Mitochondrial Respiratory Complex I Regulates Neutrophil Activation and Severity of Lung Injury

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Rationale: Mitochondria have important roles in intracellular energy generation, modulation of apoptosis, and redox-dependent intracellular signaling. Although reactive oxygen species (ROS) participate in the regulation of intracellular signaling pathways, including activation of nuclear factor (NF)- κ B, there is only limited information concerning the role of mitochondrially derived ROS in modulating cellular activation and tissue injury associated with acute inflammatory processes.

Objectives: To examine involvement of the mitochondrial electron transport chain complex I on LPS-mediated NF- κ B activation in neutrophils and neutrophil-dependent acute lung injury.

Methods: Neutrophils incubated with rotenone or metformin were treated with bacterial lipopolysaccharide (LPS) to determine the effects of mitochondrial complex I inhibition on intracellular concentrations of reactive oxygen species, NF- κ B activation, and proinflammatory cytokine expression. Acute lung injury was produced by intratracheal injection of LPS into control, metformin, or rotenone-treated mice.

Measurements and Main Results: Inhibition of complex I with either rotenone or the antihyperglycemic agent metformin was associated with increased intracellular levels of both superoxide and hydrogen peroxide, as well as inhibition of LPS-induced I κ B- α degradation, NF- κ B nuclear accumulation, and proinflammatory cytokine production. Treatment of LPS-exposed mice with rotenone or metformin resulted in inhibition of complex I in the lungs, as well as diminished severity of lung injury.

Conclusions: These results demonstrate that mitochondrial complex I plays an important role in modulating Toll-like receptor 4-mediated neutrophil activation and suggest that metformin, as well as other agents that inhibit mitochondrial complex I, may be useful in the prevention or treatment of acute inflammatory processes in which activated neutrophils play a major role, such as acute lung injury.

Keywords: acute lung injury; neutrophil; mitochondria; reactive oxygen species; nuclear factor- κ B

Mitochondria have important roles in cellular function, including participation in energy generation and modulation of apoptosis (1). In addition, mitochondria produce large amounts of reactive oxygen species (ROS) and are important participants in redox-dependent intracellular signaling (2). There are

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Little is known about how mitochondria participate in neutrophil activation and neutrophil-driven inflammation.

What This Study Adds to the Field

These results demonstrate that mitochondrial complex I plays an important role in modulating Toll-like receptor 4-mediated neutrophil activation and suggest that metformin, as well as other agents that inhibit mitochondrial complex I, may be useful in the prevention or treatment of acute inflammatory processes in which activated neutrophils play a major role, such as acute lung injury.

four carriers of electron transport, complexes I to IV, embedded in the lipid bilayer of the inner mitochondrial membrane. Complex I (NADH: ubiquinone oxidoreductase) transfers electrons from NADH (nicotinamide adenine dinucleotide reduced) to ubiquinone (coenzyme Q [CoQ]). The energy released by this process results in the transfer of protons across the mitochondrial membrane. Under baseline conditions, approximately 5% of electrons escape from the mitochondrial electron transport chain and, in the presence of oxygen, result in the formation of superoxide anion. Superoxide is rapidly converted into hydrogen peroxide by mitochondrial superoxide dismutase or by spontaneous disproportionation. Complex I is considered to be a major site of electron leak and subsequent superoxide production (3, 4).

Activated neutrophils produce large amounts of ROS, a beneficial function in host defense, where such activity is involved in eradication of bacteria and other pathogens. However, excessive production of ROS by neutrophils can be deleterious, contributing to organ system dysfunction in inflammatory conditions, such as sepsis or acute lung injury (5–10). In addition to generating ROS, neutrophils also appear to be affected by ROS present in the extracellular and intracellular environments. For example, whereas increased intracellular levels of superoxide in neutrophils lead to activation of nuclear factor (NF)- κ B as well as production of proinflammatory cytokines, exposure of neutrophils to hydrogen peroxide inhibits bacterial lipopolysaccharide (LPS)-induced proteasomal degradation of the NF- κ B inhibitory subunit I κ B- α , nuclear translocation of NF- κ B, and expression of NF- κ B-dependent cytokines (11).

Recent reports have shown the presence of a mitochondrial network in neutrophils that participates in regulation of cell shape and chemotaxis (12). However, little is known about the role of mitochondrially derived ROS in modulating neutrophil function. To examine this issue, we treated isolated neutrophils and mice with inhibitors of mitochondrial complex I, which are known to increase mitochondrial ROS generation (3, 4). We found that treatment of neutrophils with rotenone, a potent and

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specific inhibitor of complex I, or metformin, an antihyperglycemic agent that is frequently used in patients with diabetes (13) and which also modulates mitochondrial ROS formation by inhibition of complex I (14), resulted in increased ROS formation, decreased NF- κ B activation, and diminished production of proinflammatory cytokines in LPS-stimulated neutrophils. We also demonstrated that rotenone and metformin effectively reduced the severity of LPS-induced acute lung injury in mice.

METHODS

Mice

Male C57BL/6 mice, 8 to 12 weeks of age, were purchased from Jackson Laboratory (Bar Harbor, ME). The mice were kept on a 12:12-hour light–dark cycle with free access to food and water. All experiments were conducted in accordance with institutional review board–approved protocols (Institutional Animal Care and Use Committee, UAB).

Reagents

Escherichia coli 0111:B4 endotoxin (LPS), hydrogen peroxide, dihydroethidium (DHE), rotenone, metformin, catalase, pegylated catalase (PEG-CAT) CoQ₁, NADPH, carbonyl cyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP), and thenoyltrifluoroacetone (TTFA) were obtained from Sigma (St. Louis, MO). 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) was purchased from Invitrogen (Carlsbad, CA). Cytokine ELISA kits were obtained from R&D Systems (Minneapolis, MN).

