

Signaling Role of Intracellular Iron in NF- κ B Activation*

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Iron chelators inhibit endotoxin-induced NF- κ B activation in hepatic macrophages (HMs), suggesting a role for the intracellular chelatable pool of iron in NF- κ B activation. The present study tested this hypothesis. Analysis of Fe⁵⁹-loaded HMs stimulated with lipopolysaccharide (LPS), revealed a previously unreported, transient rise in intracellular low molecular weight (LMW)·Fe⁵⁹ complex ([LMW·Fe]_i) at ≤ 2 min returning to the basal level within 15 min. The [LMW·Fe]_i response preceded I κ B kinase (IKK) (≥ 15 min) and NF- κ B (≥ 30 min) activation. Iron chelators (1,2-dimethyl-3-hydroxypyridin-4-one and *N,N'*-bis-2-hydroxybenzylethylenediamine-*N,N'*-diacetic acid) abrogated the [LMW·Fe]_i response and IKK and NF- κ B activation. The [LMW·Fe]_i response was also observed in tumor necrosis factor α (TNF α)-stimulated HMs and RAW264.7 cells treated with LPS and interferon- γ but not in primary rat hepatocytes or myofibroblastic cells exposed to LPS or TNF α . Both [LMW·Fe]_i response and IKK activation in LPS-stimulated HMs were inhibited by diphenylene iodonium (nonspecific inhibitor for flavin-containing oxidases), L-N⁶-(1-iminoethyl)lysine (selective iNOS inhibitor), and adenoviral-mediated expression of a dominant negative mutant of Rac1 or Cu,Zn-superoxide dismutase, suggesting the role of NO and O₂⁻ in mediating the iron signaling. In fact, this inhibition was recapitulated by a cell-permeable scavenger of ONOO⁻, 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron (III) chloride. Conversely, ONOO⁻ alone induced both [LMW·Fe]_i response and IKK activation. Finally, direct addition of ferrous iron to cultured HMs activated IKK and NF- κ B. These results support a novel signaling role for [LMW·Fe]_i in IKK activation, which appears to be induced by ONOO⁻ and selectively operative in macrophages.

NF- κ B is the prototypic transcription factor in eukaryotic cells known to play a pivotal role in transactivation of promoters for genes involved in inflammation, immune responses, and anti-apoptotic mechanisms (Ref. 1, for review). At least two levels of redox regulation of NF- κ B appear to exist: one in the nucleus and another in the cytoplasm. The former involves direct redox modification of specific cysteine residues in the DNA binding domain of NF- κ B. In particular, oxidation of cysteine at the position 62 in p50 inhibits DNA binding activity (2). Conversely, reduction of NF- κ B by thioredoxin and Ref-1 appear to increase its activity (3, 4). For the cytosolic regulation, upstream signaling that is yet to be determined, leads to activation of IKK,¹ resulting in phosphorylation of two serine residues (Ser-32 and Ser-36) on I κ B α , its polyubiquitination, and degradation by 26 S proteasome. Treatment of the cells with the antioxidant, *N*-acetyl-L-cysteine or pyrrolidine dithiocarbamate prevents activation of NF- κ B (5–7), whereas addition of H₂O₂ or the generation of O₂⁻ causes activation of NF- κ B in certain cell types (5, 6, 8). TNF α -induced activation of NF- κ B is abrogated by inhibition of ROS production by the electron transport chain in mitochondria suggesting oxidant stress from this organelle as a signal for this mode of activation (9). Inhibition of NADPH oxidase blocks or attenuates activation of NF- κ B in monocytic cells (10, 11), whereas inhibitors for 5-lipoxygenase reduce both ROS generation and NF- κ B activation in lymphoid cells (10), indicating the possible cell type-dependent differences in the source of ROS signals for activation of this transcription factor. It is also important to note that intracellular ROS generation may not be necessary for NF- κ B activation in all cell types. Indeed, in epithelial cell lines, no detectable increase in ROS generation is observed in association with activation of NF- κ B (12). Moreover, anti-oxidants and metal chelators are often ineffective in inhibiting NF- κ B activation in these cells (13). Thus, it appears that a tighter association exists between oxidant stress and NF- κ B activation in monocytic or lymphoid cells.

Hepatic macrophages (HMs), *i.e.* Kupffer cells, represent the

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¹ The abbreviations used are: IKK, I κ B kinase; HMs, hepatic macrophages; [LMW·Fe]_i, intracellular low molecular weight-iron complex; TNF α , tumor necrosis factor α ; LPS, lipopolysaccharide; NF- κ B, nuclear factor- κ B; AP-1, activator protein 1; ROS, reactive oxygen species; O₂⁻, superoxide anion; L1, 1,2-dimethyl-3-hydroxypyridin-4-one; HBED, *N,N'*-bis-2-hydroxybenzylethylenediamine-*N,N'*-diacetic acid; NO, nitric oxide; L-NIL, L-N⁶-(1-iminoethyl)lysine; ONOO⁻, peroxynitrite; FeTTPS, 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron (III) chloride; Cu,Zn-SOD, copper-zinc superoxide dismutase; SNAP, *S*-nitroso-*N*-acetylpenicillamine; iNOS, inducible nitric-oxide synthase; NAC, *N*-acetylcysteine; DPL, diphenylene iodonium; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; GST, glutathione *S*-transferase.

largest population of macrophages, accounting for as much as 70–80% to total body macrophages. In addition to their crucial role in the first line of defense against invading microorganisms or bacterial products via splanchnic circulation, HMs represent the major site for both regulation of inflammatory and immune responses and metabolism of iron. For the former, HMs release a wide array of soluble factors, including cytokines and chemokines, that are mostly encoded by NF- κ B-responsive genes as well as growth factors, lipid metabolites, and gaseous mediators (O_2^- , H_2O_2 , and NO). For the latter function, hepatic and splenic macrophages are largely responsible for removal of senescent red blood cells, breakdown of hemoglobin, and recycling of iron for synthesis of new heme or non-heme iron proteins. These two disparate functions of inflammation/immunity and iron metabolism appear mutually related based on several lines of evidence. First, iron is known to affect cell-mediated immune response (14). Excessive iron load in macrophages reduces their functions, including phagocytosis, respiratory burst, and cytokine expression (15–17), the effects likely mediated by iron-mediated toxicity (18). On the other hand, at non-toxic concentrations, iron promotes macrophage differentiation (19) and functions, including anti-microbial effects (20) and TNF-mediated cytotoxicity (21). Iron also increases interleukin-1 secretion by macrophages stimulated by interferon- γ and LPS (22). On the contrary, the treatment with iron chelators inhibits LPS-mediated NF- κ B activation and expression of TNF α and interleukin-6 by cultured peritoneal macrophages (23) and HMs (24).

The finding that iron chelators inhibit activation of NF- κ B in macrophages suggests a role for a chelatable pool of iron in the signal transduction for this molecular event. In testing this hypothesis, the present study disclosed a novel and transient rise in the intracellular level of low molecular weight-iron complex ($[LMW\cdot Fe]_i$) in macrophages stimulated with LPS or TNF α . This response appears specific to macrophages and precedes IKK activation and increased NF- κ B binding. The $[LMW\cdot Fe]_i$ response is tightly correlated with NF- κ B activation and abrogated by inhibition of iNOS and NADPH oxidase or overexpression of SOD. Our results support the notion that $ONOO^-$ is the effector molecule for inducing $[LMW\cdot Fe]_i$ release. Last, addition of ionic iron to HMs caused activation of IKK, establishing that iron can serve as an independent agonist and as a potential signaling molecule for IKK activation.

