Nitric oxide synthase-2 regulates mitochondrial Hsp60 chaperone function during bacterial peritonitis in mice

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ABSTRACT

Nitric oxide synthase-2 (NOS2) plays a critical role in reactive nitrogen species generation and cysteine modifications that influence mitochondrial function and signaling during inflammation. Here, we investigated the role of NOS2 in hepatic mitochondrial biogenesis during Escherichia coli peritonitis in mice. NOS2−/− mice displayed smaller mitochondrial biogenesis responses than Wt mice during E. coli infection according to differences in mRNA levels for the PGC-1α coactivator, nuclear respiratory factor-1, mitochondrial transcription factor-A (Tfam), and mtDNA polymerase (Poly). NOS2−/− mice did not significantly increase mitochondrial Tfam and Poly protein levels during infection in conjunction with impaired mitochondrial DNA (mtDNA) transcription, loss of mtDNA copy number, and lower State 3 respiration rates. NOS2 blockade in mitochondrial-GFP reporter mice disrupted Hsp60 localization to mitochondria after E. coli exposure. Mechanistically, biotin-switch and immunoprecipitation studies demonstrated NOS2 binding to and S-nitrosylation of Hsp60 and Hsp70. Specifically, NOS2 promoted Tfam accumulation in mitochondria by regulation of Hsp60–Tfam binding via S-nitrosylation. In hepatocytes, site-directed mutagenesis identified 237Cys as a critical residue for Hsp60 S-nitrosylation. Thus, the role of NOS2 in inflammation-induced mitochondrial biogenesis involves both optimal gene expression for nuclear-encoded mtDNA-binding proteins and functional regulation of the Hsp60 chaperone that enables their importation for mtDNA transcription and replication.

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Multiple organ failure in severe bacterial infections is a major cause of mortality [1], and nitric oxide (NO) produced by the inducible NO synthase (NOS2) is considered a damaging factor [2]. Among the deleterious effects of NO are mitochondrial damage and loss of electron transport function, generally coinciding with high rates of NOS2 activity, whereas physiological NO levels are usually deemed protective [3]. The calcium-independent NOS2 isoform has the highest capacity for L-arginine catalysis and is the principal source of NO in inflammation [4], but this requires enzyme induction during the host response, which in macrophages and other immune cells yields bactericidal levels of NO [5,6].

Quite apart from microbial killing, NOS2 is integral to host defense [7], but the enzyme's salutary effects are confounded by collateral cellular damage. NOS2−/− mice show variable susceptibility to infections, indicating a high specificity of action for NOS2 [8]. For instance, hepatic and pulmonary injury by lipopolysaccharide (LPS) in NOS2−/− mice is comparable to that of wild-type (Wt) mice [7], and NOS2 contributes to hypotension in LPS-induced shock [9], but NOS2−/− mice do not consistently demonstrate improved survival [7,10]. NOS2 also promotes tissue repair, for example, through angiogenesis [11], but these protective mechanisms, especially in the liver, which orchestrates critical early phase cytokine production, are poorly understood.

NOS2 produces vasodilation and hypotension via guanylate cyclase [9], but many toxic effects are generated by peroxynitrite (ONOO−) and related products [12]. NO also produces posttranslational protein effects via S-nitrosylation (SNO), and SNO protein function is exemplified by reversible modification of over 100 proteins [13]. However, loss of regulation leads to biological dysfunction via abnormal spatial or temporal interactions of NO with various target proteins [14].
In mitochondria, NO produces various effects on electron transport, including inhibition of Complex I by SNO protein formation and inhibition of Complex IV by the formation of NO-transition-metal complexes [15–17]. Respiration is inhibited by ONOO− [18] and by endogenous S-nitrosothiols, which S-nitroso[γ]lulate mitochondrial proteins [19]. Moreover, membrane SNO proteins, ordinarily reduced by glutathione, may persist when the mitochondrial glutathione pool becomes oxidized. Although excessive or prolonged NO exposure is proapoptotic [20], physiological NO is antiapoptotic, in part through cGMP activity and caspase-3 inhibition [21]. NO also promotes antiapoptotic gene expression, e.g., mtHsp70 (mortalin) opposes oxidant-induced apoptosis [22], and mitochondrial biogenesis [23–25].