Neutrophil Isolation and Culture

Bone marrow neutrophils were isolated as described previously (15, 16). Briefly, bone marrow cell suspensions were isolated from the

femur and tibia of a mouse by flushing with RPMI 1640 medium/5% fetal bovine serum (FBS). The cell suspension was passed through a glass wool column and collected by subsequent washing with phosphate-buffered saline (PBS) containing 5% FBS. Negative selection to purify neutrophils was performed by incubation of the cell suspension with biotinylated primary antibodies specific for the cell surface markers F4/80, CD4, CD45R, CD5, and TER119 according to the manufacturer's procedure (www.stemcell.com/technical/13309-PIS.pdf; Stem Cell Technologies, Vancouver, BC, Canada) for 15 minutes at 4°C followed by subsequent incubation with anti-biotin tetrameric antibody (100 μ l) (Stem Cell Technologies) for 15 minutes. The complex of antitetrameric antibodies and cells was then incubated with colloidal magnetic dextran iron particles (60 μ l) (Stem Cell Technologies) for an additional 15 minutes at 4°C. The T cells, B cells, red blood cells, monocytes, and macrophages were captured in a column surrounded by a magnet, allowing the neutrophils to pass through. Neutrophil purity, as determined by Wright-Giemsa–stained cytopsin preparations, was consistently greater than 97%. The purified neutrophils were cultured in RPMI 1640 medium containing FBS (0.5%) and treated as described in the figure legends. Neutrophil viability after experimental treatments was determined using trypan blue staining and was consistently greater than 99%.

Alveolar Macrophage Isolation and Culture

Alveolar macrophages were isolated from the lungs of C57BL/6 mice by cannulating the trachea with a blunt 20-gauge needle and then lavaging the lungs five times with 1 ml of iced PBS/ethylenediaminetetraacetic acid (EDTA) (5 mM). Macrophage purity, as determined by Wright-Giemsa–stained cytopsin preparations, was consistently greater than 95%. Cells were washed two times with RPMI 1640/0.5% FBS, cultured in RPMI 1640 medium containing FBS (0.5%) for 2 hours, and then treated as described in the figure legends. Neutrophil viability

Figure 1. Rotenone inhibits mitochondrial respiratory complex I and induces dihydroethidium (DHE) and 2',7'-dichlorodihydrofluorescein (DCFH) oxidation in neutrophils. (A) Enzymatic activity of respiratory complex I in cell extracts (100 μ g protein/ml) was measured for a period of 5 minutes and then during the 5 minutes immediately after addition of rotenone (0, 1, or 10 μ M) ($\lambda = 340$ nm). The inhibition of complex I activity by rotenone was calculated as the % rate of nicotinamide adenine dinucleotide (NADH) oxidation obtained from control samples ($n = 3-4$, *** $P < 0.001$ when compared with control). (B) Representative confocal images of DHE or DCF fluorescence in neutrophils. In this experiment, cells were treated with rotenone (0 or 10 μ M) for 1 hour, loaded with DHE (10 μ M) or DCFH (10 μ M) for 30 minutes, and images were acquired using a laser scanning confocal microscope. (C, D) Mean DHE and DCF fluorescence was obtained from three or more randomly chosen fields of neutrophils treated with rotenone (0–10 μ M) and expressed as pixel intensity/cell (mean $\pm$ SD; *** $P < 0.001$ compared with untreated neutrophils).

after experimental treatment was determined using trypan blue staining and was consistently greater than 99%.

Assays of Respiratory Complex I and Citrate Synthase Activities

Respiratory complex I activity in neutrophils and in mitochondria-enriched fractions obtained from mouse lungs or livers was measured spectrophotometrically (UV-2501PC Shimadzu; Shimadzu, Scientific Instruments, Columbia, MD) and corrected by subtraction of the rotenone-insensitive rates (17). Briefly, neutrophils (30×10^6 cells/ml) or mitochondria-enriched fractions from lungs or livers were lysed in potassium/phosphate buffer by 10 repeated freeze-thaw cycles using liquid nitrogen. Complex I activity was then determined in potassium/phosphate buffer containing bovine serum albumin (BSA) (2.5 mg/ml), NADH (100 μ M), potassium cyanide (KCN) (1 mM), CoQ_1 (50 μ M), and 20–100 μ g of protein by following the rotenone-sensitive oxidation of NADH ($\lambda = 340$ nm) initiated by CoQ_1 (50 μ M). Data were acquired every 5 seconds for 5 minutes after initiation of the reaction, including 5 minutes after the addition of rotenone (0, 1, or 10 μ M) or metformin (0, 30, or 40 mM). Basal complex I activity was approximately 10 or approximately 30 $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ in samples obtained from lung or liver, respectively. Citrate synthase was measured by using the coupled reaction among oxaloacetate, acetyl-CoA, and 5,5'-dithiobis(2,4-nitrobenzoic acid) (18).

Imaging of Dichlorofluorescein and Dihydroethidium Fluorescence

Intracellular levels of hydrogen peroxide or superoxide were determined using the redox-sensitive probes DCFH-DA or dihydroethidium (DHE) in conjunction with fluorescent microscopy (19, 20). Briefly, neutrophils (1.5×10^6 /well) were incubated in a four-well chambered cover glass (Nalge, Naperville, IL), and treated with rotenone for 60 minutes or metformin for 2.5 hours. The cells were then incubated with DCFH-DA (10 μ M) or DHE (10 μ M) for 30 minutes and fluorescent

microscopic images acquired using double bidirectional scans of live neutrophils with a Leica DMIRBE inverted epifluorescence/Nomarski microscope outfitted with Leica TCS NT laser confocal optics (Leica, Inc., Exton, PA). The pinhole setting was 0.2 Airy units and laser excitation was set for 5% to avoid dye photooxidation. The levels of fluorescence were averaged using SimplePCI software (Compix, Cranberry Township, PA). Images were processed using IPLab Spectrum and Adobe Photoshop (Adobe Systems, San Jose, CA) software.

