Lipoplysaccharide Stimulates Mitochondrial Biogenesis via Activation of Nuclear Respiratory Factor-1*

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Exposure to bacterial lipopolysaccharide (LPS) in vivo damages mitochondrial DNA (mtDNA) and interferes with mitochondrial transcription and oxidative phosphorylation (OXPHOS). Because this damage accompanies oxidative stress and is reversible, we postulated that LPS stimulates mtDNA replication and mitochondrial biogenesis via expression of factors responsive to reactive oxygen species, i.e. nuclear respiratory factor-1 (NRF-1) and mitochondrial transcription factor-A. In testing this hypothesis in rat liver, we found that LPS induces NRF-1 protein expression and activity accompanied by mRNA expression for mitochondrial transcription factor-A, mtDNA polymerase γ, NRF-2, and single-stranded DNA-binding protein. These events restored the loss in mtDNA copy number and OXPHOS gene expression caused by LPS and increased hepatocyte mitotic index, nuclear cyclin D1 translocation, and phosphorylation of pro-survival kinase, Akt. Thus, NRF-1 was implicated in oxidant-mediated mitochondrial biogenesis to provide OXPHOS for proliferation. This implication was tested in novel mtDNA-deficient cells generated from rat hepatoma cells that overexpress NRF-1. Depletion of mtDNA (100 clones) diminished oxidant production and caused loss of NRF-1 expression and growth delay. NRF-1 expression and growth were restored by exogenous oxidant exposure indicating that oxidative stress stimulates biogenesis in part via NRF-1 activation and corresponding to recovery events after LPS-induced liver damage.

Lipoplysaccharide (LPS) produces deleterious effects on mitochondria in the liver through reactive oxygen species (ROS) generation (1, 2), glutathione depletion (3, 4), mtDNA damage (4), and impairment of OXPHOS (5). These effects are associated with early receptor-mediated cytokine responses, e.g. production of interleukin-1 and tumor necrosis factor-α (TNF-α), which initiate the early immune response (6). The hepatic response to LPS also includes activation of genes of mitochondrial recovery (4). Of particular interest is that TNF-α accelerates ROS production by mitochondria (7, 8), and exogenous ROS mimic aspects of TNF-α activity (9, 10). Furthermore, low TNF-α levels typically induce hepatocytes proliferation but not cell death (11, 12), whereas intense TNF receptor-1 stimulation produces cytotoxicity (13).

Mitochondria play crucial roles both in cell survival and death, and mitochondrial damage may initiate apoptosis, necrosis, or both. Mitochondria rely on an intrinsic genome (mtDNA) that is replicated and transcribed semi-autonomously but whose maintenance requires nuclear factors. The 16-kbp circular mtDNA contains limited genetic information relative to nuclear DNA; it encodes two rRNAs, 22 tRNAs, and 13 polypeptides of the electron transport chain (ETC). Mitochondrial transcription is regulated by mitochondrial transcription factor A (mtTFA) that contains domains for high mobility group-box proteins critical for DNA promoter binding and transcriptional activation. Nuclear high mobility group-box proteins enhance transcription and structural organization of chromatin and exhibit dual DNA binding ability (14, 15). mtTFA initiates transcription by binding to mtDNA upstream of control elements of both heavy and light strand promoters (16). The mtTFA knockout mouse shows mtDNA depletion and lack of OXPHOS, and dies early in embryogenesis, establishing the role of mtTFA in coordinating mitochondrial-nuclear genomic activity and regulation of mtDNA copy number (17).

Regulation of mtTFA transcription is not well understood, but loss of mitochondrial transcription inhibits cell growth because of lack of energy for cell replication. Two redox-responsive transcription factors, nuclear respiratory factors-1 and -2 (NRF-1 and NRF-2), have been implicated in its expression (18, 19), as well as that of several ETC proteins (18, 20). NRF-1 is phosphorylated, translocates to the nucleus, and binds to DNA as a homodimer. Because NRF-1 is encoded in the nucleus but controls synthesis of ETC components, it is a candidate for linking nuclear and mitochondrial genome activity during repair of damage to both mitochondria and cells. A role for NRF-1 in the duality of mtTFA function in mtDNA replication and transcription has not been explored in the context of LPS damage, which led to the present investigation. The implication is that elucidating a role for NRF-1 in communication between nucleus and mitochondrion after LPS-induced mitochondrial damage could provide new insights into the molecular mechanisms underlying a major clinical problem, organ failure of severe sepsis.
MATERIALS AND METHODS