EXPERIMENTAL PROCEDURES

Materials—Cell lines employed in this study included RAW264.7 cells obtained from the American Tissue Culture Collection (Rockville, MD), R37 cells, stably Nramp1G169 expressing RAW264.7, and R21 cells stably transfected RAW264.7 with a vector encoding the gene in an antisense orientation, both provided by Dr. C. H. Barton (University of Southampton, UK). Fetal bovine serum was obtained from Sigma Chemical Co. (St. Louis, MO). Adenoviral vectors, AdN17Rac1 (Ad.DN-Rac1), Ad.CuZn-SOD, and Ad.LacZ, expressing dominant negative mutant Rac1, Cu,Zn-SOD, or β -galactosidase, respectively, were obtained from Dr. J. F. Engelhardt (University of Iowa). Recombinant murine interferon- γ , TNF α , and a TNF α enzyme-linked immunosorbent assay kit were purchased from R&D Systems (Minneapolis, MN). Anti-human I κ B α and antibodies against p50 and p65 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Iron 59 ($FeCl_3$), [γ - ^{32}P]ATP, and [α - ^{32}P]dATP were obtained from PerkinElmer Life Sciences (Boston, MA). Peroxynitrite ($ONOO^-$) and its scavenger 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron (III) chloride ($FeTTPS$) were obtained from Calbiochem (San Diego, CA). *S*-Nitroso-*N*-acetylpenicillamine (SNAP) was purchased from Molecular Probes, Inc. (Eugene, OR). Pronase was obtained from Roche Applied Science (Indianapolis, IN). Nitrocellulose filters were obtained from Bio-Rad (Hercules, CA). ECL kit was obtained from Amersham Corp. (Arlington Heights, IL). Size-exclusion column was purchased from Millipore Corp. (Bedford, MA). L-N G -(1-Iminoethyl)lysine (L-NIL) was obtained from Alexis Biochemicals (San Diego, CA). Goat anti-rabbit IgG-horseradish peroxidase, collagenase type IV, arabinogalactan, LPS (*Escherichia coli* 055:B5),

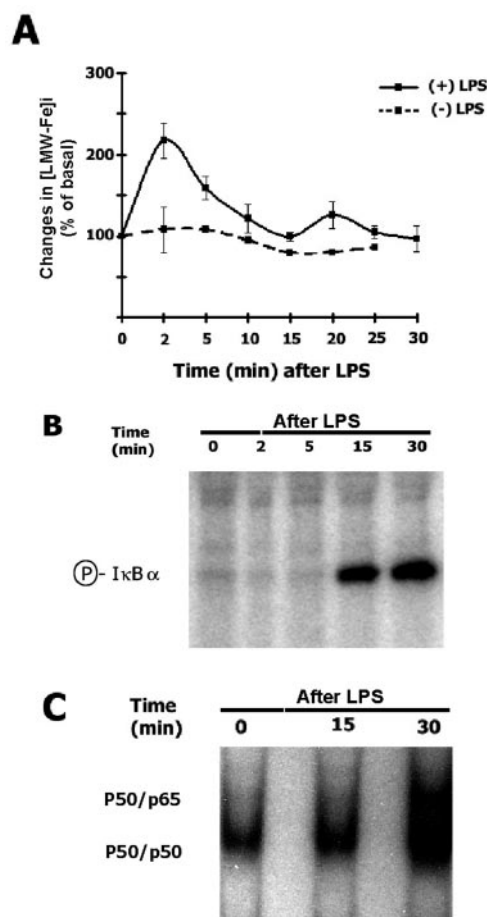


FIG. 1. LPS induces a transient rise in the intracellular level of low molecular weight-iron complexes ($[LMW\cdot Fe]_i$), which precedes activation of IKK and NF- κ B. A, cultured hepatic macrophages (HMs) were loaded with Fe^{59} , washed, and stimulated with LPS (500 ng/ml) in PBS. Cell lysate collected at different time points was subjected to the size exclusion column (<5000 kDa), followed by scintillation counting of the filtrate. The $[LMW\cdot Fe]_i$ data are expressed as the percentage to the basal level following standardization by total cellular Fe^{59} radioactivity. Note a distinct and transient rise in $[LMW\cdot Fe]_i$ at 2 min. B, HMs were similarly stimulated with LPS, and cell lysate was collected for an IKK assay with GST-I κ B α as substrate. Note the induction of IKK activity commences at 15 min. C, nuclear proteins were extracted from LPS-treated HMs for electrophoretic mobility shift assay using a κ B probe. Note an increase in NF- κ B binding occurs at 30 min. The data presented in A represent means \pm S.D. from six experiments. IKK and NF- κ B binding assays were performed on samples from at least four separate experiments.

sodium bathophenanthroline disulfonate, *N*-acetylcysteine (NAC), diphenylene iodonium (DPI), and phosphatase inhibitor mixture were purchased from Sigma Chemical Co. (St. Louis, MO). Iron chelators, 1,2-dimethyl-3-hydroxypyridin-4-one (L1) and *N,N'*-bis-2-hydrobenzylethylenediamine-*N,N'*-diacetic acid (HBED) were generous gifts from Dr. Gary Brittenham (College of Physicians and Surgeons, Columbia University, NY).

Cell Preparation—HMs were isolated from male Wistar rats by the Non-parenchymal Liver Cell Core of the Research Center for Alcoholic Liver and Pancreatic Diseases as previously published (24, 25). Briefly, the liver was digested *in situ* by sequential perfusion with Pronase and collagenase, and non-parenchymal liver cells were fractionated by discontinuous gradient ultracentrifugation using arabinogalactan. An HM-enriched fraction was further purified by the adherence method to achieve the final purity exceeding 95%. After 3 days of culture, the cells were treated with LPS (100 or 500 ng/ml) or TNF α (10 ng/ml) in serum-free DMEM in the presence or absence of various inhibitors as described below for subsequent collection of cell lysate for IKK assay and nuclear protein extraction for NF- κ B electrophoretic mobility shift assay. Hepatocytes were isolated using the standard collagenase digestion technique (26) by the Cell Culture Core of the USC Research

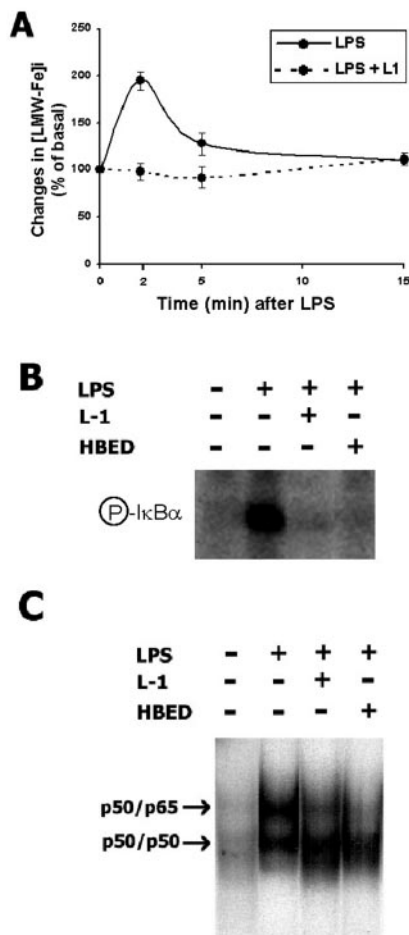


FIG. 2. Iron chelators block the [LMW-Fe]_i response, IKK activity, and NF- κ B binding in LPS-stimulated HMs. A, note the pretreatment of cultured HMs with L1 abrogates LPS-induced increase in the [LMW-Fe]_i. B, the treatment of HMs with L1 or HBED completely prevents LPS-induced IKK activity. C, similarly, L1 and HBED prevent increased binding of the p50/p65 heterodimer to the probe. The data presented in A represent means \pm S.D. from four experiments. IKK and NF- κ B binding assays were performed on samples from at least four separate experiments.