Isolation of Mitochondria

Liver and lung mitochondria were isolated according to standard procedures with minor modifications (21). Briefly, liver or lung homogenates were incubated in STE buffer containing sucrose (250 mM), Tris pH 7.4 (10 mM), and ethyleneglycol-bis-(β -aminoethyl ether)- N,N' -tetraacetic acid (EGTA) (2 mM), followed by centrifugation ($1,000 \times g$) for 3 minutes at 4°C. Supernatants were then collected and centrifuged ($10,000 \times g$) for 10 minutes at 4°C. The resultant pellets were suspended in STE buffer and centrifuged ($10,000 \times g$) for 10 minutes at 4°C. The pink and light brown pellets surrounding the dark brown center were aspirated and the remaining pellet suspended in STE buffer (1 ml) and centrifuged ($10,000 \times g$) for 15 minutes at 4°C. Finally, the cell pellet was suspended in potassium/phosphate buffer and stored at -80°C .

Purification of Nuclear Proteins

Nuclear proteins were purified from 7×10^6 neutrophils lysed in 100 μ l of buffer containing Tris pH 7.5 (10 mM), NaCl (10 mM), MgCl_2 (3 mM), Nonidet P40 (NP 40) (0.02%), EGTA (1 mM), sodium orthovanadate (1 mM), sodium fluoride (50 mM), and the protease inhibitors phenylmethylsulfonyl fluoride (100 μ M), leupeptin (10 μ g/ml), aprotinin (10 μ g/ml), pepstatin A (5 μ g/ml), and okadaic acid (1 nM). The lysed cells were centrifuged ($2,700 g$) for 10 minutes at 4°C. The supernatant containing the cytosol was collected and then centrifuged

Figure 2. Effects of rotenone on LPS-induced p65 (Ser-536) phosphorylation, p65 nuclear accumulation, and cytokine production by neutrophils. (A) Representative Western blots show rotenone-dependent inhibition of LPS-induced p65 phosphorylation and p65 nuclear accumulation. Neutrophils were incubated with rotenone (0 or 10 μ M) for 2 hours followed by culture with LPS (0 or 1 μ g/ml) for 1 hour. Nuclear extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis Western blot analysis with antibodies specific for phospho-p65 (Ser-536) or total p65. The Western blots show the levels of GAPDH (glyceraldehyde phosphate dehydrogenase), a purely cytoplasmic protein, and tubulin, a nuclear protein, in the cytosol and nuclear extracts, respectively. (B) Mean $\pm$ SD nuclear p65 band optical density ($n = 3$, $^{**}P < 0.01$). (C, D) Neutrophils were exposed to rotenone (0, 1, 3, or 10 μ M) for 2 hours followed by treatment with LPS (100 ng/ml) for 5 hours and then tumor necrosis factor (TNF)- α (C), and macrophage inflammatory protein (MIP)-2 (D) MIP-2 concentrations in the culture media were determined using ELISA assays ($n = 3$, mean $\pm$ SD; $^{**}P < 0.01$ or $^{***}P < 0.001$ compared with cells treated with LPS alone).

(20,800 g) for 15 minutes at 4°C to obtain the cytosolic fraction. The pellet containing nuclei was washed three times by gentle resuspension in 150 μ l wash buffer ([1,4-piperazinebis (ethane sulfonic acid)] pH 6.8 [10 mM], sucrose [300 mM], $MgCl_2$ [3 mM], EGTA [1 mM], NaCl [25 mM], sodium orthovanadate [1 mM], sodium fluoride [50 mM], and protease inhibitors), and centrifuged (2,700 g) for 5 minutes at 4°C. Nuclear proteins were obtained using nuclear extraction buffer (Tris pH 7.4 [50 mM], NaCl [150 mM], NP 40 [0.5%, vol/vol], EDTA [1 mM], EGTA [1 mM], okadaic acid [1 nM], and protease inhibitors). The nuclear lysates were then sonicated and centrifuged (10,000 $\times$ g) for 15 minutes at 4°C. The protein concentration of the supernatant was determined using Bradford reagent (BioRad, Hercules, CA) with BSA as a standard.

Western Blot Analysis

Briefly, neutrophils (3.5×10^6 /well), macrophages (2×10^5 /well), or isolated nuclei were lysed using buffer containing Tris pH 7.4 (50 mM), NaCl (150 mM), NP-40 (0.5%, vol/vol), EDTA (1 mM), EGTA (1 mM), okadaic acid (1 nM), and protease inhibitors. The cell lysates were sonicated and centrifuged at 10,000 $\times$ g for 15 minutes at 4°C. Protein concentration in the supernatants was determined using the Bradford reagent (BioRad) with BSA as a standard. Samples were mixed with Laemmli sample buffer and boiled for 5 minutes. Equal amounts of proteins were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred onto polyvinylidene fluoride (PVDF) membranes (Immobilon P; Millipore, Billerica, MA). The membranes were probed with specific antibodies to I κ B- α (Cell Signaling, Beverly, MA), p65 (Santa Cruz Biotechnology, Santa Cruz, CA), or actin (Sigma), followed by detection with horseradish peroxidase-conjugated goat anti-rabbit IgG. Bands were visualized by enhanced chemiluminescence (Super Signal; Pierce Biotechnology, Rockford, IL) and quantified by AlphaEaseFC software (Alpha Inno-

tech, San Leandro, CA). Each experiment was carried out two or more times using cell populations obtained from separate groups of mice.