**Animal Procedures**

Animal protocols were approved by the Duke University Animal Care and Use Committee. Male Sprague-Dawley rats weighing 300–400 g were injected with a single dose of intraperitoneal LPS (Escherichia coli, 055 B5; Difco, Inc.) dissolved in 1 ml of sterile pyrogen-free 0.9% sodium chloride at a sub-lethal dose (1 mg/kg) chosen for significant but reversible hepatic injury (4). Control animals were injected with an equal volume of 0.9% sodium chloride. Animals were killed 6, 24, and 48 h after injection, the abdomen was opened, and the livers were excised quickly and placed in cold isolation buffer. Fresh liver (4 g) was used to isolate mitochondria, and the remainder was snap-frozen in 2% paraformaldehyde at 4 °C until paraffin embedding, sectioning, and staining for light microscopy.

**Mitochondrial Isolation and Cell Fractionation**

Highly purified, tightly coupled liver mitochondria were prepared as reported by discontinuous gradient centrifugation (1–4). Nuclei were obtained by centrifugation of liver homogenate at 1000 × g for 20 min and further purified by centrifugation through 1.75 M sucrose. Protein concentration was measured by optical density. mtDNA was purified by centrifugation through 1.75 M sucrose. A DNA fragment of 390 bp was used to isolate mitochondria, and the remainder was snap-frozen in 2% paraformaldehyde and stored at −80 °C. For microscopy livers were flushed with phosphate-buffered saline, pH 7.0, and perfusion-fixed with 4% paraformaldehyde. The livers were removed, immersed in fixative for 2 h, and stored in 2% paraformaldehyde at 4 °C until paraformin embedding, sectioning, and staining for light microscopy.

**Cytoplasmic RNA was extracted with TRIzol (Invitrogen), and 1 μg from each sample was reverse-transcribed (in a total volume of 20 μl) using Moloney murine leukemia virus reverse transcriptase (180 units; Promega) in a reaction buffer containing random hexamer primers, dNTPs, and ribonuclease inhibitor RNasin (Promega). Gene transcripts were amplified in triplicate using gene specific primers (Table I). 18 S rRNA was used to control for variation in efficiency of RNA extraction, reverse transcription, and PCR for nuclear and mitochondrial mRNA gene expression. Quantification of amplified mRNA was done by densitometry normalized to the 18 S rRNA mRNA signal density for each sample using image analysis software (Bio-Rad).

**Cell Lines and mtDNA Depletion**

Rat hepatoma cells obtained from American Type Culture Collection (H4IIE; Manassas, VA) were characterized in Dulbecco’s modified Eagle’s medium supplemented with 4.5 mg/ml glucose, 50 mg/ml uridine, and 100 mg/ml pyruvate to compensate for loss of respiration. Experiments were performed on cells 24 h post-confluence. One h before adding test agents cells were switched to phenol red-free Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum at 37 °C with 5% CO2. To deplete cells of mtDNA (ρ0 cells) normal H4IIE cells were cultured for 60 or more passages in the presence of 20 μg/ml EB in Dulbecco’s modified Eagle’s medium supplemented with 4.5 mg/ml glucose, 50 mg/ml uridine, and 100 mg/ml pyruvate to compensate for loss of respiration. Experiments were performed on cells 24 h post-confluence. One h before adding test agents cells were switched to phenol red-free Dulbecco’s modified Eagle’s medium with 0.5% fetal bovine serum (Invitrogen). Oxidative damage to mtDNA wild type and mtDNA-deficient (ΔH4IIE) cells was produced with tertiary butyl hydroperoxide (t-BH). The t-BH (10–100 μM) diluted in serum-free medium was added to confluent monolayers in 6-well plates. Viability was assessed after t-BH release, staining with 0.4% trypan blue dye, and counting non-viable and total cells on a hemocytometer. PI3K inhibitor LY294002 was used at 50 μM. All measurements were performed in duplicate, and the experiments were repeated three times.
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Southern Analysis of mtDNA
Total DNA from H4IE and respiration-deficient H4IE clones was isolated, and 10-µg samples were digested to completion and separated by agarose electrophoresis. DNA was transferred to a nylon membrane as described (24) and hybridized to the cytochrome b cloned probe labeled using Ready-to-Go DNA labeling beads (Amersham Biosciences) and [γ-32P]dCTP (Amersham Biosciences).