Center for Liver Diseases. The cells were cultured in DMEM with 10% FCS and used immediately after overnight culture for the experiments described below. RAW264.7 cells were obtained from the American Tissue Culture Collection and cultured in DMEM with 25 mM glucose and 10% FCS. Rat hepatic myofibroblastic cells were isolated from a rat with biliary liver fibrosis by the method described for HMs and by collecting a fraction from the gradient interface between the medium and 1.035. They became spontaneously immortalized, and subsequently cell clones were established by the limiting dilution method. They are cultured in DMEM with 10% FCS.

Fe⁵⁹ Labeling and [LMW-Fe]_i Measurement—Freshly isolated HMs, hepatocytes, liver myofibroblastic cells, or RAW264.7 cells were plated onto a 60-mm dish. The cells were cultured with 5 μ Ci/ml FeCl₃ for 14–16 h in 5 ml of DMEM containing 5% fetal bovine serum and antibiotics. Labeled cells were sequentially washed with 5 ml of warm PBS once, PBS containing 100 μ M bathophenanthroline sulfate once, and PBS twice. The washed cells were treated with LPS (500 ng/ml) or TNF α (10 ng/ml) in 5 ml of warm PBS for 2, 5, 10, or 20 min in the presence or absence of iron chelators (L1, 100 μ M; HBED, 100 μ M), N-acetylcysteine (NAC, 500 μ M), a nonspecific inhibitor for flavin-containing oxidases, including NADPH oxidase and iNOS (DPI, 1 μ M), a selective iNOS inhibitor (L-NIL, 20 μ M), and a cell-permeable decomposition catalyst for ONOO⁻ (FeTTPS, 200 μ M). These inhibitors were added 0.5–4 h prior to addition of LPS. For adenoviral-mediated expression of a dominant negative mutant Rac1 (Ad.N17Rac1), Cu,Zn-SOD (Ad.CuZn-SOD), or β -galactosidase (Ad.LacZ) as a control, the cultured HMs were infected with the vectors at the multiplicity of infection of 50–24 h before addition of LPS. The cells were also treated

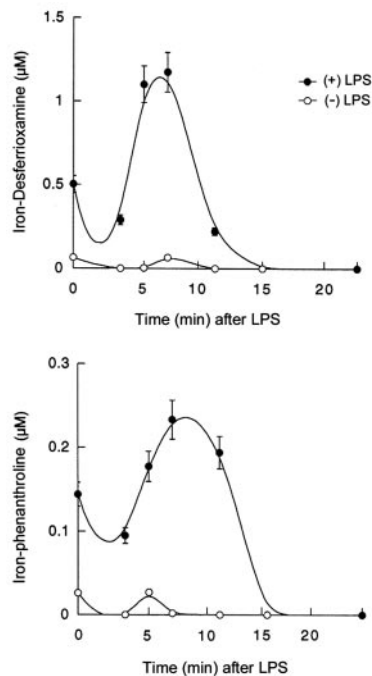


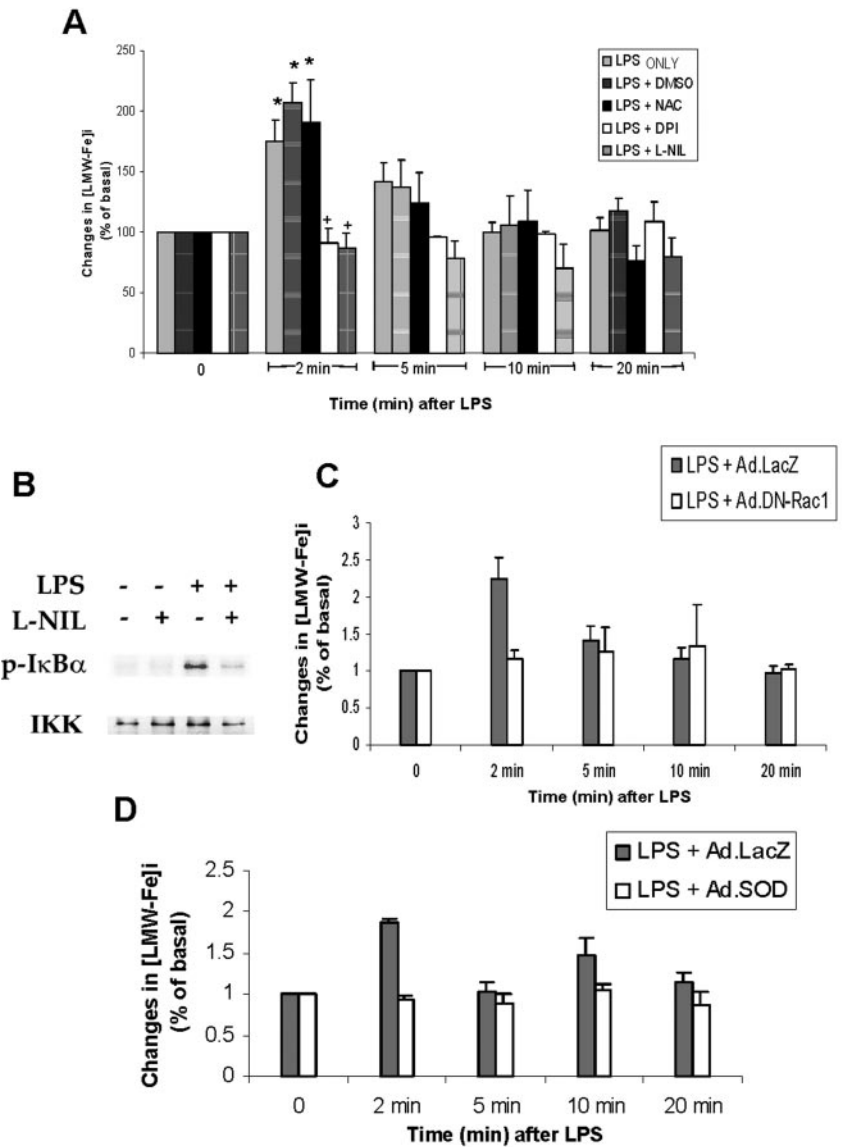
FIG. 3. The release of iron is confirmed in the intact HMs. To confirm the intracellular release of iron in the intact HMs without detergent lysis of the cells, the cells were stimulated with LPS, and the formation of complexes between iron and added chelators (desferrioxamine (top) and 1–10 phenanthroline (bottom)) was monitored spectrophotometrically in cell suspension. Note the similar transient rise in the formation of the iron-chelator complex using either chelator. The peak is slightly shifted right between 5 and 7 min as compared with the response detected with the Fe⁵⁹ method. The data represent means \pm S.D. from three separate experiments.

with a spontaneous nitric oxide donor (SNAP) to examine their effects on [LMW-Fe]_i. The incubation was stopped at the respective time point by removing PBS completely and adding 200 μ l of lysis buffer containing 1.4 M NaCl, 0.1 M HEPES (pH 7.4), 1.5% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride. A low molecular mass fraction (<5000 Da) was prepared by a centrifugation of the lysate in a size-exclusion column (Millipore, MA) at 8600 \times g for 30 min at 4 $^{\circ}$ C. Radioactivity of ultrafiltrate or total lysate was determined by a liquid scintillation counter.