In Vivo Acute Lung Injury Model

To induce acute lung injury, *Escherichia coli* 0111:B4 LPS (1 mg/kg) in PBS was administered into the oropharynx (22). Briefly, mice were anesthetized with isoflurane, the tongue was gently extended, and LPS in PBS (50 μ l) was instilled into the distal part of the oropharynx.

In pretreatment experiments, mice were given rotenone (8 mg/kg) in saline/dimethyl sulfoxide (1:1) or saline/dimethyl sulfoxide injection intraperitoneally 4 hours before intratracheal LPS administration. Previously, a similar dosage of rotenone was reported in C57BL/6 mice (23). Metformin (250 mg/kg) in saline or saline alone (200 μ l) was injected (intraperitoneally) starting 4 hours before LPS administration. In additional experiments, metformin (125 mg/kg) in saline or saline alone was given (intraperitoneally) 30 minutes and 8 hours after oropharyngeal administration of LPS. These doses of metformin were previously used in murine models examining efficacy or toxicity of metformin (24–26). There were no deaths associated with rotenone, metformin, or LPS administration.

Harvest of Lungs and BAL

Lungs were harvested 24 hours after LPS administration. BAL fluid was obtained by cannulating the trachea with a blunt 20-gauge needle and then lavaging the lungs three times with 1 ml of iced PBS.

Wet-to-Dry Lung Weight Ratios

The wet-to-dry ratio was determined as reported previously (27). Separate groups of mice were used to measure wet-to-dry ratios and for BAL fluid acquisition. All mice used for lung wet-to-dry weight ratios were of identical ages. Lungs were excised, rinsed briefly in PBS, blotted, and then weighed to obtain the “wet” weight. Lungs were then dried in an oven at 80°C for 7 days to obtain the “dry” weight.

Figure 3. Rotenone inhibits LPS-mediated NF- κ B inhibitory subunit (I κ B)- α degradation in neutrophils. (A) A representative Western blot shows dose-dependent inhibition of LPS-induced I κ B- α degradation by rotenone. Neutrophils were incubated with rotenone (0–10 μ M) for 2 hours and then cultured with LPS (0 or 1 μ g/ml) for 60 minutes. Cell lysates were subjected to SDS-PAGE and probed with I κ B- α or actin-specific antibodies. (B) Mean $\pm$ SD optical densitometry from three independent experiments. Data are expressed as I κ B- α fold increase compared with cells treated with LPS alone ($*P < 0.05$). (C) Neutrophils were pretreated with rotenone (0 or 10 μ M) for 1 hour, and time-dependent degradation of I κ B- α was determined during the 60 minutes after addition of LPS (1 μ g/ml) to the cultures. A second experiment provided similar results. (D, E) Neutrophils were treated with thenoyltrifluoroacetone (TTFA) (0 or 10 μ M), carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) (0 or 1 μ M), catalase (CAT) (0 or 600 U/ml) or pegylated catalase (PEG-CAT) (125 U/ml) for 60 minutes followed by stimulation with LPS (1 μ g/ml). Representative Western blots of I κ B- α and actin are shown. Two additional experiments provided similar results. (F) Mean $\pm$ SD optical densitometry from experiments described in (E). Data are expressed as I κ B- α fold control (untreated). ($***P < 0.01$) compared with cells treated with LPS alone.

Myeloperoxidase Assay

Myeloperoxidase activity was assayed as reported previously with minor modifications (28). In brief, lung tissue was homogenized in 1 ml of potassium phosphate buffer, pH 6.0 (50 mM), containing a reducing agent, N-ethylmaleimide (10 mM), for 30 seconds on ice. The homogenate was centrifuged (12,000 g) for 30 minutes at 4°C and washed twice in ice-cold buffer. The pellet was then resuspended and sonicated on ice for 90 seconds in $\times 10$ volume of hexadecyltrimethylammonium bromide (HTAB) buffer (HTAB [0.5%], potassium phosphate pH 6.0 [50 mM]). Samples were incubated in a water bath for 2 hours at 56°C and then centrifuged (12,000 g) for 10 minutes. The supernatant was collected for assay of myeloperoxidase activity as determined by measuring the H_2O_2 -dependent oxidation of 3,3'-dimethoxybenzidine dihydrochloride ($\lambda = 460$ nm).

Cytokine ELISA

ELISAs were used to measure cytokines in BAL fluid or in culture media from LPS-stimulated neutrophils. Levels of tumor necrosis factor (TNF)- α , macrophage inflammatory protein (MIP)-2, IL-6, or keratinocyte-derived chemokine (KC) were determined using commercially available ELISA kits (R&D Systems), according to the manufacturer's instructions and as previously described (15, 16).

Measurement of Glucose in Plasma

Glucose content in blood plasma was determined using an Accu-Chek Advantage glucometer (model 768; Boehringer Mannheim, Nutley, NJ).

Histochemistry

Mice were killed and 1 ml of paraformaldehyde (10%) was injected into the lungs by cannulating the trachea with a blunt 20-gauge needle.

The lungs were excised, rinsed briefly in PBS, blotted, and incubated in 10% paraformaldehyde for 24 hours followed by paraffin embedding and processing for hematoxylin-eosin staining.

Statistical Analyses

For each experiment, neutrophils were isolated and pooled from groups of mice ($n = 3-6$) and all conditions were studied at the same time. One-way analysis of variance, the Tukey-Kramer multiple comparisons test (for multiple groups), or Student's t test (for comparisons between two groups) was used. $P < 0.05$ was considered to be statistically significant.

RESULTS

Rotenone Inhibits Mitochondrial Complex I and Increases ROS Generation by Neutrophils

Rotenone is a potent inhibitor of mitochondrial respiratory complex I and increases superoxide production by this organelle in many types of cells (29, 30). However, whether rotenone can inhibit complex I and alter mitochondrially generated ROS production in neutrophils has not previously been investigated. Therefore, the effect of rotenone on complex I activity was measured in neutrophil extracts by following rotenone-sensitive oxidation of NADH initiated by CoQ_1 . Figure 1A shows that neutrophils possess active complex I that is dose-dependently inhibited by rotenone.