The ambiguity of NOS2 as an immune defense factor led us to investigate its impact on hepatic mitochondria in gram-negative peritonitis in mice, in which hepatocellular injury is reflected by increases in serum transaminases, alkaline phosphatase, and total bilirubin, and the histology shows prominent inflammatory cell infiltration, microabscess formation, cholestasis, hepatocyte vacuolization, and autophagy [26,27]. In this setting, hepatic NOS2 induction is a protective factor [28], and we expected NOS2−/− mice to demonstrate contrasting effects on mitochondrial equipoise because functional damage from NO would be opposed by activation of mitochondrial biogenesis. NOS2−/− mice did develop multiple deficits, including limited up-regulation of several transcriptional regulatory elements for mitochondrial biogenesis, but, uniquely, showed an impaired S-nitrosoylation of Hsp60 that governed its regulatory elements for mitochondrial biogenesis. NOS2−/− mice did develop multiple deficits, including limited up-regulation of several transcriptional regulatory elements for mitochondrial biogenesis, but, uniquely, showed an impaired S-nitrosoylation of Hsp60 that governed its interactions with mitochondrial Tfam, the major mitochondrial DNA transcription factor, and Poly (A) and the catalytic core of the mtDNA polymerase. Thus, the work reveals the novel mechanistic principle that NOS2 facilitates Hsp60 chaperone interactions with the proteins required to maintain mtDNA transcription and replication after activation of the host antibacterial defenses.

Materials and methods

Mouse studies

The studies were conducted in 20- to 25-g male mice on a protocol approved by the Duke University Institutional Animal Care and Use Committee. Wt C57BL/6j and inbred genetically engineered NOS2−/− mice on a C57BL/6j background were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and maintained in a pathogen-free barrier facility. Liver bacteria were grown from lyophilized Escherichia coli (serotype 086a:K61; American Type Tissue Culture Collection, Rockville, MD, USA), quantified as described [29], and either implanted in a fibrin clot into the peritoneum [30] or heat-inactivated at 65°C and frozen at −80°C. Heat-killed E. coli were thawed once and diluted with sterile, endotoxin-free NaCl to a concentration of 1 × 109/ml and 0.5 ml was injected into the peritoneum. The mice also received 1.0 ml of 0.9% NaCl subcutaneously when the bacteria were administered.

Mitochondrial isolation

Liver mitochondria were prepared by two methods. For respiration studies, liver homogenates prepared using a Dounce homogenizer were centrifuged at 1000 g for 10 min and the supernatants placed on a 0.25% sucrose cushion and centrifuged at 15,000 g for 15 min. State 3 and State 4 respiratory capacity and respiratory control ratios (RCR) were measured at 35°C using calibrated Clark electrodes [31,32]. Purified mitochondria were obtained from the liver homogenate by discontinuous Percoll gradient centrifugation using a method for highly coupled organelles [33]. The mitochondria-rich band at the interface between Percoll layers was harvested, collecting organelles of the same density. Mitochondria were washed once, resuspended, and placed on ice. Mitoplast and outer membrane fractions were prepared using the method of Greenawalt [34]. Nuclei were obtained from liver homogenates by centrifugation at 1000 g for 20 min and then through 1.75 M sucrose at 40,000 g for 1 h. Protein content was determined using bichinonic acid with BSA as a standard (Pierce, Rockford, IL, USA).

Nuclear expression of mitochondrial genes

Cytoplasmic RNA was extracted with TRIzol RNA isolation kits (Invitrogen, San Diego, CA, USA) [31]. Total RNA (1 μg) was reverse-transcribed using oligo(dT) and with gene-specific primers for mouse NADH dehydrogenase subunits 1 (ND1) or cytochrome b. Real-time RT-PCR was performed on the ABI Prism 7000 using the SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). After PCR, the samples were subjected to melting curve analysis. The threshold cycle (Ct) was determined in the exponential phase and a ΔCt method used to quantify mRNAs levels for cytochrome b, ND1, NRF1, NRF2, PGC-1α, Pol, Tfam, and NOS2. Amplification efficiency was checked with an internal 18S rRNA reference over a range of 0.9–90 ng of RNA and gene expression was quantified using ABI Prism 7000 SDS software. Each sample was assayed in triplicate and the mean values are reported.