Measurement of Intracellular Chelatable Iron in HMs—The chelatable pool of iron in HMs was measured as previously described (27). Briefly, primary cultures of rat HMs were washed with PBS twice, scraped gently, and incubated in PBS (5 \times 10⁶/3 ml) to which an iron chelator, desferrioxamine or 1,10-phenanthroline, was added to form a stable complex with iron. The formed complexes were spectrophotometrically detected by their absorption at 430 and 510 nm, respectively. The kinetic of complex formation was assessed with a Beckman spectrophotometer equipped with a flow cuvette connected with a vessel with constant stirring. The blank absorption was obtained by using the mixture of all reagents except chelators.

IKK Assay—To assay the activity of IKK, HMs cultured in a 100-mm dish were treated with LPS (500 ng/ml) for 15–30 min in the presence and absence of the iron chelators, L-NIL, or FeTTPS, washed once with PBS, and lysed with a lysis buffer (20 mM Tris-HCl, pH 7.5, 20 mM NaF, 20 mM β -glycerophosphate, 0.5 mM Na₃VO₄, 2.5 mM metabisulfite, 5 mM benzamide, 1 mM EDTA, 0.5 mM EGTA, 10% glycerol and protease inhibitors, 300 mM NaCl, and 1.5% Triton X-100). The lysates were immediately frozen in liquid nitrogen and stored at -80 $^{\circ}$ C until assayed. IKK activity was determined as previously described (28). HMs were also treated with SNAP, ONOO⁻, and FeSO₄ at the indicated concentrations to test their direct effects on IKK. Briefly, IKK was immunoprecipitated by IKK α antibodies and protein G-Sepharose. The assay was performed at 30 $^{\circ}$ C for 1 h in buffer containing 20 mM Tris-HCl, pH 7.5, 20 mM MgCl₂, 2 mM dithiothreitol, 20 μ M ATP, 2 μ g of GST-I κ B α , and [γ -³²P]ATP (0.5 μ Ci). The reaction was stopped by addition of Laemmli buffer and was resolved by 10% SDS-PAGE followed by a transfer onto a nitrocellulose membrane. Phosphate incor-

FIG. 4. *A*, diphenylene iodonium (*DPI*, 1 μ M), a nonspecific inhibitor for flavin-containing oxidases such as NADPH oxidase and iNOS, and *L-NIL*-N6-(1-iminoethyl)-lysine, a selective iNOS inhibitor (*L-NIL*, 20 μ M), abrogate the LPS-induced [LMW-Fe]_i response in HMs. *B*, *L-NIL* also inhibits LPS-stimulated IKK activity in HMs (upper panel) while not affecting the IKK level as shown by immunoblotting (lower panel). The data in *A* represent means \pm S.D. from four experiments. *, $p < 0.05$ as compared with the basal; +, $p < 0.05$ as compared with LPS plus Me₂SO. Expression of a dominant negative mutant of Rac1 (*C*) or Cu,Zn-SOD (*D*) blocks LPS-induced [LMW-Fe]_i response in HMs. Cultured HMs were infected with an adenoviral vector expressing a dominant negative mutant Rac1 (Ad.DN-Rac1), Cu/Zn-SOD (Ad.SOD), or LacZ (Ad.LacZ) as a control. Twenty-four hours later, the cells were stimulated with LPS (500 ng/ml) for the [LMW-Fe]_i analysis as described under "Experimental Procedures." The data were obtained from four and three sets of experiment for *C* and *D*, respectively. *, $p < 0.05$ as compared with the basal; +, $p < 0.05$ as compared with Ad.LacZ.



porated into GST-IkBa was visualized by analyzing the membrane with a PhosphorImager (Amersham Biosciences).

Immunoblot Analysis—For immunoblot analysis of IKK and nitrated IkBa, cytosolic extracts were prepared as previously reported (29). Proteins were resolved on a 10% SDS-polyacrylamide gel (SDS-PAGE), transferred to a nitrocellulose membrane, incubated with anti-IKK α antibodies, and detected by chemiluminescence. For immunoblot analysis of nitrated IkBa, IkBa was first immunoprecipitated with anti-IkBa antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), and the immune complex was resolved on a 10% SDS-PAGE and transferred to a membrane for immunoblot analysis using anti-nitrotyrosine antibodies (Upstate Biotechnology, Lake Placid, NY).

Nuclear Protein Extraction and Electrophoretic Mobility Shift Assay—To examine DNA binding by NF- κ B, nuclear proteins were extracted using the method of Schreiber *et al.* (30) from cultured HMs, RAW264.7 cells, hepatic myofibroblastic cells, or hepatocytes exposed to LPS or TNF α in the presence or absence of the iron chelators. The extracts (5 μ g) were incubated in a reaction mixture (20 mM HEPES, pH 7.6, 100 mM KCl, 0.2 mM EDTA, 2 mM dithiothreitol, 20% glycerol, 200 μ g/ml poly(dI-dC)) on ice with the double-strand κ B consensus sequence (31) or the κ B site from TNF α promoter (32) labeled with ³²P. After a 20-min incubation, the reaction mixture was resolved on a 6% non-denaturing polyacrylamide gel, and the gel was dried for subsequent autoradiography. For the supershift assays, antibodies against p50 and p65 (Santa Cruz Biotechnology) were added to the reaction mixture for an additional 30 min.

Analysis of Nramp1^{G169}-expressing RAW264.7—A recent study (33) demonstrates that RAW264.7 cells stably transfected with a vector

expressing a wild type Nramp1^{G169} (a transformant designated as R37) have lower cellular iron load and a reduced chelatable iron pool as compared with those stably transfected with a vector encoding the gene in an antisense orientation (designated as R21). We have obtained these two stable transformants from Dr. C. H. Barton's laboratory (University of Southampton, UK) and tested whether the altered chelatable pool of iron in R37 affects the [LMW-Fe]_i signaling, IKK activation, NF- κ B binding, and TNF α release following LPS stimulation. TNF α immunoassay was performed with a commercially available mouse TNF α enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN). For the measurement of total non-heme iron content, R37 or R21 cells (3×10^7) were washed with ice-cold PBS twice. The cells were treated with 1 ml of solution of 10% (w/v) trichloroacetic acid dissolved in 3% (v/v) thioglycolic acid and 1.94 M HCl, vortexed, and put on ice for 10 min. After centrifugation at 14,000 \times g for 5 min at 4 $^{\circ}$ C, 100 μ l of supernatant was incubated for 30 min at 37 $^{\circ}$ C with the equal volume of 0.42 mM bathophenanthroline sulfonate in 2 M sodium acetate. The reaction mixture was then measured for the absorbance at 550 nm on a microtiter plate scanning spectrophotometer (Power Wave 200TM, Bio-Tek Instruments, Winooski, VT). An iron atomic absorption standard solution (Aldrich) was used as iron standard. The labeling with Fe⁵⁹ was performed as described for HMs, the specific activity of Fe⁵⁹ in the cells was determined by dividing the radioactivity by iron content, and the chelatable pool of iron was determined by measuring the mobilization of Fe⁵⁹ radioactivity into the low molecular weight fraction following the treatment of the cell lysate with L1 (100 μ M) for 5 min at room temperature.