Because rotenone potently inhibited mitochondrial complex I activity in neutrophils, we next examined the role of complex I on ROS production. To detect superoxide generation, neutrophils

Figure 4. Effects of metformin on complex I activity and dihydroethidium (DHE) or 2',7'-dichlorodihydrofluorescein (DCFH) oxidation in neutrophils. (A) Mitochondrial complex I activity was determined in control cell extracts (100 μ g/ml) and after addition of metformin (0, 30, or 40 mM). Complex I activity was calculated as the % rate of NADH oxidation obtained from control samples (* $P < 0.05$ compared with control). (B, C) Representative images and averages of DHE or DCF fluorescence of neutrophils incubated with metformin (500 μ M) for 2.5 hours or left untreated (control). Mean DHE or DCF fluorescence was obtained from three or more randomly chosen fields. Data are shown as a pixel intensity/cell (mean $\pm$ SD, ** $P < 0.01$ compared with control).

were treated with rotenone and then loaded with the cell-permeable probe DHE (10 μ M). As shown in Figures 1B and 1C, treatment of neutrophils with rotenone resulted in a robust increase in DHE fluorescence. Consistent with the rotenone-induced increase in superoxide production, there were also elevated levels of intracellular hydrogen peroxide, as determined by the probe DCFH-DA, in rotenone-treated neutrophils (Figures 1B and 1D).

Prolonged inhibition of mitochondrial complex I by rotenone has been shown to induce apoptosis in nonmyelocytic cell populations (30–33). In contrast, rotenone did not induce apoptosis of human neutrophils (34). Cell death after exposure of mouse neutrophils to rotenone (10 μ M) for 6 hours was consistently less than 2%, as determined by trypan blue exclusion assay (data not shown).

Rotenone-dependent Inhibition of Complex I Alters LPS-induced Nuclear Translocation of NF- κ B and Cytokine Production by Neutrophils

Mitochondrially generated ROS participate in the regulation of multiple signal transduction pathways (19, 24, 35, 36), including NF- κ B activation (37). Because rotenone treatment caused an increase in intracellular levels of both superoxide and hydrogen peroxide (Figures 1B, 1C, and 1D), we determined if LPS-mediated phosphorylation of the NF- κ B p65 subunit, p65 nuclear accumulation, and proinflammatory cytokine production were altered in neutrophils exposed to rotenone. As shown in Figures 2A and 2B, culture of neutrophils with LPS resulted in

increased levels of Ser-536 phosphorylation and nuclear accumulation of the NF- κ B p65 subunit, whereas rotenone pretreatment inhibited these effects. Moreover, exposure of neutrophils to rotenone was associated with a dose-dependent decrease in LPS-induced secretion of the NF- κ B-dependent cytokine TNF- α as well as release of MIP-2, a CXC chemokine whose transcription is also regulated by NF- κ B (38) (Figures 2C and 2D). Rotenone alone had no effect on NF- κ B nuclear accumulation and cytokine production by neutrophils.

Because I κ B- α has a central role in maintaining cytoplasmic sequestration of NF- κ B (39, 40), we examined the effect of rotenone on I κ B- α levels in neutrophils. As shown in Figures 3A–3D, rotenone inhibited LPS-mediated I κ B- α degradation. In contrast, treatment of neutrophils with thenoyltrifluoroacetone (TTFA), a specific inhibitor of complex II (41, 42), had no effect.

Inhibition of the mitochondrial electron chain can affect ATP generation and result in inefficient I κ B- α phosphorylation and degradation (43). To examine this possibility, cells were treated with carbonyl cyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP), an uncoupler of mitochondrial respiration that results in inhibition of mitochondrial ATP synthesis (44). As shown in Figure 3D, pretreatment of neutrophils with FCCP (5 μ M) for 60 minutes had no effect on LPS-induced I κ B- α degradation. This result indicates that the decrease in mitochondrially generated ATP associated with complex I inhibition is not responsible for effects on I κ B- α degradation.

To further explore whether the ROS generated by complex I inhibition are responsible for modulation of I κ B- α degradation,

Figure 5. Metformin inhibits LPS-mediated p65 (Ser-536) phosphorylation, nuclear factor (NF)- κ B nuclear translocation, and cytokine production by neutrophils. Neutrophils were incubated with metformin (0 or 250 μ M) for 2.5 hours followed by treatment with or without LPS (1 μ g/ml) for 60 minutes. (A) Representative Western blots of phospho-p65 (pSer-536), total p65 obtained from whole cell lysates, and nuclear levels of p65. (B) mean $\pm$ SD optical band densities for the levels of nuclear p65 ($n = 3$, $^{**}P < 0.01$ comparing LPS-treated neutrophils with untreated controls, and $^{*}P < 0.05$ for the comparison with LPS- and metformin-treated cells). (C, D) Metformin (0 or 250 μ M) was added to neutrophil cultures for 2.5 hours, followed by treatment with LPS (100 ng/ml) for 5 hours. TNF- α and MIP-2 levels in the supernatants are shown ($n = 3$, mean $\pm$ SD, $^{***}P < 0.001$ for LPS-treated neutrophils compared with untreated control cells or for LPS-treated neutrophils compared with cells incubated with both metformin and LPS).

neutrophils were pretreated with catalase or PEG-CAT and then cultured with rotenone and LPS. Of note, PEG-CAT, unlike unmodified catalase, has been demonstrated to transit rapidly to the intracellular space and participate in the dismutation of mitochondrially derived H_2O_2 (45, 46). As shown in Figures 3E and 3F, treatment of neutrophils with PEG-CAT, but not catalase, abolished the inhibitory effects of rotenone on LPS-induced $I\kappa B-\alpha$ degradation. These results suggest that the mechanism through which mitochondrially derived ROS, and specifically H_2O_2 , inhibit the nuclear translocation of NF- κB and expression of NF- κB -dependent cytokines is by preventing $I\kappa B-\alpha$ degradation with resultant cytosolic retention of NF- κB .