Mitochondrial Membrane Potential and ROS Production
Mitochondrial membrane potential was compared in wild type and ΔH4IE cells using a cell permeant cationic fluorescent indicator, rhodamine-123 (5 µM; Molecular Probes), which is sequestered by active mitochondria in proportion to membrane potential (23). ROS production was estimated in cells immediately after 3 h of exposure to z-BH using dihydrodorhodamine-123 (Molecular Probes, Eugene, OR). Dihydropodamine-123 accumulates primarily in mitochondria and is oxidized by ROS to rhodamine-123 (29). Rhodamine-123 fluorescence (excitation wavelength, 490 nm; emission, 535 nm) was measured on a microplate reader (Safire; Tecan US, Research Triangle Park, NC), and signal intensity was expressed qualitatively in relative units.

Western Analysis
Protein samples (nuclear or cytoplasmic) were separated by SDS-PAGE and prepared for immunoblot analysis (1). Nonspecific binding sites were blocked with TBST (Tris-buffered saline/Tween) containing 5% nonfat dry milk for 12 h at 4 °C. Membranes were incubated with antibody as follows: polyclonal rabbit anti-PCNA (1:1000; Pharmingen, San Diego, CA), polyclonal rabbit anti-cyculin D1 and Cdk4 (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), monoclonal mouse anti-β-actin (1:5000; Sigma), polyclonal rabbit anti-NRF-1 (1:3000; see Ref. 20), anti-tubulin (1:1000; Sigma), rabbit polyclonal anti-P-Akt (1:1000; Cell Signaling Technology, Beverly, MA), and rabbit polyclonal anti-Akt (1:1000; Cell Signaling Technology). After five washes in TBST, membranes were incubated in a 1:10,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit or mouse IgG (Amersham Biosciences). The membranes were developed by enhanced chemiluminescence (Amersham Biosciences). Protein expression was quantitated on digitized images from the center of the dynamic range and normalized to β-actin or tubulin in the same sample. At least four samples were used for each densitometry measurement.

Electrophoretic Mobility Shift Assay (EMSA)
EMSA for NRF-1 was performed with [γ-32P]ATP polynucleotide kinase-labeled oligonucleotides annealed to double-strand oligonucleotides. Binding reactions were carried out on 20 µg of nuclear extract from liver or hepatoma cell nuclei. Binding assays were performed in a volume of 20 µl with 5 µg of bovine serum albumin and 1 µg of sonicated salmon sperm DNA. Preliminary experiments were performed to determine the range of nuclear protein that generated a linear signal response for the gel shift on autoradiographs. Control binding experiments were performed under conditions of mutant NRF-1 probe. For supershifts, the reactions included 1 µl of undiluted goat anti-NRF-1 antiserum. For competition, excess unlabeled oligonucleotide was incubated with nuclear extract before adding labeled oligonucleotide. After 20 min at room temperature, reaction mixtures were loaded on prerun 5% native polyacrylamide for 2.5 h at 10 V/cm, and the gels were dried and exposed to x-ray film. The DNA oligonucleotide sequences used for EMSA were as follows (NRF-1 recognition sites are underlined): human mtTFA NRF-1A, 5′-CGGCTTCCCGGGCGGCTGCAATT-3′; NRF-1B, 5′-GGCGGGAATTTGGGCGCAGCUGCGTG-3′. Control oligonucleotides were synthesized as follows (mutated regions underlined): muNRF-1C, 5′-CGCCTCTCGAAAAATGAAACCAATT-3′; muNRF-1D, 5′-GGGGCGAAATTTGTTCATTGTCGG.

Statistics
Data were expressed as mean ± S.E. Statistics were performed by ANOVA followed by Tukey’s post hoc comparison using computer software. A p ≤ 0.05 was considered significant. n values refer to independent replicates. Regression analysis was performed using Statview (SAS, Version 5.0.1).