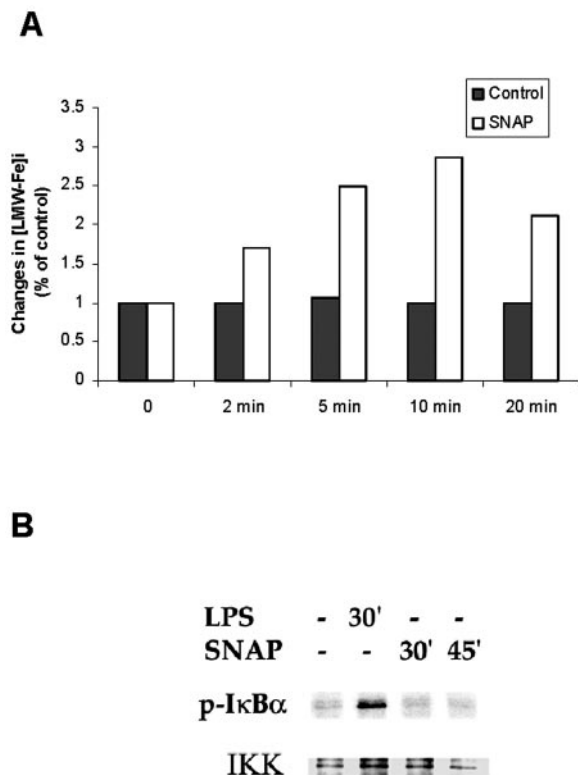


FIG. 5. SNAP induces a gradual and sustained increase in LMW-Fe⁵⁹ (A) and does not activate IKK (B) in HMs. Fe⁵⁹-loaded, cultured HMs were treated with SNAP (500 μ M) to determine their effects on the [LMW-Fe]_i and IKK activity as described under "Experimental Procedures." Note a gradual and sustained increase in [LMW-Fe]_i during 2–20 min after addition of the NO donor, SNAP (A). SNAP does not activate IKK at 30 and 45 min, while LPS shows strong induction of IKK activity at 30 min (B).

RESULTS

LPS-induced [LMW-Fe]_i—We previously showed that the treatment of cultured HMs with an iron chelator, L1, prevents LPS-induced NF- κ B activation (24), suggesting the role of the chelatable pool of iron in activation of this transcription factor. To examine this possibility, HMs were loaded with Fe⁵⁹, washed stringently, and stimulated with LPS to determine time-dependent changes in the Fe⁵⁹ radioactivity in the low molecular weight cytosolic fraction ([LMW-Fe]_i). Addition of LPS (500 ng/ml) resulted in a transient rise in [LMW-Fe]_i at \leq 2 min, followed by a decline to its basal level by 10–15 min (Fig. 1A). The radioactivity of [LMW-Fe]_i, separated by a size exclusion column with the molecular mass cutoff of 5000 Da, was 1–2% of total cell-associated radioactivity and increased 2-fold by LPS stimulation. Because the earliest time point we were able to process the cells for this analysis was 2 min, this response might have taken place earlier than 2 min. Kinetic analysis of this event in relation to IKK and NF- κ B activation revealed that the [LMW-Fe]_i response preceded the beginning of increased IKK activity at 15 min and that of NF- κ B binding at 30 min (Fig. 1, B and C). Addition of an iron chelators, L1 (100 μ M, Fig. 2A) or HBED (100 μ M, data not shown), prior to LPS stimulation completely abrogated the response as well as IKK (Fig. 2B) and NF- κ B (Fig. 2C) activation. Because the assessment of a cytosolic labile pool of iron after detergent lysis of the cells may cause the release of iron from solubilized vesicles, organelles, or proteins, we have also employed another approach to determine changes in the chelatable pool of iron in intact cells. This method involved chelation of iron with desferrioxamine or 1,10-phenanthroline and spectrophotometric

determination of the iron-chelator complexes in cell suspension. Using this method, we confirmed a transient rise in the chelatable iron level with either chelator following LPS stimulation (Fig. 3). However, the response curve shifted to right with a peak occurring between 5 and 7 min, instead of 2 min or earlier when detected by the Fe⁵⁹ method. This delay probably reflected the fact that the chelators were not reaching liberated iron immediately and that this spectrophotometric method has a higher detection threshold than the radioactive method. Nevertheless, the early, transient iron response in LPS-stimulated HMs was confirmed by this method in the intact cells.

[LMW-Fe]_i Response Is Unique to Macrophages—We next tested whether TNF α , another agonist for NF- κ B activation, causes the [LMW-Fe]_i response in HMs. After addition of TNF α , the identical, transient response to that seen after LPS treatment was reproduced at 2 min (data not shown). We then tested whether the [LMW-Fe]_i response is observed in other cell types when NF- κ B is activated. The [LMW-Fe]_i was examined in cultured hepatocytes treated with LPS or TNF α . Despite the fact that these treatments caused robust NF- κ B activation, no [LMW-Fe]_i response was observed (data not shown). Next, we examined rat liver myofibroblastic cells stimulated with TNF α and RAW264.7 cells, the murine macrophage cell line, treated with interferon γ (10 units/ml) and LPS (100 ng/ml). Again, no significant changes occurred in the [LMW-Fe]_i in myofibroblastic cells, although NF- κ B binding was increased (data not shown). On the other hand, RAW264.7 cells showed a conspicuous [LMW-Fe]_i peak at 2 min after the treatment preceding activation of NF- κ B (data not shown). Therefore, these results suggest that the [LMW-Fe]_i response is unique to macrophages.

Inhibition of iNOS and NADPH Oxidase Abrogates Inhibit [LMW-Fe]_i Rise—To further examine the association between the [LMW-Fe]_i response and NF- κ B activation and to explore potential mechanisms underlying induction of the [LMW-Fe]_i response, we tested whether the known inhibitors of NF- κ B activation could regulate the [LMW-Fe]_i increase. Cultured HMs were stimulated with LPS in the presence of *N*-acetylcysteine (NAC, 500 μ M), diphenylene iodonium (DPI, 1 μ M), a nonspecific inhibitor for flavin-containing oxidases such as NADPH oxidase and iNOS, and L-NIL (20 μ M), a selective iNOS inhibitor. DPI and L-NIL effectively blocked the [LMW-Fe]_i rise, whereas NAC or Me₂SO as a vehicle had no effect (Fig. 4A). DPI was shown to inhibit NF- κ B activation in HMs (11), whereas NAC did not inhibit activation and nuclear translocation of NF- κ B but suppressed NF- κ B-mediated transcription in HMs (34). Thus, our results for [LMW-Fe]_i correlate with these findings. L-NIL was shown to attenuate LPS-induced NF- κ B binding in RAW264.7 cells (35), and our study demonstrates suppression by L-NIL of LPS-induced IKK activity in HMs (Fig. 4B). Thus, these results further tighten the relationship between the [LMW-Fe]_i response and activation of NF- κ B, supporting the signaling role of [LMW-Fe]_i. They also suggest the involvement of iNOS and NADPH oxidase in facilitating this signaling event. Because DPI is not a specific inhibitor, to further test the role of NADPH oxidase, we next used an adenoviral vector expressing a dominant negative mutant of Rac1 (Ad.DN-Rac1) and a vector expressing β -galactosidase (Ad.LacZ) as a control. Ad.DN-Rac1 has recently been used to block activation of NADPH oxidase and subsequent activation of NF- κ B in LPS-stimulated macrophages via its dominant negative effect on the recruitment of the regulatory units of the oxidase to the membrane (36). HMs were infected with Ad.DN-Rac1 followed by stimulation with LPS, and the [LMW-Fe]_i rise was measured. As shown in Fig. 4C, Ad.DN-Rac1 infection abrogated the [LMW-Fe]_i rise, whereas Ad.LacZ did not. This suggests the importance of Rac1-mediated NADPH oxidase