Metformin Inhibits Complex I Activity and LPS-induced Neutrophil Activation

Metformin, a biguanide frequently used in the treatment of diabetes, has been shown to block complex I, increase ROS production by mitochondria, and alter intracellular signaling pathways (24), but its activity on Toll-like receptor/IL-1 receptor (TLR/IL-1R)-induced cellular activation has not been explored in neutrophils. As shown in Figure 4A, addition of metformin to neutrophil cell extracts significantly inhibits complex I activity.

Metformin increased DCF and DHE fluorescence in neutrophils (Figures 4B and 4C), indicating that metformin increases intracellular levels of ROS. In a similar manner to rotenone, metformin decreased LPS-induced Ser-536 phosphorylation of the NF- κB p65 subunit, nuclear accumulation of NF- κB , and cytokine production (Figure 5). In addition, metformin dose-dependently prevented degradation of $I\kappa B-\alpha$ in LPS-stimulated neutrophils (Figures 6A–6C). This effect of metformin was reversed by preincubation of neutrophils with PEG-CAT, demonstrating that it was specific for hydrogen peroxide (Figure 6D).

The inhibitory effects of complex I inhibition by rotenone (Figure 3) or metformin (Figures 6A–6D) on $I\kappa B-\alpha$ degradation

were not limited to neutrophils, as similar findings were present in LPS-stimulated alveolar macrophages (Figure 6E).

Rotenone Pretreatment Attenuates LPS-induced Acute Lung Injury

To examine the effects of mitochondrial complex I inhibition on LPS-induced acute lung injury, mice were pretreated with rotenone 4 hours before LPS administration. Rotenone administration resulted in significant inhibition ($\sim 30\%$) of complex I activity in enriched mitochondrial fractions from lung homogenates (Figure 7A). To ensure that the reduction in complex I activity was due to rotenone specific effects and was not an artifact of rotenone affecting the mitochondrial purification process, mitochondrial citrate synthase activity was measured. In previous studies (47, 48), mitochondrial citrate synthase activity has been shown to be resistant to oxidant-induced inactivation and therefore has been used as an indicator of mitochondrial content in enriched fractions. As shown in Figure 7, no alterations of citrate synthase activity were found in mitochondrial fractions from rotenone-treated as compared with vehicle-treated mice.

Rotenone-treated mice had less severe lung injury than those given vehicle alone before LPS. In particular, wet-to-dry ratios, pulmonary myeloperoxidase concentrations, and neutrophil numbers in BAL fluid were significantly reduced in mice that received rotenone before LPS administration (Figure 7B). There were no significant differences in wet-to-dry ratios between control mice given vehicle or rotenone alone, without LPS administration (data not shown). Pulmonary and BAL concentrations of MIP-2, TNF- α , IL-6, and KC were also significantly decreased in mice pretreated with rotenone before LPS instillation (Figure 7C).

Metformin Prevents LPS-induced Acute Lung Injury

Because a presumed mechanism of metformin's metabolic actions *in vivo* is inhibition of respiratory complex I (14), complex I

Figure 6. Effects of metformin on LPS-induced $I\kappa B-\alpha$ degradation. (A, B) A representative Western blot and optical densitometry (mean $\pm$ SD) of $I\kappa B-\alpha$ and actin from neutrophils incubated with metformin (0–500 μM) for 2.5 hours followed by LPS (0 or 1 $\mu g/ml$) for 60 minutes. Data are expressed as $I\kappa B-\alpha$ fold increase compared with cells treated with LPS alone ($n = 3$, $*P < 0.05$). (C) Effects of metformin on LPS-dependent degradation of $I\kappa B-\alpha$. Cells were preincubated with metformin (0 or 500 μM) for 2.5 hours followed by LPS (1 $\mu g/ml$) treatment for the indicated time period. A representative Western blot of $I\kappa B-\alpha$ and actin is shown. (D) Effects of pegylated catalase (PEG-CAT) on metformin-dependent inhibition of LPS-induced $I\kappa B-\alpha$ degradation. Neutrophils were incubated with PEG-CAT (0 or 125 U/ml) and metformin (0 or 500 μM) for 2.5 hours followed by treatment with LPS (0 or 1 $\mu g/ml$) for 60 minutes. (E) Effects of metformin or rotenone on LPS-mediated $I\kappa B-\alpha$ degradation in alveolar macrophages. Representative Western blots show the level of $I\kappa B-\alpha$ in cell extracts obtained from macrophages treated with metformin (0 or 500 μM) for 2.5 hours or rotenone (0 or 10 μM) for 60 minutes followed by exposure to LPS (0 or 100 ng/ml) for 1 hour. A second experiment provided similar results.