RESULTS
Oxidative Stress and Hepatic Cell and Mitochondrial Injury after LPS—LPS depletes hepatic glutathione (GSH) and increases glutathione disulfide (GSSG/GSH) even in highly coupled mitochondria (4); this stress occurred within 6 h and recovered by 48 h (not shown). GSH recovery was associated with increased expression of the regulatory heavy catalytic subunit of γGCS (24). The mRNA for γGCS heavy chain increased 4-fold by Northern analysis at 24 h after LPS (0.46 ± 0.10 at 0 h versus 1.87 ± 0.12 at 24 h; see Fig. 1A, p < 0.05). Competitive PCR demonstrated transient decreases in mtDNA copy number coincident with GSH depletion/recovery (Fig. 1B). mtDNA copy number decreased significantly 6 h after LPS, stabilized at 24 h, and had recovered by 48 h. This coincided with an increased hepatocyte mitotic index (Fig. 1C).

Nuclear and Mitochondrial mRNA—Mitochondrial transcription was checked using gene-specific primers for mitochondrial mRNA for four mitochondrial proteins (Fig. 2A), COX I, ND1, and ATPase 6 referenced to nuclear 18 S rRNA. At 24 h significant decreases were found in ATPase 6, COX I, and ND1 transcripts (22 to 27%), which recovered at 48 h. Because mitochondrial mRNA is transcribed as single polycistronic molecules from mtDNA heavy chain, similar COX I, ND1, and ATPase 6 mRNA contents indicate near steady-state conditions. Message for nuclear-encoded Complex IV subunits (cytochrome c oxidase, COX IV, COX V), and cytochrome c were analyzed by RT-PCR normalized to the stable nuclear-encoded 18 S rRNA (Fig. 2B). COX IV and cytochrome c mRNA doubled 24 h after LPS whereas COX V mRNA did not change relative to 18 S rRNA.

Fig. 1. Hepatic oxidative stress and mtDNA damage in LPS-treated rats. A, expression of γGCS (heavy chain) in the liver of LPS-treated rats (0, 6, and 24 h). A representative ethidium bromide-stained gel is shown; RNA loading and integrity is shown by intact 28 S and 18 S rRNA bands. B, mtDNA copy number by competitive PCR. Top band corresponds to 590-bp mtDNA amplified from each sample; bottom band corresponds to the 415-bp competitive DNA fragment. Table indicates equivalent optical density points of amplified PCR signal (competitive DNA template, mtDNA) versus logarithm of number of DNA copies added. Data represent four independent studies. Values are mean ± S.E. (n = 4); *, p < 0.05. C, representative liver histology from LPS-treated rats. a, 0 h shows a control section of normal liver, b, section 24 h after LPS shows a necrotic focus (top center) with inflammatory cells, c, section 48 h after LPS shows numerous mitotic figures (arrowsheads).
approximately 70% of the increase in NRF-1 protein
increase in NRF-1 binding was evident at 6 h after LPS expo-
factors NRF-1 and NRF-2 are involved in regulating expression
of nuclear genes encoding major mitochondrial proteins that
Mitochondrial gene expression was measured using RT-PCR and specific
oligonucleotide primers for rat mitochondrial ATPase 6, COX1, and ND1. Nuclear mRNA for 18 S rRNA was used to control for RNA loading and
efficiency of RT-PCR. Mitochondrial gene expression is shown in right panel after normalization to 18 S rRNA. Data represent four independent
studies. Values are mean ± S.E. (n = 4; * p < 0.05). B, steady-state nuclear mRNA transcripts in LPS-treated rats. Gel Star-stained 2% agarose
gels demonstrating RT-PCR products from rat liver are shown in the left panel. RNA was prepared from control liver (0 h) and after LPS (6–48 h).
Nuclear gene expression for cytochrome c, COX IV, and COX V was measured with RT-PCR using constitutive 18 S rRNA to control for RNA
loading and amplification (right panel). Data are representative of four independent studies. Values are mean ± S.E. (n = 4; * p < 0.05).