Mitochondrial DNA copy number

MtDNA was isolated from purified mitochondria and copy number quantified by real-time PCR on a 7700 sequence detector system (Applied Biosystems). Amplifications were performed on 10 ng total mtDNA using primers designed for cytochrome b (cyt b-s and cyt b-as) with ABI Probe Design software (Applied Biosystems). One copy of linearized pC2MT−ct b vector was used as a standard for simultaneously mtDNA quantification [35]. The cytochrome b probe, 5′-FAM-TTCTCCAGCAAGACTCAG-TAMRA-3′, contained FAM (6-carboxyfluorescein) at the 5′ end, as an reporter dye, and at the 3′ end, TAMRA (6-carboxytetramethylrhodamine) as a quencher dye selected from a highly conserved region of the mouse cytochrome b gene. Serial dilutions of 102 to 1010 copies of the cytochrome b plasmid were prepared to establish the standard curve. Samples were tested in triplicate for mtDNA at 1:100 and 1:1000 dilutions and the number of mtDNA copies was determined relative to known standards and expressed on a log scale.

Western analysis

Total, nuclear, and mitochondrial proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes [35]. Membranes were incubated with validated polyclonal rabbit antibodies against mouse PGC-1α, TFam, and Poly [31] or with antibodies to caspase-3 (Cell Signaling, Beverly, MA, USA), SOD2, or NOS2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). β-Actin was used as a loading control for total or nuclear protein and anti-porin for mitochondrial protein (Sigma, St. Louis, MO, USA). After five washes in Tris buffer with Tween20 (TBST), the membranes were incubated in horseradish peroxidase (HRP)-conjugated goat anti-rabbit or anti-mouse IgG (Amersham, Arlington Heights, IL, USA). Blots were developed using enhanced chemiluminescence (ECL) and protein quantification was performed on digitized images in the mid-dynamic range and expressed relative to the loading control.

Apoptosis assay

For in situ apoptosis studies, the anti-caspase-3 antibody (Cell Signaling) was used. After perfusion–fixation with 10% formalin, processing, and sectioning, apoptosis was assessed using the TUNEL assay kit and the manufacturer’s instructions (Promega, Madison, WI, USA). Alexa Fluor 594-labeled streptavidin was used to develop the signal for labeled nuclei. Photomicrographs were taken on a Nikon Eclipse 50i fluorescence microscope at 400× and the number of apoptotic nuclei was counted per 100 hepatocytes.
Mitochondrial proteins (100 µg) were incubated overnight at 4°C with 1 µg of Hsp60 antibody (Santa Cruz) or preimmune serum (negative control) followed by 2 h with 20 µl of protein A–Sepharose beads (Santa Cruz; prewashed and suspended 1:1 in phosphate-buffered saline). After incubation, the beads were washed four times with lysis buffer, and the proteins were separated by SDS–PAGE, transferred to Immobilon-P (Millipore), and analyzed by immunoblotting with anti-Poly, anti-Tfam, or anti-NOS2 antibodies (Santa Cruz). Horseradish peroxidase-conjugated secondary antibodies were applied and the signals developed with ECL. Hsp60 and Hsp70 coimmunoprecipitated proteins were checked by Western blotting with validated antibodies.

Results

The fibrin-clot model

Implantation of infected fibrin clots into the mouse peritoneum produces dose-dependent hepatic damage with focal necrosis and apoptosis by 24–48 h [30]. More pronounced and diffuse hepatic apoptosis was encountered in NO2−/− mice than in Wt mice, in which it was sporadic and closely associated with inflammatory foci (Fig. 1A). By day 3, nonneutrophilic TUNEL-positive cell counts in control and infected Wt mice were 2.1 and 5.5%, respectively, whereas the counts in NO2−/− mice were 3.4 and 11.2%, respectively (P<0.05 between strains). In NO2−/− mice, apoptosis involved caspase-3 activation (Fig. 1B), and lethality was higher in NO2−/− mice than in Wt mice (Fig. 1C). Because NO2−/− mice demonstrated early cell death and increased mortality, and differences in bacterial clearance in the mouse strains can have an impact on survival, we performed mechanistic studies with nonlethal heat-inactivated E. coli to eliminate this bias and improve the specificity for NO2 effects by delta-function activation of Toll-like receptor-4 (TLR4) [29,39].