Figure 7. Effects of rotenone on LPS-induced acute lung injury. (A) Complex I and citrate synthase activity were measured in mitochondria-enriched fractions obtained from lungs of C57BL/6 mice treated with rotenone (0 or 8 mg/kg intraperitoneally) 24 hours previously ($n = 3$, means $\pm$ SEM, $**P < 0.01$ compared with control). (B) Rotenone treatment diminishes the severity of LPS-induced acute lung injury. Mice were pretreated with rotenone (8 mg/kg intraperitoneally) or saline and then 4 hours later were given LPS (1 mg/kg) intratracheally. Lung wet-to-dry ratios, myeloperoxidase (MPO) concentrations, total cell or neutrophil numbers in bronchoalveolar lavage (BAL) fluid, and cytokines in BAL fluids (C) were determined 24 hours after LPS administration. Fold increase above values present in mice receiving saline alone are shown in case of lung wet-to-dry ratios ($n = 6$, means $\pm$ SEM, $*P < 0.05$ comparing LPS-treated mice with mice given metformin and LPS). KC = keratinocyte-derived chemokine; MIP-2 = macrophage inflammatory protein-2.

activity was measured in mitochondria-enriched fractions obtained from murine liver and lungs. In hepatic extracts, basal complex I activity was approximately $30 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$, and there was nearly complete inhibition in the presence of rotenone (Figure E1A of the online supplement). Metformin, when given to unmanipulated mice or when administered to LPS-treated mice either before or after LPS administration, decreased complex I activity in the liver and lungs (Figure 8A and Figure E1B), demonstrating *in vivo* inhibitory effects on complex I.

The severity of lung injury, as determined by lung edema and neutrophil accumulation into the pulmonary interstitium and airspaces, was significantly reduced in mice that received metformin starting either before (Figure E2) or after administration of LPS (Figures 8A and 8B). Levels of cytokines and

chemokines, specifically MIP-2 and IL-6, were also significantly decreased in the lungs of mice that had received metformin after LPS administration (Figure 8C). Similar results were obtained from mice given metformin before LPS injection (Figure E3). There were no significant differences in the wet-to-dry ratios between control mice given saline or metformin alone, without LPS administration (data not shown). As shown in Figure 9, there was decreased LPS-induced neutrophil accumulation and interstitial edema in metformin-treated mice as compared with those given saline.

Recent studies in critically ill patients have shown that strict control of circulating glucose levels results in better clinical outcomes and fewer complications (49, 50). Because metformin is used to reduce elevated serum glucose levels in patients with type 2 diabetes, it is possible that its bene-

Figure 8. Metformin treatment diminishes the severity of LPS-induced acute lung injury. C57BL/6 mice were given LPS (1 mg/kg) intratracheally followed by vehicle intraperitoneally 0.5 and 8 hours post-LPS, or two doses of metformin (125 mg/kg intraperitoneally) given 0.5 and 8 hours post-LPS. The lungs were obtained for mitochondrial purification 24 hours after LPS administration ($n = 3$, means $\pm$ SEM, $**P < 0.01$ compared with LPS alone). (A) Effects of metformin on complex I activity *in vivo*. Complex I and citrate synthase activity was measured in mitochondria-enriched fractions obtained from lungs of LPS- or LPS/metformin-treated mice. (B) shows lung wet-to-dry ratios, myeloperoxidase (MPO) concentrations and total cell or neutrophil numbers in bronchoalveolar lavage (BAL) fluid determined 24 hours after LPS administration. Fold increase above values present in mice receiving saline alone are shown for lung wet-to-dry ratios ($n = 6$ mice in each experimental group, means $\pm$ SEM, $*P < 0.05$ or $**P < 0.01$ comparing LPS-treated mice with mice given metformin and LPS). (C) TNF- α , MIP-2, IL-6, and KC concentrations measured in BAL fluids 24 hours post-LPS administration ($n = 6$ mice in each experimental group, means $\pm$ SEM, $*P < 0.05$ compared with LPS alone).

ficial effects on acute lung injury were due to actions in controlling glucose levels rather than on complex I. However, LPS-exposed mice treated with metformin had higher circulating glucose concentrations than did those in the control group, where there was a significant decrease ($\sim 40\%$) in plasma glucose 24 hours after LPS administration (control, 385 ± 26 mg/dl; LPS, 216 ± 32 mg/dl; metformin/LPS, 345 ± 34 mg/dl). Moreover, in mice not given LPS, metformin had no effects on plasma glucose levels (metformin, 374 ± 11 mg/dl).

DISCUSSION

Complex I, also known as NADH-ubiquinone oxidoreductase, is the first of four electron carriers in the mitochondrial transport chain and, with complex III, is a major site of mitochondrial superoxide production (3). Under normal metabolic conditions, approximately 5% of electrons can escape from the mitochondrial electron chain and generate superoxide in the

presence of oxygen. Although increased production of superoxide from complex I has been postulated to occur as a result of reverse electron flux (4), the precise mechanism of complex I-mediated superoxide formation has not been fully elucidated.

The present studies show that inhibition of mitochondrial respiratory complex I in neutrophils is associated with profound effects on TLR4-induced neutrophil function, including increases in intracellular ROS levels as well as diminished NF- κ B activation and proinflammatory cytokine production. Although inhibition of respiratory complex I, with associated increase in ROS production, has been demonstrated using isolated mitochondria, in cell culture models and *in vivo* (3, 4, 24, 51), mitochondrial complex I has not previously been shown to be involved in the regulation of intracellular levels of ROS in neutrophils. In these experiments, both rotenone and metformin inhibited complex I activity and exposure to either of these agents produced rapid increases in superoxide and H_2O_2 concentrations in neutrophils. Inhibition of complex I was also found in the lungs of mice treated with metformin or rotenone.

Figure 9. Effects of metformin on LPS-induced neutrophil accumulation in the lungs. Representative histologic sections show decrease in the accumulation of interstitial and intraalveolar neutrophils in LPS/metformin-treated mice compared with mice given LPS and vehicle. Hematoxylin-eosin staining was performed using lungs obtained 24 hours after LPS administration from mice treated with LPS (1 mg/kg, intratracheally) and vehicle 0.5 and 8 hours post-LPS or from mice treated with metformin (125 mg/kg, intraperitoneally) administered 0.5 and 8 hours post-LPS.