Mitochondrial DNA Replication—Mitochondrial biogenesis requires mtDNA replication under nuclear regulation. To investigate mtDNA regulation, we examined three nuclear genes encoding proteins for mtDNA replication (Fig. 3). DNA polymerase \( \gamma \) (Poly) replicates mtDNA, mitochondrial single-stranded DNA-binding protein (mtSSB) serves DNA replication, repair, and recombination, and mtTFA is the only currently known activator of mammalian mitochondrial transcription. Poly, mtSSB, and mtTFA transcripts increased significantly by 6 h after LPS, further at 24 h, and by 48 h had declined toward pre-injury baseline.

**LPS Induced Protein Binding of NRF-1**—The transcription factors NRF-1 and NRF-2 are involved in regulating expression of nuclear genes encoding major mitochondrial proteins that regulate mtDNA transcription and replication (18). We investigated the effect of LPS on NRF-1 activation by EMSA. An increase in NRF-1 binding was evident at 6 h after LPS exposure. Approximately 70% of the increase in NRF-1 protein binding was lost 24 h after LPS exposure (Fig. 4A). To confirm specificity of NRF-1 binding, we performed supershift assays with anti-NRF-1 antibody, and a mutated oligonucleotide sequence for NRF-1 interfered with protein binding (Fig. 4B).

The murine NRF-1 peptide sequence revealed an Akt phosphorylation motif (RXRXX(S/T)) at position 104–109. Therefore, we assessed Akt activation by Western blot using anti-Akt and anti-phospho-Akt antibodies. To activate Akt fully, phosphorylation of two sites is necessary, one in the activation domain, and the other in the COOH-terminal hydrophobic motif (25). Phospho-Akt increased significantly 6 h after LPS treatment and declined by 24 h but remained above baseline (Fig. 4C).

**LPS Induced Expression of NRF-2**—We analyzed changes in NRF-2 mRNA by RT-PCR in liver after LPS. NRF-2 is also a nuclear regulatory protein for mitochondria; its mRNA was expressed at low levels in control liver (Fig. 4D), but transient NRF-2 mRNA induction was found within 6 h of LPS injection followed by a decline over the next 24 h (Fig. 4D). Up-regulation of NRF-2 expression is novel, and probable transcriptional regulation of this gene has not been demonstrated before.

**Cell Growth Regulatory Proteins**—Cell cycle activation evaluated with a PCNA marker was increased significantly (p < 0.05) by LPS at 24 and 48 h (Fig. 5A). To determine whether \( G_1 \) cyclin expression accompanied recovery from LPS injury, cyclin D1 and cyclin-dependent kinase-4 (Cdk4) expression were checked by Western analysis in liver nuclei and cytoplasm (Fig. 5B). At 24 h, cytoplasm and nuclear cyclin D1 protein levels were stable and nearly undetectable, but \( G_1 \) cyclin was found after 48 h in both cytoplasm and nuclei (Fig. 5B). In comparison, Cdk4 constitutively present in control cytoplasm was equivocal in the nucleus. Cytoplasmic Cdk4 was unaffected by LPS, but the nucleoprotein was expressed significantly by 48 h (p < 0.05) (Fig. 5B). Nuclear translocation of D1 and Cdk4 indicate cell cycle progression.

**Respiration-deficient Cells**—To assess the relationship between mitochondria and hepatic cell proliferation we selected a rat hepatoma (H4IIE) cell line that constitutively expresses NRF-1 and mtTFA and generated mtDNA depleted clones. Southern analysis of DNA from parental (H4IIE) and EB derivative cells identified two clones (ΔH4IIE and ΔH4IIE) as mtDNA-deficient cells (>98% reduction of the mtDNA content) (Fig. 6A). These cells grew slowly, depended on glycolysis, and required pyruvate and uridine (not shown). Both clones responded similarly, but the reported data were obtained from
clone ΔH4IIE. To assess nuclear genes that control mitochondrial biogenesis, we examined mRNA for transcription factors NRF-1 and mtTFA in H4IIE and ΔH4IIE cells. Fig. 6B shows expression of NRF-1 and mtTFA relative to 18 S rRNA mRNA by RT-PCR in control and mtDNA-depleted cells. Depletion of mtDNA significantly down-regulated biogenesis gene expression in ΔH4IIE cells compared with H4IIE cells (Fig. 6C). Deletion of mtDNA decreased mitochondrial transmembrane potential and ROS generation relative to control cells as determined using fluorescence techniques (Fig. 7). The ΔH4IIE cells, like H4IIE cells, took up the fluorescent dye rhodamine-123 initially but lost it rapidly, whereas H4IIE cells retained the dye (Fig. 7A). The effect of mtDNA depletion on ROS generation was assessed by oxidation of dihydrodihoramide (Fig. 7B). In comparing parent (H4IIE) with mtDNA-deficient (ΔH4IIE) cells, significantly less fluorescence was detected in ΔH4IIE cells, but t-BH exposure caused both cell types to accumulate fluorescence signal in the mitochondria.

