Co-regulation of nuclear respiratory factor-1 by NFκB and CREB links LPS-induced inflammation to mitochondrial biogenesis

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Accepted 20 April 2010
Journal of Cell Science 123, 2565-2575
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doi:10.1242/jcs.064089

Summary

The nuclear respiratory factor-1 (NRF1) gene is activated by lipopolysaccharide (LPS), which might reflect TLR4-mediated mitigation of cellular inflammatory damage via initiation of mitochondrial biogenesis. To test this hypothesis, we examined NRF1 promoter regulation by NFκB, and identified interspecies-conserved κB-responsive promoter and intronic elements in the NRF1 locus. In mice, activation of Nrf1 and its downstream target, Tjαm, by Escherichia coli was contingent on NFκB, and in LPS-treated hepatocytes, NFκB served as an NRF1 enhancer element in conjunction with NFκB promoter binding. Unexpectedly, optimal NRF1 promoter activity after LPS also required binding by the energy-state-dependent transcription factor CREB. EMSA and ChIP assays confirmed p65 and CREB binding to the NRF1 promoter and p65 binding to intron 1. Functionality for both transcription factors was validated by gene-knockdown studies. LPS regulation of NRF1 led to mtDNA-encoded gene expression and expansion of mtDNA copy number. In cells expressing plasmid constructs containing the NRF-1 promoter and GFP, LPS-dependent reporter activity was abolished by gene-knockdown studies. LPS regulation of p65 and CREB binding to the activity after LPS also required binding by the energy-state-dependent transcription factor CREB. EMSA and ChIP assays confirmed p65 and CREB binding to the NRF1 promoter and p65 binding to intron 1. Functionality for both transcription factors was validated by gene-knockdown studies. LPS regulation of NRF1 led to mtDNA-encoded gene expression and expansion of mtDNA copy number. In cells expressing plasmid constructs containing the NRF-1 promoter and GFP, LPS-dependent reporter activity was abolished by cis-acting κB-element mutations, and nuclear accumulation of NFκB and CREB demonstrated dependence on mitochondrial H2O2. These findings indicate that TLR4-dependent NFκB and CREB activation co-regulate the NRF1 promoter with NFκB intronic enhancement and redox-regulated nuclear translocation, leading to downstream target-gene expression, and identify NRF-1 as an early-phase component of the host antibacterial defenses.

Key words: Mitochondrial biogenesis, Nuclear respiratory factor-1, Reactive oxygen species, Toll-like receptor-4

Introduction

The impact of activation of innate immunity on the regulation of energy production, despite its importance in cell survival, is poorly understood, especially with respect to cis-acting elements that bind to gene promoters involved in the transcriptional program of mitochondrial biogenesis. An example is the full spectrum of processes involved in immunity, inflammation, proliferation and apoptosis that are activated by the pleiotropic transcription factor NFκB and lead to a broad array of effects (Ghosh and Karin, 2002). The five NFκB family members, p50, p52, p65 (RelA), cRel and RelB, share a Rel-homology domain that is responsible for dimerization and for the DNA binding that imparts transcriptional activity (Hayden and Ghosh, 2008). In classical NFκB activation, Toll-like receptor (TLR) ligands, such as the TLR4 cognate lipopolysaccharide (LPS) and NFκB-dependent cytokine secretion, particularly TNFα, lead to IkK activation, resulting in phosphorylation of IkB, followed by ubiquitylation by the E3 ligase (Skag et al., 2009). Ubiquitylated IkB is degraded, allowing NFκB to enter the nucleus to activate transcription.

Further processing is required for NFκB-activated gene expression; for instance, p65 phosphorylation enables transcription by allowing interactions with acetyltransferase co-activators such as CBP and p300 (Zhong et al., 1998). There is also crosstalk between NFκB and other transcription factors, including CREB, p53, IRF3 and IRF7, that use CBP/p300 (Martin et al., 2005; Yang et al., 2004). Some NFκB-regulated genes require chromatin modification; constitutive and immediately accessible (CIA) promoters require no modification, whereas regulated and late accessibility (RLA) promoters depend on stimulus-specific chromatin remodeling for differential control of inflammatory gene expression (Ramirez-Carrozzi et al., 2006). Known connections between NFκB and physiological processes are actively expanding; for instance, NFκB is associated with regulation of mammalian aging (Adler et al., 2007), and in myoblasts, the classical NFκB pathway inhibits differentiation, whereas in newly contractile myotubes, selective IkKα activity and the p52-RelB non-canonical pathway stimulate mitochondrial biogenesis by unknown mechanisms (Bakkar et al., 2008).