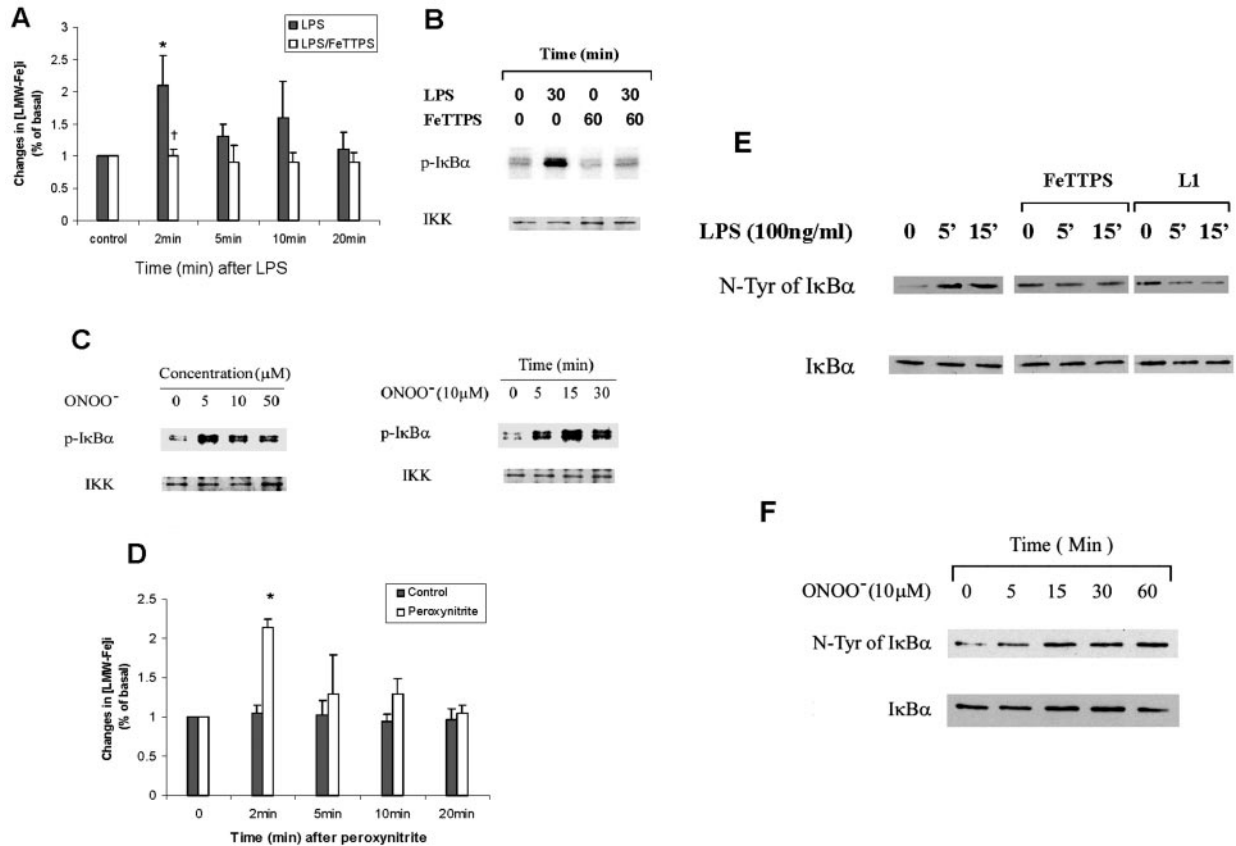


FIG. 6. LPS-induced [LMW-Fe]_i response (A) and IKK activity (B) in HMs are blocked with FeTTPS, a cell-permeable decomposition catalyst for ONOO⁻. FeTTPS (200 μM) was added 30 min prior to addition of LPS to cultured HMs for both the [LMW-Fe]_i analysis and IKK assay. Direct addition of peroxynitrite induces IKK activity in dose- and time-dependent manners in HMs (C). Peroxynitrite (10 μM) also induces an increase in [LMW-Fe]_i (D). LPS induces nitration of IκBα from 5 to 15 min, and this effect is abrogated by FeTTPS or L1 (E). Peroxynitrite also increases IκBα nitration at 15–60 min (F). Representative data from at least three separate experiments are shown for IKK activity (B and C) and IκBα nitration (E and F). The data from four and five separate experiments are shown as means ± S.D. for A and D, respectively. *, *p* < 0.05 as compared with the basal.

activation in generating the [LMW-Fe]_i rise. To test whether O₂⁻ is required for generation of the [LMW-Fe]_i, we overexpressed Cu,Zn-SOD in HMs using an adenoviral vector (Ad.SOD). This expression vector was also successfully employed to demonstrate the importance of O₂⁻ in LPS-induced NF-κB activation in macrophages (36), and indeed our experiment demonstrated complete inhibition of the [LMW-Fe]_i rise by Ad.SOD infection (Fig. 4D).

Peroxynitrite Is Responsible for [LMW-Fe]_i Signaling—The results presented so far suggest that NO produced by iNOS and NADPH oxidase-derived O₂⁻ are involved in LPS-stimulated [LMW-Fe]_i rise and subsequent IKK and NF-κB activation in HMs. Indeed, both radical species are required for this iron-mediated signaling pathway, because NO alone produced by addition of SNAP to the cells caused no transient rise in [LMW-Fe]_i but a gradual and sustained increase in this parameter (Fig. 5A). In addition, SNAP did not activate IKK (Fig. 5B). Thus, these results indicate that ONOO⁻, a radical species generated from NO, and O₂⁻ may be effector molecules for induction of [LMW-Fe]_i response. To test this possibility, HMs were stimulated with LPS in the presence of FeTTPS, a cell-permeable decomposition catalyst for ONOO⁻. The treatment abrogated LPS-induced [LMW-Fe]_i rise (Fig. 6A) and IKK activation (Fig. 6B). Finally, HMs were directly treated with ONOO⁻. Peroxynitrite at the concentrations of 5–50 μM activated IKK as early as 5 min (Fig. 6C). This induction of IKK activity is earlier than the timing observed for LPS stimulation, suggesting that ONOO⁻ is indeed an intermediate signal-

ing molecule. More importantly, ONOO⁻ induced [LMW-Fe]_i rise (Fig. 6D).

LPS and Peroxynitrite Increase Tyrosine Nitration of IκBα—Peroxynitrite is recently shown to activate NF-κB by the mechanism that appears to involve nitration of IκBα (29). If this nitration occurs at Tyr-42, this may abrogate negative regulation of IκBα degradation facilitated by phosphorylation of this tyrosine residue (37), resulting in accelerated IκBα degradation. Alternatively, any nitrated IκBα may be subjected to its preferential degradation (38). Thus, we then examined whether the treatment of cultured HMs with LPS or ONOO⁻ results in nitration of IκBα. For this analysis, HM cytosolic extracts were immunoprecipitated with an antibody against IκBα followed by immunoblotting with an anti-nitrotyrosine antibody. As shown in Fig. 6E, LPS induced nitration of IκBα at 5 and 15 min, and this effect was abrogated when the cells were pretreated with FeTTPS, a decomposition catalyst for ONOO⁻ or L1, an iron chelator. The treatment of the cells with ONOO⁻ (10 μM) also increased nitration of IκBα from 15 to 60 min (Fig. 6F). Thus these results support the previous findings on the role of IκBα nitration as a signaling event for IκBα degradation besides IKK-mediated phosphorylation of this inhibitory protein (29) and suggest that ONOO⁻ and iron are involved in this signaling process in the LPS-stimulated cells.