Strong oxidants such as t-BH affect cell growth and viability by both direct oxidation and through secondary ROS generation (26). In H4IIE and ΔH4IIE cells, viability was decreased significantly in a concentration-dependent manner after exposure to t-BH at concentrations of 50 μM or above for 24 h (not shown). However, lower concentrations of t-BH had either no effect or stimulated growth. To avoid cytotoxicity, proliferation studies were performed using 15 μM t-BH, which enhanced ΔH4IIE cell growth (and survival) significantly, restoring it almost to the level of control cells. H4IIE cells exhibited slight increases in growth rate at 24 to 48 h with t-BH (Fig. 7C).

Translocation of mtDNA Regulatory Proteins—Translocation and binding of transcription factors NRF-1 and -2 induce mtTFA expression in response to oxidative damage of mtDNA (27, 28). Therefore, we probed nuclear extracts of H4IIE and ΔH4IIE cells with anti-NRF-1 by Western analysis after exposure to t-BH. The t-BH increased NRF-1 expression in both cell lines within 1 h and was pronounced at 3 h. NRF-1 expression extended through 6 h in ΔH4IIE but decreased in control cells (Fig. 8A).

Activation of Akt—Akt is activated by phosphorylation, and oxidant-activated PI3K binds with high affinity and specificity to the Akt PH domain. We assessed Akt by Western analysis after oxidant treatment of cells grown in serum-free medium for 12 h to reduce basal phosphorylated Akt. Using antibody that detects only serine-473 phospho-Akt, serum control showed Akt phosphorylation within 15 min, a maximal increase after 1 h that was sustained for 6 h (not shown). To determine whether t-BH promotes Akt phosphorylation, cells were incubated with t-BH in serum-deficient medium for 1 to 3 h, and phospho-Akt accumulation was compared in ΔH4IIE and H4IIE cells. To evaluate the involvement of PI3K in t-BH-induced Akt phosphorylation, cells were pretreated with 50 μM PI3K-specific inhibitor LY294002 for 3 h showed Akt phosphorylation within 15 min, a maximal increase after 1 h that was sustained for 6 h (not shown). To determine whether t-BH promotes Akt phosphorylation, cells were incubated with t-BH in serum-deficient medium for 1 to 3 h, and phospho-Akt accumulation was compared in ΔH4IIE and H4IIE cells. To evaluate the involvement of PI3K in t-BH-induced Akt phosphorylation, cells were pretreated with 50 μM PI3K-specific inhibitor LY294002 for 3 h. Subsequent immunoblots demonstrated that LY294002 blocked t-BH-mediated Akt phosphorylation, indicating the involvement of PI3K in t-BH-induced Akt phosphorylation (Fig. 8B).

Role of PI3K in NRF-1 Activation—To determine whether PI3K was involved in activating NRF-1 binding, multiple experiments were carried out to determine the effects of PI3K inhibitors on NRF-1 binding activity. H4IIE and ΔH4IIE cells incubated with t-BH and 50 μM LY294002 for 3 h showed NRF-1 activity suppression by lack of DNA binding on EMSA. A representative gel with supershift analysis (Fig. 8C) demonstrates that PI3K is necessary for oxidant to activate NRF-1 especially in ρ0 hepatoma cells.