Another transcription factor, nuclear respiratory factor-1 (NRF-1), is essential for the integration of nuclear- and mitochondrial-encoded gene transcription (Evans and Scarpulla, 1989). NRF-1 dimerizes and binds to palindromic promoter sites of mitochondrial genes (Virbasius et al., 1993), interacting with peroxisome proliferator-activated receptor γ co-activator-1α (PGC-1α) and PGC-1-related co-activator (PRC), which can operate in concert with CREB to activate target genes involved in mitochondrial function (Vercauteren et al., 2006; Wu et al., 1999). Among the several hundred NRF-1 target genes are subunits of all five mitochondrial respiratory complexes (MRCs), assembly factors, parts of the mtDNA transcription and replication machinery, components of heme biosynthesis and mitochondrial protein import, and mtDNA transcription factors A (Tfam) and B (TFB1M and TFB2M, mtTFB1 and mtTFB2) (Kelly and Scarpulla, 2004). Tfam and the mtTFBs transcribe the mitochondrial genome, leading to an increase in mitochondrial-encoded subunits of the MRC (Scarpulla, 2008). The Nrf1−/− mouse shows mtDNA depletion and
inactivated in the livers of mice injected with a single i.p. dose of heat-
NF\(\kappa\)B was confirmed by suppression of NF\(\kappa\) effect of BAY11 on NF\(\kappa\) mRNA levels. In wild-type mice, 7085, followed by
E. coli only the 6 hour activation in vivo mice with heat-inactivated
viability (Huo and Scarpulla, 2001).

**Results**

**NRF-1 and mtDNA transcription and replication via NF\(\kappa\)B activation in vivo**

The effect of NF\(\kappa\)B activation on Nrf1 expression was characterized in the livers of mice injected with a single i.p. dose of heat-
inactivated E. coli \((5 \times 10^9 \text{ c.f.u.})\) by measuring sequential Nrf1 mRNA levels. In wild-type mice, Nrf1 mRNA analysis by real
time RT-PCR showed that hepatic mtDNA levels increase significantly 6-24 hours after E. coli administration (Fig. 1A). To test whether NF\(\kappa\)B activation regulates NRF-1 production, mice were treated with the irreversible \(\kappa\)B kinase inhibitor, BAY11-
7085, followed by E. coli. BAY11 significantly delayed and attenuated the increase in Nrf1 mRNA (Fig. 1A). The inhibitory effect of BAY11 on NF\(\kappa\)B was confirmed by suppression of E. coli-induced NOS2 expression (data not shown). To confirm that NF\(\kappa\)B participates in Nrf1 gene expression, we challenged p50\(^{-/-}\) mice with heat-inactivated E. coli. The absence of p50 inhibited only the 6 hour Nrf1 gene expression (Fig. 1A), implicating p50 in
initial Nrf1 induction and one or more other subunits in the complete
early-phase response.

NRF-1 stimulates nuclear-encoded Tfam expression by binding to
NRF-1-response elements in the promoter region (Virbasius and
Scarpulla, 1994). Tfam is then imported into mitochondria and
increases mtDNA transcription and replication (Scarpulla, 2002).
The mRNA levels for Tfam and two mitochondrial-encoded proteins,
COI and NDI, were analyzed by real time RT-PCR to determine
whether Nrf1 induction by NF\(\kappa\)B activation causes Tfam transcription
and mitochondrial-encoded target gene expression. In wild-type
mice, Tfam mRNA levels increased at 24 and 48 hours after E. coli administration, and the response was blocked by addition of
BAY11 (Fig. 1B). Tfam expression was also delayed in p50\(^{-/-}\) mice until 48 hours (Fig. 1B). mRNA levels of mtDNA-encoded COI
\((\text{Mtco1})\) and NDI \((\text{Mtnd1})\) increased 24-48 hours after E. coli
administration; this was inhibited in BAY11-treated mice and delayed
in p50\(^{-/-}\) mice (Fig. 1C-D). We also examined downstream NF\(\kappa\)B-
mediated effects of NRF-1 induction by checking hepatic mtDNA
copy number, and found an increased copy number at 48 and 72
hours after E. coli administration that was inhibited in wild-type
mice by BAY11 and delayed in p50\(^{-/-}\) mice (Fig. 1E).