A possible mechanism for the antiinflammatory effects of complex I blockade may be through increasing intracellular concentrations of H_2O_2 . Superoxide produced by complex I is predominantly generated in the mitochondrial matrix and, as a charged species, superoxide is not readily diffusible across mitochondrial membranes. However, superoxide rapidly undergoes dismutation to H_2O_2 within the mitochondria and, in contrast to superoxide, H_2O_2 is capable of diffusing across membranes and can transit from the mitochondria into the cytoplasm where it participates as a second messenger in the regulation of NF- κ B and other intracellular signaling pathways. Previous studies, including those from our laboratory, have demonstrated that exposure of neutrophils to extracellular H_2O_2 , which is associated with rapid increases in its intracellular concentrations, resulted in inhibition of LPS-induced I κ B- α degradation, NF- κ B activation, and expression of NF- κ B-dependent cytokines, such as TNF- α , MIP-2, and IL-8 (11, 15, 52). In the present experiments, treatment of LPS-stimulated neutrophils with PEG-CAT abrogated the inhibitory effects of rotenone or metformin on I κ B- α degradation. These results are consistent with recent reports showing that PEG-CAT decreases intracellular levels of H_2O_2 , including mitochondrially derived H_2O_2 (45, 46).

Inhibition of complex I activity, in addition to enhancing mitochondrial ROS generation, also results in decreased ATP production by mitochondria and subsequent effects on ATP-dependent intracellular processes. Although such inhibition of mitochondrial ATP generation could affect I κ B- α degradation, our experiments found that rotenone or metformin, but not FCCP, a known uncoupler of mitochondrial respiration, inhibited LPS-induced I κ B- α degradation. These results indicate that enhanced mitochondrial ROS, and not suppression of ATP generation, are responsible for the inhibitory effects of complex I blockade on LPS-induced I κ B- α degradation, nuclear translocation of NF- κ B, and expression of NF- κ B-dependent proinflammatory cytokines. In addition, we examined whether other components of the mitochondrial respiratory sequence could also affect I κ B- α degradation in a manner similar to that observed with complex I inhibition. However, inhibition of complex II with TTFA had no effects on LPS-induced I κ B- α degradation, indicating that inhibition of complex I has specific effects not generalizable to other ROS-generating mitochondrial components.

Several steps in the NF- κ B signaling cascade have been shown to be regulated by H_2O_2 . H_2O_2 -dependent increases in S-glutathionylation of IKK β (53), oxidation of I κ B- α at methionine 45 (54), cysteine thiol oxidation of the p50 subunit of NF- κ B (55), as well as inhibition of proteasome-dependent degradation of I κ B- α result in diminished NF- κ B signaling. However,

not all of these inhibitory effects of H_2O_2 have been found in all cell types (56–58). For example, the ability of H_2O_2 to reduce LPS-induced proteasomal activity, which was recently described in neutrophils, was not present in macrophages (11). Nevertheless, in the present studies, blockade of complex I with rotenone or metformin had similar inhibitory effects on I κ B- α degradation in LPS-stimulated alveolar macrophages and neutrophils, suggesting that this is a general antiinflammatory mechanism. These results also suggest that effects of rotenone and metformin on pulmonary cell populations other than infiltrating neutrophils, such as alveolar macrophages, may contribute to the benefit of complex I inhibition in acute lung injury.

In the present studies, rotenone and metformin reduced the severity of LPS-induced acute lung injury, an inflammatory process in which activated neutrophils that infiltrate the pulmonary interstitium and migrate into the airways play an important role (27, 59). Although rotenone has been shown to effectively block respiratory complex I, its *in vivo* use is limited by dopaminergic neuron injury followed by the appearance of Parkinson-like symptoms in rodent models as well as in humans. In these experiments, metformin had beneficial effects even when administered after exposure of the lungs to LPS, suggesting that the role of mitochondrially derived ROS in contributing to pulmonary injury is not limited to the immediate period after TLR4 engagement. Metformin is commonly used in patients with diabetes (13) and complex I inhibition has been suggested as a central mechanism for its beneficial effect in this clinical setting (14). Metformin has a broad spectrum of effects that include improving insulin-mediated glucose metabolism, inhibition of adipose tissue lipolysis, reduction in circulating free fatty acids, and diminished very low density lipoprotein production. However, little is known concerning the effects of metformin on acute inflammatory processes. Recently, metformin has been shown to prevent LPS-induced liver dysfunction after partial hepatectomy, including the accumulation of neutrophils into the liver as well as elevations in hepatic levels of IL-6 and IFN- γ (25). Similarly, metformin reduced hepatic inflammation in animal models of nonalcoholic steatohepatitis as well as in humans with this condition (60, 61). Metformin has also been shown to decrease the proinflammatory response of human umbilical vein endothelial cells when exposed to cytokines *in vitro* (62). Of note, despite its known effects on glucose metabolism, mice treated with metformin in these experiments had higher plasma glucose levels than did those receiving placebo, indicating that the antiinflammatory effects of metformin were not due to improved glucose control in the treated animals.

The inhibitory effects of metformin on complex I activity appear to be related to its time-dependent accumulation in

mitochondria, where millimolar ranges of concentration may be achieved (14). Although metformin has been shown to have both direct and indirect effects on complex I activity, the primary mechanisms responsible are not fully characterized (14, 63). Nevertheless, the present studies demonstrate that mitochondrial complex I activation plays an important role in modulating LPS-mediated neutrophil activation, degradation of I κ B- α , nuclear translocation of NF- κ B, and neutrophil-dependent cytokine production, as well as inflammatory processes, such as acute lung injury, in which activated neutrophils play a major role. Metformin is a safe and widely used agent for the therapy of non-insulin-requiring diabetes. The present results suggest that metformin, as well as other agents that inhibit mitochondrial complex I activity, may be useful in the prevention or treatment of acute inflammatory processes, such as acute lung injury.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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