FIG. 4. LPS induced expression and nuclear binding of NRF-1 and Akt in liver. A, DNA binding specificity of NRF-1 in liver nuclear extract. NRF-1 32P-labeled consensus oligonucleotides were incubated with 20 μg of nuclear protein after optimizing the binding conditions. Lanes 1 and 2 indicate reactions with radiolabeled oligo alone. Lanes 3–8 represent reactions with liver nuclear extract; arrow denotes NRF-1 complex. Nonspecific complex (NS) is also indicated. B, supershift analysis of NRF-1 binding activity. Nuclear proteins were isolated from rat livers treated with LPS for 6 h. Before gel-shift assay, protein extracts were incubated with 1 μl of NRF-1 antibody. The position of specific bands is indicated by the arrow; the supershifted (SS) band is indicated by the arrow. The specificity of NRF-1 binding activity was confirmed by the absence of NRF-1 complex band in the negative control (muNRF-1 probe). C, induction of P-Akt in the liver after LPS. Western blots were performed using antibody to P-Akt (top). Anti-total Akt antibody serves as internal control (bottom). D, mRNA expression of NRF-2. Messenger RNA levels of NRF-2 were determined by semiquantitative RT-PCR according to “Materials and Methods” (top) and normalized to 18 S rRNA (bottom). Data are representative of four studies.
Mitochondria are not autonomous with respect to mtDNA replication; the nucleus coordinates nuclear and mitochondrial gene expression to meet the need for OXPHOS. For example, thyroid hormone increases mitochondrial mRNA in liver and muscle in hypothyroid rats (33, 34) and increases steady-state nuclear mitochondrial F1-ATPase and adenine nucleotide translocase transcripts (35). In this study, mtDNA template damage after LPS significantly decreased mitochondrial mRNA, whereas selected nuclear-encoded mRNA for OXPHOS proteins increased, e.g. cytochrome c and COX IV. In concert, nuclear mRNA for regulatory proteins of mtDNA transcription increased including mtSSB, mtTFA, and DNA Poly. These proteins maintain and replicate the mitochondrial genome, and mtTFA is pivotal in both processes (36). mtTFA level varies with mtDNA copy number in mitochondrialopathies (36, 37), and mtTFA gene disruption demonstrates that mtTFA is essential for biogenesis and embryogenesis (17).

The increase in mRNA expression (~5-fold) for mtTFA supports increased protein and mtTFA-DNA binding activity with LPS (4), consistent with activation of the mtTFA promoter. Transcription of mtTFA is regulated in part by NRF-1 and by NRF-2 (18); the increase in NRF-1 protein and binding to the cis element here are consistent with this notion. The promoter regions of nuclear genes involved in mitochondrial biogenesis (mtTFA, mtSSB, DNA Poly) reveal consensus motifs recognized by NRF-1 (and/or NRF-2) implicating them in nuclear-mitochondria regulation (38). Regulation by NRF-1 and NRF-2 are also revealed in studies of subunit 2 of succinate dehydrogenase (39). It is not yet clear, however, why some mitochondrial genes appear to be regulated by one or both factors, whereas others do not have NRF response elements.

New mitosis induced by LPS is detectable by histology only after evidence of biogenesis has been established (4). Biogenesis maintains the volume density of mitochondria, and proliferation requires additional energy. The liver compensates for necrosis with cell division, and expression of PCNA and G1/S cyclin (cyclin D1 and Cdk4) was detected within 48 h after LPS injury. Cyclin D1 mediates progression from the quiescent gap (G0) phase of the cycle through G1 (40), and cyclin D1 and Cdk presence in the nucleus after LPS indicates binding associated with mitosis-regulating retinoblastoma kinase activity (41). Regulation of cyclin D1/Cdk4 complex formation and translocation varies among cells (41), but in late G1 and during S phase PCNA (cyclin A) binds DNA polymerase-δ (42, 43), and its expression is sensitive to proliferation (44) and consistent with liver regeneration (45). Though the mechanisms coordinating biogenesis and cell division are unknown, NRF-1 activation suggests a role for redox signaling by ROS.

LPS induces ROS generation by liver mitochondria associated with TNF-α production during pre-replication, which although potentially dangerous, also promotes cell survival and proliferation (40–45). TNF-α regulates PI3K/Akt (46), and Akt inhibits apoptosis and stimulates cell growth (47, 48). Akt contains a central motif with Ser or Thr specificity, and the NH₂ terminus includes pleckstrin homology domain essential for lipid-protein or protein-protein interactions. On activation, Akt induces anti-apoptotic effects through phosphorylation of Bad (49) and caspase-9 (50). Akt stimulates cyclin D1, which promotes cell growth (51) and protects murine hepatocytes from TNF-α and Fas (CD95)-mediated apoptosis (52). A key implication of Akt activation by LPS is that it may prevent apo-
ptosis, e.g. mediated by TNF, but not necrosis. Akt activation would thus promote replication in surviving hepatocytes. LPS also activates PI3K/Akt in human monocytes to limit TNF-β/H9251 induction (53).