**Identification of \(\kappa\)B sites in the NRF-1 locus**

Since NRF-1 expression was induced in an NF\(\kappa\)B-dependent
manner, we explored how LPS and E. coli stimulate NRF-1 gene
expression. Despite mounting evidence that the immune system
activates NRF1 (Piantadosi and Sulinman, 2006; Sulinman et al.,
2003; Sulinman et al., 2005), there are no reports of the gene having
functional \(\kappa\)B-binding sites. We searched for NF\(\kappa\)B and CREB
consensus binding sequences using web-based rVISTA to identify
conserved sequences for specific transcription factors by linking
them to the TRANSFAC database (Loots and Ovcharenko, 2004).
Analysis of the mouse and human proximal 1.5kb of the
NRF1 locus with expanded sequences (DNAsis and Genomatix) identified potential NF\(\kappa\)B-response elements (kBREs) within the conserved NRF1 5‘-promoter sequence. A schematic of the NRF1 locus with expanded sequences is shown in Fig. 2A where the regions at \(-500\) to \(-120\) of the mouse and \(-920\) to \(-150\) of the human upstream of the NRF-1 transcription
start site (TSS) bear sequences identified with a high likelihood for
NF\(\kappa\)B binding by exhibiting 90-100% identity with the canonical

![Fig. 1. NF\(\kappa\)B-dependent activation of Nrf1 and downstream NRF1 target-gene expression in mice.](image-url)

Timed experiments for the effects of administration of heat-
inactivated E. coli in wild-type BAY11-
treated mice and p50\(^{-/-}\) mice. (A) Hepatic
Nrf1 mRNA expression determined by real
time RT-PCR. (B) Hepatic Tfam mRNA
expression by real time RT-PCR.
(C) Hepatic mitochondrial COI \((\text{Mtco1})\)
mRNA expression by real time RT-PCR.
(D) Hepatic mitochondrial NDI \((\text{Mtnd1})\)
mRNA expression by real time RT-PCR.
(E) Hepatic mitochondrial DNA copy
number determined by real time PCR.
Values are means ± s.e. of 4-6 mouse livers
\((^{*P}<0.05)\).
NFXB enhancer sequence, 5′-GGRRNNYYCC-3′ (where R is a purine, Y is a pyrimidine and N is any nucleic acid). Comparative sequence analysis, effective for finding functional coding and non-coding elements in vertebrates (Loots et al., 2002), identified 11 NFXB sites in the non-coding region; one in the promoter region is interspecies-conserved whereas three conserved sites are located in NRF1 coding elements in vertebrates (Loots et al., 2002), identified 11 sequence analysis, effective for finding functional coding and non-purine, Y is a pyrimidine and N is any nucleic acid). Comparative

Fig. 2. Bioinformatics analysis of the 5′-proximal region of the NRF1 gene promoter and conserved region of intron 1 in the mouse and human. (A) Sequences were aligned between human and mouse using rVISTA 2.0. The middle histogram represents the interspecies DNA conservation within the 5′-UTR segment. CNS (interspecies conservation more than 75%) is emphasized in red. (B) The first three exons (E) and the first three introns (I) for the NRF1 gene are shown. NFXB consensus sequences for human and mouse NRF1 genes identified by Genomatix and DNAsis are displayed on the blue line under the histograms. Detailed sequences spanning promoter and intron NFXB motifs are depicted at the bottom. Red letters indicate the NFXB consensus sequences. (C) Detailed sequences spanning promoter and conserved region of intron 1 in the mouse and human. (D) Western blot for p65 in HepG2 cells transfected with control siRNA or p65 siRNA. (E) NFXB-1 expression in HepG2 and HL-1 cells before and 8 hours after incubation with LPS+TNFα. Cells with BAY11 (50 μM) were pretreated for 1 hour before addition of LPS+TNF. Cells with control siRNA or siRNA targeting p65 (si-p65) were transfected 48 hours before LPS+TNF treatment for 8 hours. NRF1 mRNA expression was determined by q-RT-PCR. Values with error bars are means ± s.e. of four replicates (*P<0.05).
When nuclear probes for the intronic region (I1-I4) were used, a shift in the DNA-protein signal from E. coli-treated mice was observed (Fig. 3D). The signal intensity was highest with I1, and was greatly attenuated in nuclear extracts from untreated mice (Fig. 3D, lane C). Supershift experiments with anti-RelA or anti-cRel antibody showed that the P2-binding factor was primarily p65, and to a lesser extent, cRel, but no RelB supershift was detected (Fig. 3E). This indicated a specific NFκB interaction with the predicted binding site in the Nrf1 promoter.

