Circulation 103, 2296–2302
11. Bowie, A., and O’Neill, L. A. J. (2000) Biochem. Pharmacol. 59, 13–23
12. Mercurio, F., and Manning, A. M. (1999) Oncogene 18, 6163–6171
13. Mercurio, F., Zhu, H., Murray, B. W., Shevchenko, A., Bennett, B. L., Li, J., Young, D. R., Barbosa, M., Mann, M., Manning, A., and Rao, A. (1997) Science 278, 860–866
14. Slanecovski, I., and Baltimore, D. (1997) Cell 91, 299–302
15. Cohen, L., Henzel, W. J., and Bazerosi, P. A. (1998) Nature 395, 292–296
16. Rothwarf, D. M., Zandi, E., Natoli, G., and Karin, M. (1998) Nature 395, 297–300
17. Zandi, E., Rothwarf, D. M., Delhaene, M., Hayakawa, M., and Karin, M. (1997) Cell 91, 243–252
18. Ha, H., Hester, L. D., and Snyder, S. H. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 3270–3275
19. Hassa, P. O., Covic, M., Hasan, S., Imhof, R., and Hettiger, M. O. (2001) J. Biol. Chem. 276, 45588–45597
20. Digio, C. A., Grammas, P., Giacomelli, F., and Wiener, J. (1988) Tissue Cell. 20, 477–492
21. Marello-Berg, F. M., Peek, E., Lüdington, E. A., Stauss, H. J., and Lechler, R. I. (2000) Immunol. Methods 244, 205–215
22. Dahl, N. O., Niu, J., Browning, D. D., Ye, R. D., and Voyno-Yasenetskaya, T. (2000) J. Biol. Chem. 275, 20867–20870
23. Nguyen, D., and Stangel, M. (2001) Brain Res. Dev. Brain Res. 129, 77–81
24. DUNSTAN, C. A., SALAFRANZA, M., ASHIKARI, S., XIA, Y., FENG, L., and Harrisson, J. K. (1996) J. Biol. Chem. 271, 32770–32776
25. Goh, A. E., Huang, M. R., Wang, S., Zhou, Y., and Oberg, K. (1997) Biochim. Biophys. Acta 1326, 171–177
26. Chandrasekar, B., Mitchell, D. H., Colston, J. T., and Freeman, G. L. (1999) Circulation 99, 427–433
27. Godambe, S. A., Chaplin, D. D., Takora, T., and Bellone, C. J. (1994) J. Immunol. 153, 143–152
28. Singh, I. S., Viscardi, R. M., Kalivokanou, I., Calderwood, S., and Hasday, J. D. (2000) J. Biol. Chem. 275, 9841–9848
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29. Clark, R. A., Li, S. L., Pearson, D. W., Leidal, K. G., Clark, J. R., Denning, G. M., Reddick, R., Krause, K. H., and Valente, A. J. (2002) J. Biol. Chem. 277, 32369–32378
30. Murray, D. R., Prabhakar, S. D., and Chandrasekhar, B. (2000) Circulation 101, 2338–2344
31. Foukas, L. C., Daniele, N., Ktori, C., Anderson, K. E., Jensen, J., and Shepherd, P. R. (2002) J. Biol. Chem. 277, 37124–37130
32. White, J. R., Lee, J. M., Young, P. R., Hertzberg, R. P., Jurwiz, A. J., Chakravarthy, M. A., Widdowson, K., Foley, J. J., Martin, L. D., Griswold, D. E., and Sarau, H. M. (1998) J. Biol. Chem. 273, 10695–10698
33. Sarau, H. M., Widdowson, K. L., Palovich, M. R., White, J. R., Underwood, D. C., and Griswold, D. E. (2001) Prog. Respir. Res. 31, 293–296
34. Damaj, B. B., McColl, S. R., Neote, K., Hebert, C. A., and Naccache, P. H. (1996) J. Biol. Chem. 271, 20540–20544
35. Damaj, B. B., McColl, S. R., Mahana, W., Crouch, M. F., and Naccache, P. H. (1996) J. Biol. Chem. 271, 12783–12789
36. Ahuja, S. K., and Murphy, P. M. (1996) J. Biol. Chem. 271, 20545–20550
37. Hall, D. A., Bresford, I. J., Browning, C., and Giles, H. (1999) Br. J. Pharmacol. 126, 810–818