Nramp1 Overexpression Modulates [LMW-Fe]_i Signaling—Nramp1 is a 90- to 100-kD membrane-spanning protein that is known to confer the natural resistance of macrophages to infection by intracellular pathogens (39) through mechanisms

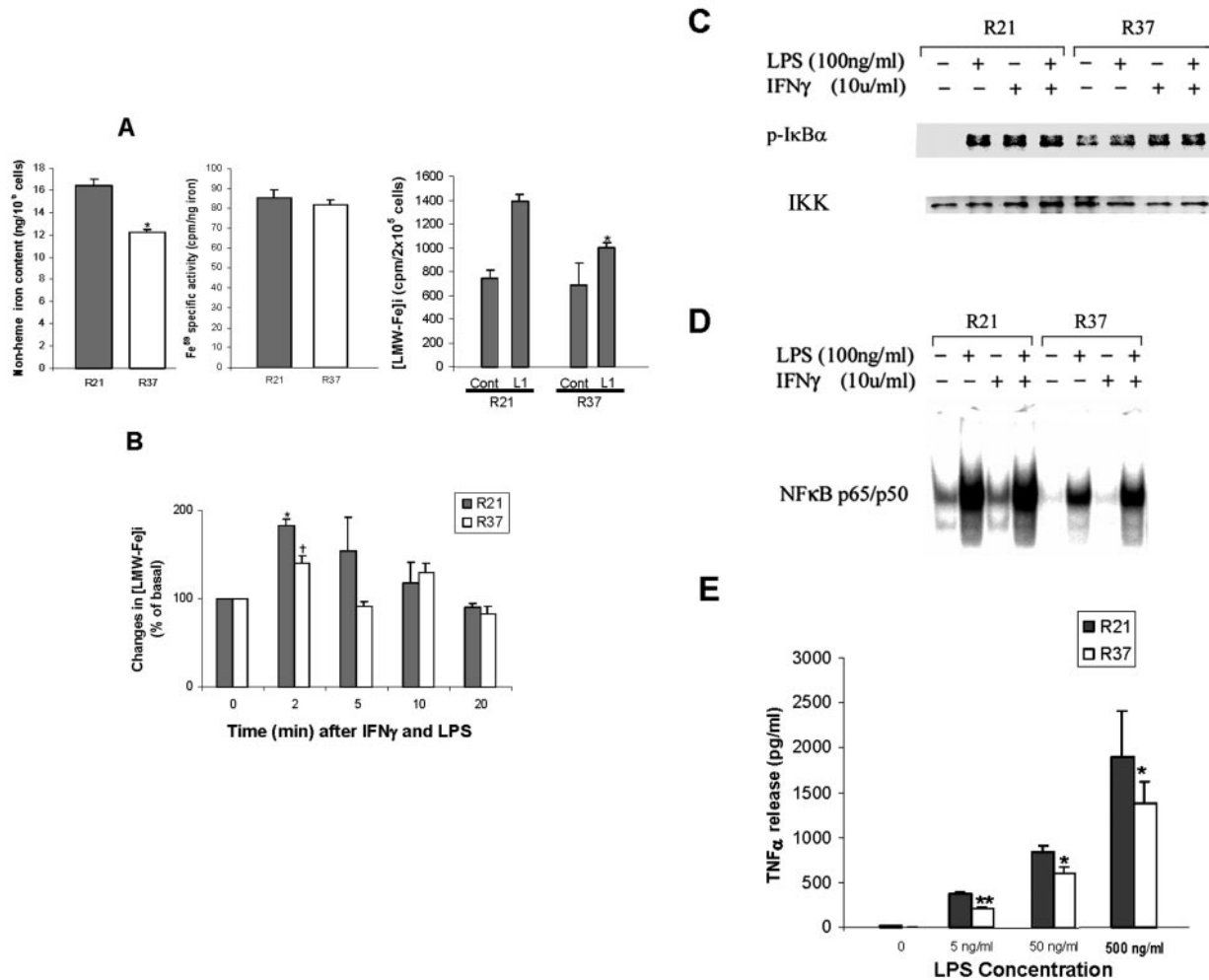


FIG. 7. Overexpression of Nramp1 in RAW264.7 cells reduces total cellular non-heme iron content, the chelatable pool of iron, LPS-induced LMW-Fe⁵⁹ response, IKK activity, NF- κ B binding and TNF α release. RAW264.7 cells stably transfected with a vector encoding wild type Nramp1 in a sense (R37) or antisense (R21) orientation were compared. The non-heme iron content is reduced in R37 cells (A, left panel, *, $p < 0.05$ compared with R21, $n = 3$), whereas the specific activity achieved with Fe⁵⁹ is comparable between the two cell lines (A, middle panel). Note L1-induced mobilization of iron into the low molecular weight fraction (LMW-Fe⁵⁹) is significantly lower in R37, demonstrating the chelatable pool of iron is reduced in these cells (A, right panel, **, $p < 0.01$ compared with R21, $n = 3$). They were also examined for LPS-stimulated changes in the [LMW-Fe]_i following Fe⁵⁹ loading. The data are expressed as the percentage of the basal level of [LMW-Fe]_i following standardization by total cellular Fe⁵⁹ radioactivity. Note the [LMW-Fe]_i response is attenuated in R37 cells (B, *, $p < 0.05$ as compared with the basal; **, $p < 0.05$ as compared with R21, $n = 3$). LPS-induced IKK activity (C), NF- κ B binding (D), and TNF α release (E, *, $p < 0.05$; **, $p < 0.01$ compared with R21, $n = 3$) are also reduced in R37 cells.

that are believed to regulate iron transport into or out of late endosomes (Ref. 40, for review). A recent study (33) using a stable transformant of RAW264.7 cells expressing high level of Nramp1 revealed that these cells (R37) have less total iron content and a reduced chelatable pool of iron as compared with the mock transfected cells (R21). We considered that the use of these cells would offer a unique opportunity to assess the effects of the reduced chelatable iron pool on LPS-stimulated [LMW-Fe]_i response and IKK activation. The total non-heme iron content in R37 cells was indeed reduced by 25% (Fig. 7A, left panel). Following Fe⁵⁹ loading, the specific activity achieved with the isotope was comparable between the two cell lines (Fig. 7A, middle panel). The chelatable pool of iron as assessed by an increase in the radioactivity in the low molecular weight fraction after L1 treatment of the cell lysate was significantly reduced in R37 cells (Fig. 7A, right panel), confirming the finding by Atkinson and Barton (33). When these cells were treated with LPS, R37 cells showed attenuated [LMW-Fe]_i response as compared with R21 cells (Fig. 7B), and this alteration was associated with inhibited IKK activity (Fig. 7C), NF- κ B binding (Fig. 7D), and TNF α release by R37 (Fig.

7E). Thus, these results provide an example of overexpression of a protein that regulates a chelatable pool of iron and in turn modifies iron-mediated signaling, NF- κ B activation, and expression of an NF- κ B-responsive gene.

Iron Directly Activates IKK—To test the cause and effect relationship, we next examined the effect of direct addition of ionic iron to cultured HMs on NF- κ B activation. Ferrous sulfate was added to HMs in the serum-free medium. Addition of ferrous sulfate at the concentration above 10 μ M induced IKK activity (Fig. 8A), and this effect was apparent as early as 5 min (Fig. 8B). These results demonstrate that iron can serve as an independent agonist for IKK activation.