NFκB subunit nuclear translocation was also checked at different times. Liver nuclei of E. coli-treated mice showed p65 translocation by 2 hours and cRel translocation by 6 hours (Fig. 3F). E. coli induced a minor p50 translocation at 2 hours and almost no RelB translocation.

NFκB binding to Nrf1 promoter in vivo
ChIP assays were used to examine whether NFκB bound directly to putative κB sites identified in the Nrf1 promoter and intronic regions in mouse liver after E. coli challenge. ChIP was used with anti-NFκB p65 and primer sets designed to detect the promoter and intron 1 of Nrf1 (Fig. 4A,B). Since recruitment of transcription factors precedes the maximal transcription rate (Ryser et al., 2007; Ryser et al., 2004), we monitored p65 occupancy of these Nrf1 regions at 2 hours and 6 hours after E. coli and found that p65/RelA bound most strongly at 6 hours to promoter regions R1 to R3 (Fig. 4C) and to intron 1 at R7, just downstream of exon 1 (Fig. 4D). The ChIP indicated moderate p65 promoter occupancy after E. coli treatment, but more active binding at intron 1 (Fig. 4D). Since anti-cRel antibody had shown binding to P2, we assessed the occupancy of cRel at the Nrf1 promoter, and cRel binding at position –94 to –298 was found at 6 and 24 hours (Fig. 4E). Occupancy of the Nrf1 promoter by p65 was also accompanied by DNA PolII recruitment, which is indicative of initiation of transcription (Fig. 4F).

CREB binding to Nrf1 promoter and enhanced transcription
The Nrf1 promoter region revealed CRE-binding sites at three locations: –714, –829 and –968, respectively. CREB binding was evaluated in nuclear protein extracts from control and E. coli-stimulated livers with unique oligonucleotides for the three promoter CRE motifs (Fig. 5). The EMSA indicated the formation of a single DNA-protein complex (Fig. 5A, arrow) with changes in the intensity of the complex in response to E. coli, with the highest intensity for the –829 tcTGACACCAtg sequence (Fig. 5A). Since transcriptional activation by CREB occurs when Ser133 is phosphorylated, EMSA was used with supershift assays for CREB and phosphorylated CREB to test binding at the Nrf1 promoter CRE sites. We found an oligonucleotide-protein complex supershift in liver nuclear extracts from mice treated with E. coli.
Unlabeled CRE oligonucleotide in molar excess outcompeted labeled oligonucleotide for binding, indicating specificity of the CRE complex.

A further ChIP assay in liver revealed significant CREB binding to the \(\text{Nrf1}\) promoter at two different positions (–652 to –748, –812 to –982) (Fig. 5C). Interestingly, the two sites were activated at different times in \(E.\ coli\)-treated mice and the –829 CREB site showed more binding activity at 6 hours.

To confirm \(\text{NRF1}\) as a transcriptional target of NF\(\kappa\)B p65 and CREB, one or both were silenced in HepG2 cells. We have reported NRF-1 silencing in these cells at 24-48 hours, and effective CREB silencing is demonstrated in Fig. 5D. The transfected cells were treated with LPS and TNF for 8 hours and \(\text{NRF1}\) expression was measured by real-time RT-PCR. These data showed that \(\text{NRF1}\) induction was suppressed by silencing CREB and p65 (Fig. 5E).

**LPS induces a functional \(\text{Nrf1}\) promoter**

We mapped the upstream \(\text{Nrf1}\) promoter region to expand our knowledge of potential interactions between basal transcriptional core promoter elements and specific DNA-binding and accessory transcription factors. For instance, \(\text{Nrf1}\) ARE consensus sequences bind NF-E2-related factor 2 (Nfe2l2) to enhance its expression in the heart (Piantadosi et al., 2008), but in inflammation, the presence of complex cognate TLR signaling would imply the involvement of several transcription factors in transcriptional regulation of \(\text{Nrf1}\).

Regulatory sequences upstream of the core region that might have a role in \(\text{Nrf1}\) transcription were delineated by cloning three DNA fragments spanning –1000 to +40 bp, –500 to +40 bp and a chimera –1000 to –700 plus –52 to +40 bp region of the mouse \(\text{Nrf1}\) promoter upstream of the TSS into plasmid pGlow-TOPO (Fig. 6A). The constructs were designated GFP1 (p1040-Nrf1glow), GFP2 (p540-Nrf1glow) and GFP3 (p392-Nrf1glow).