38. Jones, S. A., Wolf, M., Qin, S., Mackay, C. R., and Baggiolini, M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 6682–6686
39. Jones, S. A., Moser, B., and Thelen, M. (1995) FEBS Lett. 364, 211–214
40. Jordan J. E., Zhao, Z.-Q., and Vinten-Johansen, J. (1999) Cardiov. Res. 43, 860–878
41. Weiss, S. J. (1989) N. Engl. J. Med. 320, 365–376
42. Engler, R. L., Schmid-Schönbein, G. W., and Pavletic, S. B. (1983) Am. J. Pathol. 111, 98–111
43. Youker, K. A., Birdsall, H. H., Frangogiannis, N. G., Kumar, A. G., Lindsey, M. L., Ballantyne, C. M., Smith, C. W., Rossen, R. D., and Entman, M. L. (1997) Ann. N. Y. Acad. Sci. 832, 243–265
44. Frangogiannis, N. G., Youker, K. A., and Entman, M. L. (1996) EXS 76, 263–284
45. Williams, F. M. (1996) Pharmacol. Ther. 72, 1–12
46. Boyle, M. P., and Weisman, H. F. (1993) Circulation 88, 2872–2883
47. Entman, M. L., and Smith, C. W. (1994) Cardiovasc. Res. 28, 1301–1311
48. Entman, M. L., Youker, K., Shoji, T., Kekielka, G., Shappell, S. B., Taylor, A. A., and Smith, C. W. (1992) J. Clin. Invest. 90, 1335–1345
49. Finkel, M. S., Oddis, C. V., Jacob, T. D., Watkins, S. C., Hattler, B. J., and Simmons, R. L. (1992) Science 257, 387–389
50. Fridey, I. (1998) J. Exp. Biol. 201, 1203–1209
51. Kelly, R. A., and Smith, T. W. (1997) Circulation 95, 778–781
52. Le, J., and Vilecek, J. (1987) Lab. Invest. 56, 234–241
53. Meldrum, D. R. (1998) Am. J. Physiol. 274, R577–R595
54. Stilson, E., and Sakatvala, J. (1998) Int. J. Biochem. Cell Biol. 30, 1075–1079
55. Wolf, M., Delgado, M. B., Jones, S. A., Dewald, B., Clark-Lewis, I., and Baggiolini, M. (1998) Eur. J. Immunol. 28, 164–170
56. Yang, M., Sang, H., Rahman, A., Wu, D., Malik, A. B., and Ye, R. D. (2001) J. Immunol. 166, 6885–6892
57. Ye, R. D. (2001) J. Leukocyte Biol. 70, 829–848
58. Schraml, J., I., Ma., M., Oudes, Z. G., Barritt, D. S., and Cochrane, C. G. (1995) J. Biol. Chem. 270, 10428–10431
59. Schottelius, A. J., Mayo, M. W., Sartor, R. B., and Baldwin, A. S., Jr. (1999) J. Biol. Chem. 274, 31868–31874
60. Frangogiannis, N. G., Youker, K. A., Rossen, R. D., Pechenberger, M., Lindsey, M. H., Mendoza, L. H., Michael, L. H., Ballantyne, C. M., Smith, C. W., and Entman, M. L. (1998) J. Mol. Cell. Cardiol. 30, 2567–2576
61. Traenckner, E. B., Pahl, H. L., Henkel, T., Schmidt, K. N., Wilk, S., and Baumeier, P. A. (1995) EMBO J. 14, 2876–2883
62. Stab, C., Lim, L. H., Cuzzocrea, S., Getting, S. J., Zingarelli, B., Flower, R. J., Salzman, A. L., and Perretti, M. (1997) J. Exp. Med. 186, 1041–1049
63. Oliver, F. J., Menissier-de Murcia, J., Nacci, C., Decker, P., Andriantsitohaina, R., Muller, S., de la Rubia, G., Stoclet, J. C., and de Murcia, G. (1999) EMBO J. 18, 4446–4454
64. Kuhnle, S., Nicotera, P., Wendel, A., and Leist, M. (1999) Biochem. Biophys. Res. Commun. 263, 433–438
65. Lau, L., Pacher, P., Mabey, M. G., Virag, L., Soriano, F. G., Hasok, G., and Stahm, C. (2002) Am. J. Respir. Crit. Care Med. 165, 372–377
66. Shall, S., and de Murcia, G. (2000) Mutat. Res. 460, 1–15