The in vivo study fully supported the concept that mitochondrial biogenesis increases after LPS injury but did not provide a specific mechanism. There is evidence that ROS regulate nuclear genes (54) and that mtDNA replication and transcription depend on nuclear-encoded proteins some of which are redox-sensitive, e.g. NRF-1 (55). Stimulation of these oxidant-sensitive transcription factors by LPS raised the possibility that mitochondrial ROS regulated biogenesis. To investigate this possibility, we generated ρ° clones from rat hepatoma cells that constitutively express NRF-1 and mtTFA. These cells were found to have diminished mitochondrial ROS production and grew slowly compared with controls in accordance with studies of human ρ° cells (56). After mtDNA knockout (ΔH4IIE cells), nuclear NRF-1 translocation decreased, but upon exogenous oxidant exposure, NRF-1 translocation and growth returned to control levels. These findings clearly demonstrated that mitochondrial ROS generation influences the redox regulation of NRF-1. ΔH4IIE cells also showed a lower expression of mtTFA mRNA similar to other ρ° cells (37).

In addition to ROS, modulation of mitochondrial gene expression by other nuclear factors is possible. For example, communication between mitochondria and nucleus may involve ATP/ADP and depend on mitochondrial ATP generation (57). We could not exclude this possibility, because mtDNA depletion impairs OXPHOS, and ATP turnover should be lower in ρ° than control cells. That oxidant treatment increased NRF-1 translocation and cell growth in ΔH4IIE cells, however, indicates the importance of oxidative signaling, because the ρ° cell still responds to it.

We also found that Akt expression, which is regulated by oxidants (58), was increased by t-BH via PI3K activation in the ρ° cell. Both kinases are integral to cell growth, survival, and transformation. Because Akt is redox-sensitive, PI3K activation of Akt was a candidate for NRF-1 phosphorylation and activation. Inhibition of PI3K suppressed NRF-1 binding activity and Akt phosphorylation by t-BH, thus demonstrating involvement of PI3K/Akt in activating nuclear NRF-1-binding proteins. Although PI3K/Akt activation preceded NRF-1 acti-
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Fig. 8. Effect of t-BH on NRF-1 nuclear translocation. A, immunoblots of NRF-1. Each lane contains 20 μg of nuclear protein from H4IIE or ΔH4IIE cells treated with 15 μM t-BH and incubated for 1–6 h. Blots were probed sequentially for NRF-1 and tubulin. Signal intensity was evaluated by densitometry, and nuclear translocation of NRF-1 was determined relative to tubulin. The histogram summarizes data from four studies. B, Akt-kinase activation in mtDNA-deficient cells cultured in serum-deficient medium. A representative immunoblot demonstrates phospho-Akt (top) and total Akt (bottom) in the same extracts. Akt phosphorylation was activated by t-BH through PI3K in H4IIE and ΔH4IIE cells. Cell lysates prepared after 3 h of treatment with 15 μM t-BH were separated by electrophoresis, protein was transferred to nitrocellulose, and phospho-Akt was detected using polyclonal phospho-Akt (serine-473) antibody (top). Anti-total Akt was an internal control (bottom). C, PI3K inhibitor (LY294002) blocks activation of NRF-1. Nuclear extracts prepared from H4IIE and ΔH4IIE cells were used for shift and supershift assays with anti-NRF-1 polyclonal antibody. The autoradiogram shows the formation of the NRF-1 complex in cells cultured in the presence of t-BH (lane 3) compared with ΔH4IIE cells in the absence of t-BH (lane 4). A supershift (SS) of NRF-1 was detected with the anti-NRF-1 antibody (lanes 5 and 6). Note the absence of NRF-1 complex band in the negative control (muNRF-1 probe) lanes 9 and 10. Nonspecific complex is indicated (NS). Data are representative of three runs.

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