We expected that GFP expression from the basal promoter would be altered depending on the presence of regulatory sequence(s) in this region. For these assays, HepG2 cells were used for ease of transfection and high LPS responsiveness. After transfection with the vectors, HepG2 cells were treated for 8 hours with LPS, and GFP activity was measured 24 hours later by fluorescence microscopy or by microplate fluorometer. Our results with these constructs are shown in Fig. 6B (cell images) and Fig. 6C (histograms). In preliminary studies, the basal \(\text{NRF1}\) promoter transcription efficiency was ~1.06-fold that of control promoter-free vector (GFP0; not shown). After LPS exposure in cells transfected with GFP1, green fluorescence intensity increased 11-fold compared with untreated cells (Fig. 6B,C), whereas fluorescence increased ~7-fold in cells transfected with GFP2 vector compared with untreated cells (Fig. 6B,C), suggesting that the –500 to +40 region of \(\text{NRF1}\) augments transcription by the basal promoter. GFP1 transfection revealed an enhanced \(\text{NRF1}\) promoter transcription efficiency compared with cells transfected...
with plasmid GFP2, thus implicating additional regulatory motifs between –1000 and –500 in the sequence. The –1000 to –500 region also had CREB consensus sequences at –968, –829, –716 and –714; however, deletion of –700 to –52 as in GFP3 caused a 63.5% decrease in GFP fluorescence intensity compared with GFP1, indicating that CREB cooperates with NFκB in the 1040 bp fragment to drive optimal NRF1 promoter activity.

To assess the effect of NFκB binding on NRF1 promoter activity, transient transfection analysis with the GFP1 construct was carried out in HepG2 cells with and without BAY11 treatment. GFP1-transfected cells treated with BAY11 followed by exposure to LPS showed a ~90% lower promoter transcription efficiency compared with control LPS-treated cells (Fig. 6C). Cooperation between NRF1 and CREB showed a ~90% lower promoter transcription efficiency compared with control LPS-treated cells (Fig. 6C). Cooperation between NRF1 and CREB is required for optimal promoter activity. 

Mitochondrial H2O2 signaling of NFκB gene expression

NFκB and CREB are among the redox-regulated transcription factors that can be activated by reactive oxygen species (ROS) (Frey et al., 2008). Because innate immune effector molecules,
such as TNFα, stimulate mitochondrial ROS production, we asked whether mitochondrial ROS influence NFκB- or CREB-mediated NRF1 gene expression directly or alternatively via cytoplasmic Ca^2+ signaling. Superoxide and H_2O_2 egress via Complex III, for instance, might serve as a messenger for NFκB activation directly, or perhaps in conjunction with Ca^2+ (Sen et al., 1996). Therefore we used a mitochondrial-targeted catalase vector to investigate the role of mitochondrial H_2O_2 production in activation of NFκB and CREB. Normal mouse HL-1 atrial cardiomyocytes were transfected with mtCAT or empty vector for 48 hours, exposed to LPS+TNF for 4 hours, and nuclear extracts were analyzed for NFκB subunits by ~50-80% and phosphorylated CREB by approximately two thirds (Fig. 8A,B), implicating mitochondrial H_2O_2 release as a control point.

Since mitochondrial H_2O_2 might be involved in stress-induced Ca^{2+} signaling, the effects of Ca^{2+} mobilization on nuclear translocation of NFκB and phosphorylated CREB were evaluated using EGTA-AM, a potent cellular Ca^{2+} chelator, and thapsigargin (TG), an inhibitor of Ca^{2+}-ATPase in the ER. Since most Ca^{2+} chelators are short acting, HL-1 cells were pre-treated with EGTA-AM (20 μM) for 30 minutes followed by LPS and TNF for 2 hours and nuclear extracts probed for NFκB subunits by western blot. LPS- and TNF-induced nuclear translocation of p65, p50, and cRel was decreased, but not prevented by EGTA-AM (Fig. 8A), indicating a small Ca^{2+} effect. EGTA-AM did however block the nuclear accumulation of phosphorylated CREB (Fig. 8B).

Thapsigargin produces rapid Ca^{2+} efflux from the ER lumen (Thastrup et al., 1990), and in contrast to slow-acting ER stress-eliciting agents (Pahl and Baeuerle, 1995), rapidly activates NFκB (Pahl et al., 1996). If ER Ca^{2+} release is involved in NFκB activation after treatment with LPS and TNF, it would be important to know whether it interacts with mitochondrial H_2O_2 generation.