DISCUSSION

The present study demonstrated a novel transient rise in the intracellular labile level of iron ([LMW-Fe]_i) that leads to IKK and NF- κ B activation in response to LPS or TNF α in macrophages. This response occurs rapidly (≤ 2 min) and is transient in nature, much like the intracellular calcium response seen in agonist-induced cells that initiates calcium-mediated signal transduction. Our results suggest that both iNOS-derived NO

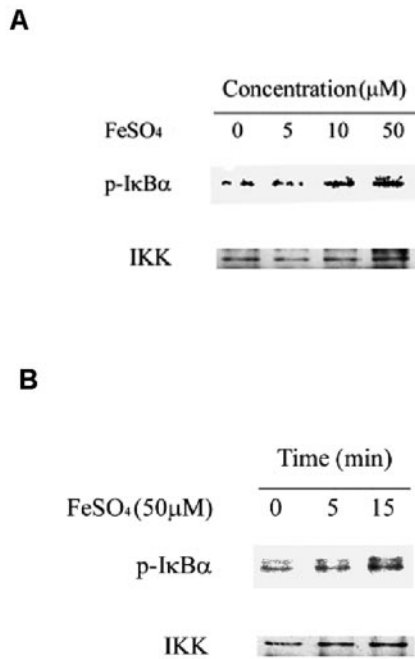


FIG. 8. **Direct addition of ferrous iron induces IKK activity in HMs in dose- and time-dependent manners.** Ferrous sulfate was added to cultured HMs at the indicated concentrations. Note that, at 10 and 50 μ M, it induces IKK activity (A). Addition of FeSO₄ (50 μ M) begins to activate IKK at 5–15 min (B).

and NADPH oxidase-derived O₂⁻ are required for this response and that ONOO⁻ is a likely effector molecule for the cytosolic release of iron. Peroxynitrite has been shown to induce expression of IL-8 by human blood cells, the effect that was inhibited with pyrrolidine dithiocarbamate, a classic inhibitor of NF- κ B (41). More recently, the direct evidence for ONOO⁻-induced NF- κ B binding was presented using human monocytes (29). Our results demonstrate for the first time that ONOO⁻ activates IKK, the upstream kinase responsible for phosphorylation of I κ B α at serine residues 32 and 36, facilitating NF- κ B nuclear translocation via polyubiquitination and subsequent degradation of I κ B α by 26 S proteasome. Furthermore, we demonstrate that iron directly activates IKK in cultured HMs. Together, these results support the notion that ONOO⁻ activates IKK through the release of iron, placing this novel [LMW-Fe]_i signaling event downstream of ONOO⁻ and upstream of IKK. We further present an intriguing example of the reduced chelatable pool of iron in Nramp1-overexpressing RAW264.7 cells that is associated with attenuation of LPS-induced [LMW-Fe]_i response, IKK activity, NF- κ B binding, and TNF α expression. This highlights the physiological importance of the [LMW-Fe]_i response that can be modified by the changes in cellular iron homeostasis rendered by one particular protein. We have previously reported an opposite example of an enhanced chelatable pool of iron in HMs. In these HMs, which were isolated from a rat model of alcoholic liver disease, the expanded chelatable pool of iron was tightly associated with increased NF- κ B binding and TNF α expression, the changes of which could be entirely normalized by the treatment with the iron chelator, L1 (25). In fact, these cells exhibit a heightened rise in the labile pool of iron after LPS stimulation as compared with the cells from control animals.² Thus, these findings collectively demonstrate that the chelatable iron pool within the cells can modulate the [LMW-Fe]_i signaling for IKK and NF- κ B activation and that this mode of regulation has physiological

and pathological relevance.

The source of [LMW-Fe]_i remains unknown. Three interesting possibilities are suggested. First, ferritin, the main intracellular site for sequestration and storage of excess iron, may be a target for oxygen (42, 43) and nitrogen (44) radicals for release of iron. The ONOO⁻ likely achieves the same effect. Second, iron-containing proteins can be an important source. Even though [•]NO is known to mobilize iron from proteins containing the [Fe-S] cluster such as IRP-1 (45), a recent study demonstrates that ONOO⁻ is more efficient than [•]NO in this effect (46). Lastly, iron may be mobilized by [•]NO or ONOO⁻ from the transitional pool prior to incorporation into ferritin (47). Regardless of the source, the [LMW-Fe]_i response has to be early and transient to serve as the signaling event, because a gradual and sustained increase in [LMW-Fe]_i seen after the treatment with a [•]NO donor, SNAP, did not activate IKK (Fig. 5, A and B). This is very analogous to the differential effects rendered by a transient *versus* sustained increase in the intracellular calcium concentration (48).

An equally important question is how the transient rise in [LMW-Fe]_i leads to IKK activation. One possibility is iron-mediated release of calcium. Oxidant stress is known to induce calcium mobilization in a variety of cell types (49–51). This mode of calcium mobilization is prevented with cell-membrane permeable iron chelators (52), suggesting the involvement of iron in this process. In fact, excessive iron loading is known to perturb mitochondrial calcium homeostasis and to induce the release of calcium from this storage site (53). More recently, micromolar concentrations of ferrous iron were shown to cause a transient release of calcium from liver mitochondria without either damaging the organelle or decreasing its membrane potential, suggesting the physiological implication of iron-mediated calcium mobilization (54). Moreover, ONOO⁻ at a low, physiologically relevant concentration (20 μ M), induced rapid intracellular mobilization of calcium in thymocytes (55). Because the current study demonstrated the release of iron by ONOO⁻ (Fig. 6D), an increase in the intracellular calcium concentration may be an immediate consequence initiating the known calcium-mediated signal transduction leading to activation of IKK. Conversely, calcium mobilization may occur upstream of iron. Currently, these possibilities are being investigated. The analogous kinetic patterns of both iron and calcium mobilization may also suggest that iron may signal via the mechanism analogous to that shown for calcium. The transiently released calcium binds to acceptor proteins such as calmodulin, and they in turn recruit and activate signaling proteins. This mode of iron-mediated signaling has never been demonstrated but cannot be ruled out.

Peroxynitrite was recently shown to activate NF- κ B in monocytes in a manner that appears to involve tyrosine nitration of I κ B α (29), possibly preventing negative regulation mediated via phosphorylation of the tyrosine residues such as Tyr-42 (37). Furthermore, nitrated I κ B α becomes a target for degradation by intracellular enzymes (38). Thus, this nitration-based mode of NF- κ B activation does not require activation of IKK and phosphorylation of Ser-32 and Ser-36 of I κ B α . In fact, our own analysis demonstrates increased nitration of I κ B α by the treatment with LPS or ONOO⁻ (Fig. 6, E and F), supporting this notion. Furthermore, the nitration induced by LPS is prevented by FeTTPS or an iron chelator, L1, suggesting the involvement of ONOO⁻ and iron in this signaling event. It has yet to be determined how nitration and phosphorylation I κ B α interact to facilitate NF- κ B activation in LPS-stimulated HMs and which sites of I κ B α are critical regulatory targets of nitration.

It is interesting that the [LMW-Fe]_i response was evident

² S. Xiong, H. She, and H. Tsukamoto, unpublished data.

only in HMs and the murine macrophage cell line but not in hepatocytes and myofibroblastic cells, despite the finding that the latter cells also activated NF- κ B in response to agonists. These results suggest that the iron-mediated signaling may be unique to the macrophage or similar cell type. This notion may not be so out of line. After all, the macrophages are the major site of both iron metabolism and the initiation of inflammatory and immune responses. They specifically utilize iron in their anti-microbial defense by releasing iron extracellularly and promoting Fenton pathway-mediated killing of microbes (20). They also express Nramp1, which regulates efflux of iron from or influx of this metal into late endosomes to help eliminate intracellular pathogens. We propose that macrophages are also evolved to uniquely develop the mechanism by which iron can be utilized as a signaling molecule to support transcriptional regulation of inflammatory and immune-related genes.

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