HL-1 cells stimulated with TG (30 nM for 30 minutes) did activate NFκB, but mtCAT transfection nearly completely blocked nuclear accumulation of p65 and cRel (Fig. 8A), implying that ER Ca^{2+} release stimulates mitochondrial H_2O_2, which ultimately mediates...
NFκB activation. TG also stimulated nuclear accumulation of phosphorylated CREB, and this effect was partially abrogated by mCAT (Fig. 8B). Confocal microscopy demonstrated the LPS- and TNF-mediated nuclear translocation of p65 and phosphorylated CREB in HL-1 cells, which was inhibited by mCAT (Fig. 8C). Ca²⁺ chelation by EGTA-AM did not block LPS+TNF-induced nuclear p65, but did block nuclear accumulation of phosphorylated CREB; the latter together with the mCAT data implies an interaction between mitochondrial H₂O₂ and ER Ca²⁺.

Discussion

A direct link between the innate inflammatory response mediated by NFκB signaling and the transcriptional activation of mitochondrial biogenesis has not been reported previously. This work has identified active intragenic NFκB-responsive cis-elements both in the promoter and in the first intron of NRF1, a pivotal transcription factor for mitochondrial biogenesis, which enables mitochondrial gene expression during the inflammatory response. These κB elements act as transcriptional enhancers that allow the LPS-TLR4 cognate and the early-phase cytokine TNFα, to activate NRF1. Also new is the aspect of synchronization between the classical NFκB pathway and the bZIP transcription factor CREB, which is integral to the regulation of pyruvate, glycogen and fatty acid metabolism (Zhang et al., 2005). This unusual mechanism of NRF1 regulation drives NRF-1 target-gene expression during the TLR4-mediated immune response.

Because TLR4 activates NFκB (da Silva Correia and Ulevitch, 2002; Li and Verma, 2002) and can be accompanied by CREB activation (Illario et al., 2008; Martin et al., 2005), it was necessary to address direct transcription factor binding to the NRF1 locus as an explanation for LPS-mediated NRF-1 expression (Suliman et al., 2003). A bioinformatics analysis revealed κB sites spanning the NRF1 locus in both the 5′-proximal (promoter) and intronic regions. Three κB sites within intron 1 are conserved across species, and we considered these potentially crucial for NRF1 expression because interspecies-conserved regions often correspond to DNase1 HS sites (Kang and Im, 2005), which could be targets for binding of transcription factors. Therefore, we focused our analysis initially on NFκB.

By EMSA, NFκB was demonstrated to function as an enhancer element within the NRF1 locus through significant p65 and weaker cRel binding at the NRF1 promoter. ChIP analysis confirmed that the NRF1 promoter site of interest is occupied primarily by p65. GFP-reporter assays using NRF1 promoter deletion and NFκB-mutation constructs in HepG2 cells demonstrated that NFκB contributes to LPS-induced reporter activity in the mouse Nrf1 promoter. The enhancer operates efficiently with a promoter derived from the 5′-proximal region, but not with a minimal promoter.

Because LPS-TLR4 cognate and the early-phase cytokine TNFα, to activate NF-κB (da Silva Correia and Ulevitch, 2002; Li and Verma, 2002) and can be accompanied by CREB activation (Illario et al., 2008; Martin et al., 2005), it was necessary to address direct transcription factor binding to the NRF1 locus as an explanation for LPS-mediated NRF-1 expression (Suliman et al., 2003). A bioinformatics analysis revealed κB sites spanning the NRF1 locus in both the 5′-proximal (promoter) and intronic regions. Three κB sites within intron 1 are conserved across species, and we considered these potentially crucial for NRF1 expression because interspecies-conserved regions often correspond to DNase1 HS sites (Kang and Im, 2005), which could be targets for binding of transcription factors. Therefore, we focused our analysis initially on NFκB.

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We confirmed the NFκB intronic functionality by GFP-reporter assays by co-transfecting HepG2 cells with NRF-1 reporter constructs and siRNA to knock down CREB or p65 after stimulation.
with LPS. These assays indicated that full NRF1 induction requires not only NFkB, but also CREB, and that these two transcription factors interact because NFkB silencing blocks the CREB effect and vice versa. It is thus possible that NFkB not only activates CREB, but that CREB participates in LPS-mediated NFkB activation for NRF1 expression.

Stress-related mtDNA damage leads to sustained mitochondrial ROS production (Suliman et al., 2005) that alters the expression of an array of genes associated with apoptotic phenotype and cell survival (Suliman et al., 2007b). Exposure to LPS alone, or LPS and TNF, in HL-1 cells activates NFkB and CREB, while stimulating the production of H2O2, a known regulator of NFkB nuclear translocation (Beg et al., 1993). In these cells, NFkB activation required mitochondrial H2O2 whereas Ca2+ had a minimal role. Mitochondrial-targeted catalase (mCAT) but not the Ca2+ chelator EGTA-AM blocked NFkB activation by the LPS signal, whereas after Ca2+ mobilization by TG, EGTA-AM, and mCAT blocked nuclear accumulation of p65 and cRel. Ca2+ chelation fully abrogated TG-mediated accumulation of phosphorylated CREB, but mCAT also partially blocked accumulation of phosphorylated CREB, placing importance on mitochondrial H2O2 in both NFkB and Ca2+-mediated CREB activation. Thus, the evidence in heart cells implicates only mitochondrial H2O2 production in the LPS- and TNF-induced nuclear translocation of NFkB and mitochondrial H2O2-associated Ca2+ mobilization in the nuclear translocation of pCREB. Both conditions are consistent with the redox regulation of NRF1 (Piantadosi and Suliman, 2006); however, the sites of intramitochondrial ROS production were not determined in this case. Moreover, the conditional participation of other redox mechanisms involving NFkB, for instance via NO and CREB (Dhakshinamoorthy et al., 2007), has not been excluded. Under comparable NFkB-activating conditions, IκK activation and accelerated loss of IκB inhibitory protein is frequently reported (Kamata et al., 2002).

Loss-of-function experiments indicated that LPS and TNF induce NRF1 transcription through NFkB-promoter and intronic-enhancement elements, thus providing an explanation for the prompt increases in NRF-1, an established integrator of nuclear-mitochondrial communication (Scarpulla, 2002), during the early phase of the antibacterial host response. Similarly, LPS and TNF increase NRF-1-regulated Tfam gene expression and subsequent increases in mtDNA-encoded COI and NDI mRNA, reflecting mitochondrial transcriptional activity befitting reports that LPS exposure doubles COI mRNA content in mouse cells (Chen et al., 2004). Following NRF-1 production, Tfam levels and mtDNA copy number also increase, which is necessary to support the capacity for oxidative phosphorylation (Scarpulla, 2008) and which might reduce oxidative stress (Stirone et al., 2005).

NRF1 induction through NFkB and CREB binding to intragenic DNA with enhancement of NRF-1-regulated Tfam transcription addresses the Tfam-regulated mitochondrial gene transcription and early mtDNA replication found in LPS-injured animals (Suliman et al., 2003), as well as the late restitution of mtDNA copy number after LPS exposure in Tlr4-null mice (Suliman et al., 2005). Tfam is required for efficient mitochondrial promoter recognition by DNA poly, and is necessary for maintenance of oxidative phosphorylation as well as for mtDNA replication during mitochondrial proliferation (Scarpulla, 2008). The NFkB-CREB signal integration for NRF1 function also reflects a rapid optimization of mitochondrial gene expression coincident with metabolic and inflammatory stressor influences on host defense and repair through classical NFkB activation, apparently without dependence on non-canonical mechanisms (Bakkar et al., 2008).

In conclusion, NRF1 promoter co-regulation by NFkB and pCREB combinatorial interactions enhanced by p65 binding to NRF1 intron 1 and with essential regulation by mitochondrial 

H2O2 represents the first known mechanism by which LPS-receptor-mediated signaling engages directly in the transcriptional control of mitochondrial biogenesis. The implications for rapid mitochondrial turnover and quality control are vital to cell survival during inflammatory pathogenesis, and interference with this mechanism would place cells at consequential risk for increased apoptosis and/or necrosis, particularly during exaggerated or prolonged activation of host antibacterial defenses.
Materials and Methods

Materials
Antibodies against p65, cRel, RelB (Cell Signaling), CREB, pCREB and TBP were obtained from Santa Cruz Biotechnology. All secondary and fluorescent antibodies were from Invitrogen. The mCAT vector was developed and characterized in our laboratory (Suliman et al., 2007a; Suliman et al., 2007b). Small interfering (si) RNA oligonucleotides were from Ambion. Mouse recombinant tumor necrosis factor-α (TNF), EGF-AM and E. coli LPS were from Sigma. Thapsigargin and BAY 11-7085 were obtained from BioMol (Plymouth Meeting, PA).

Mice
The studies were pre-approved by the Duke Institutional Animal Care and Use Committee; male C57BL/6 and p50−/− mice were obtained from Jackson Labs and used at 6–8 weeks of age. E. coli (serotype 086bK61, ATCC, Rockville, MD) was non-disruptively heat-inactivated (Suliman et al., 2005). Heat-killed bacteria were diluted with sterile 0.9% NaCl to a concentration of 5 x 10^6/ml and single 0.5 ml doses injected i.p. into mice. At the appropriate times, the livers were harvested and snap-frozen. For NFκB inhibition, mice were injected with BAY 11-7085 (20 mg/kg i.p. in 1% DMSO diluted with 0.9% NaCl). BAY 11 was administered twice to each animal, once 6 hours before E. coli injection and then 6 hours after E. coli.

Cell studies
HepG2 (human hepatocellular carcinoma) cells purchased from ATCC were maintained in RPMI 1640 medium (HyClone) containing 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Marine atrial H11032-1 cells, a generous gift from William C. Claycomb (LSU Medical Center, New Orleans, LA), were cultured in Claycomb medium with 10% FBS, 100 μM norepinephrine and 4 mM L-glutamine in gelatin- or fibronectin-coated flasks or plates. Cells were cultured at 37°C with 5% CO2 and 95% air. Cells were transfected with scrambled (negative control) or targeted siRNA using FuGene HD transfection reagent (Roche) and transfection efficiencies of 65-80% and gene suppression achieved. For pharmacological inhibition of NFκB in cells challenged with LPS+TNF, BAY 11-7085 (2 μg/ml) was used.

Bioinformatics profiling
Mouse and human Nrf1 loci were aligned and the extent of DNA sequence homology computed with the web-based Regulatory Visualization Tools for Alignment (vVISTA; www.gsd.lbl.gov/vista) (Loots and Ovcharenko, 2004; Loots et al., 2002). Promoter analysis was performed with consensus sequences for NFκB (NF-κB) and the CRE-binding sites. Liver extracts from Wt or BAY11-H11032−/− mice were obtained from Jackson Labs and screened for the presence of the insert by restriction enzyme digestion and confirmed by DNA sequencing.

Transient transfection and GFP reporter assays
HepG2 cells (5 x 10^4/well) were plated in 24-well plates and incubated overnight. Nrf1 promoter GFP constructs (1.0 μg) or CMV-GFP plasmid (0.1 μg, as positive control) were transfected into HepG2 cells using FuGene (Roche) according to the manufacturer’s instructions. The Glow-TOPO empty vector was used as a negative control (GFP0). 48 hours after transfection, cells were examined by fluorescence microscope on a Nikon fluorescence microscope and photomicrographs were captured with a charge-coupled device camera. Fluorescent intensities were recorded and analyzed on an ABI Prism 7000 sequence-detector system (Applied Biosystems). MtDNA-encoded cytochrome oxidase subunit 1 (MCO1) and NADH dehydrogenase subunit 1 (NAD1) mRNA were quantified by qRT-PCR and normalized to nuclear-encoded 18S rRNA (Suliman et al., 2007a; Suliman et al., 2007b). MtDNA copy number and respiratory proteins
MtDNA was determined by SYBR green quantitative PCR (qPCR). Fluorescence intensities were recorded and analyzed on an ABI Prism 7000 sequence-detector system (Applied Biosystems). MtDNA-encoded cytochrome c oxidase subunit 1 (MCO1) and NADH dehydrogenase subunit 1 (NAD1) mRNA were quantified by qRT-PCR and normalized to nuclear-encoded 18S rRNA (Suliman et al., 2007a; Suliman et al., 2007b).

Real-time PCR
qPCR was performed on an ABI PRISM 7000 Sequence Detection System with the comparative threshold cycle Ct method. PCR primers were designed (Applied Biosystems) and 18S rRNA served as an endogenous control. Quantification of gene expression was determined using the comparative threshold cycle Ct and RQ method.

Statistics
Grouped data are expressed as the means ± s.e. for n=6 replicates. Statistical significance was tested with the unpaired Student’s t-test or two-way analysis of variance using commercial software. Differences at P=0.05 were considered significant.

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The authors thank Craig Marshall, Susan Fields, Marta Salinas, and John Patterson for excellent technical assistance. Supported by R01 AI0664789 (C.A.P.). Deposited in PMC for release after 12 months.

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