Transcriptional program of mitochondrial biogenesis in Wt and NO2−/− mice

Transcriptional activation of nuclear-encoded activators of mitochondrial biogenesis was evaluated by real-time RT-PCR. An mRNA analysis of the NRF1 and NRF2 transcription factors and the PGC-1α nuclear coactivator of mitochondrial biogenesis indicated that all three responded in Wt and NO2−/− mice (Figs. 2A–2C), followed by transient mRNA elevations for two genes, Tfam and Polγ, required for mtDNA transcription and replication (Figs. 2D and 2E). The mRNA profiles for NRF1 and PGC-1α were attenuated in NO2−/− compared with Wt mice, and the responses were time-shifted by 1 day. NO2−/− mice showed lower mRNA levels than Wt mice for the mitochondrial transcriptional proteins in the first 3 days after challenge.

The differences in NRF1 and PGC-1α, which together regulate downstream Tfam and Polγ genes, were evaluated by nuclear Western analysis (Fig. 3A). By gel densitometry, the nuclear accumulation of NRF1 and PGC-1α was significantly delayed in NO2−/− mice by at least 1 day (Fig. 3B).

Mitochondrial function

The proliferation and function of liver mitochondria in Wt and NO2−/− mice were compared before and after E. coli administration in several ways. Because mtDNA damage can impair mitochondrial gene transcription, mtDNA content was measured by real-time PCR. In both lines of mice, hepatic mtDNA copy number decreased at day 1 after E. coli, and Wt but not NO2−/− mice recovered by day 2 (Fig. 4A). At day 3, the mtDNA deficit in NO2−/− mice remained significant, indicating clear differences between NO2−/− and Wt mice in the regulation of mtDNA copy number. To assess this difference, mRNA levels were assayed for two mtDNA-encoded
genes, cytochrome b (Cyt b) and ND1. Cyt b and ND1 mRNA levels increased significantly in Wt but not NOS2−/− mice, which is compatible with the differences in mtDNA content (Figs. 4B and 4C). In the fibrin-clot live bacteria model, hepatic State 3 respiration improved significantly over 3 days for both NADH- and FADH2-linked substrates in Wt but not in NOS2−/− mice (Fig. 4D). RCR was in the range 4–8, and although NOS2−/− mice showed a trend toward looser coupling at day 3, overall State 4 rates and respiratory coupling were not statistically different over the 3 days.

Mitochondrial proteins

The late recovery of mtDNA copy number in NOS2−/− mice may have reflected different rates of nitrosative damage and/or capacity to replicate mtDNA, e.g., because of compromised mitochondrial protein importation. First, we evaluated NOS2 localization after infection by Western analysis of whole homogenates (Fig. 5A) and liver mitochondria (Fig. 5B) and identified a significant fraction of the enzyme associated with mitochondria. As NOS2 lacks a known mitochondrial leader sequence, gentle digitonin stripping of the outer mitochondrial membrane was performed, and most of the enzyme was removed (Fig. 5C).

Next, the quantities of the mtDNA binding proteins Tfam and Polγ were examined by Western blot relative to porin (VDAC), a stable outer membrane protein (Fig. 6A). Unlike the mRNA levels, which in NOS2−/− mice were only modestly below Wt levels, mitochondrial Tfam and Polγ increased in Wt but remained unchanged in NOS2−/− mice. To assess mitochondrial oxidative stress, we checked hepatic SOD2 mRNA and mitochondrial protein levels and found that SOD2 mRNA levels increased by only ~50% in both strains after E. coli and then recovered (Fig. 6B, left). The mitochondrial SOD2 protein increased slightly more in Wt than in NOS2−/− mice, but overall, the changes were less than twofold (Fig. 6B, right).

This discrepancy suggested that NOS2 influences mitochondrial protein importation, which was examined using mRNA and protein for two critical chaperones, Hsp60 (Fig. 6C) and mtHsp70 (mortalin; Fig. 6D). The mRNA levels for these chaperones were similar in Wt and NOS2−/− mice after E. coli challenge, and protein expression was marginally increased relative to porin. Similar mitochondrial levels of both chaperones were detected in NOS2−/− and Wt mice (Figs. 6C and 6D); thus, differences in mitochondrial Hsp60 and Hsp70 protein regulation did not explain the failure of mitochondrial Tfam and Polγ to increase in NOS2−/− mice.

Fig. 1. Hepatic apoptosis and survival after live E. coli peritonitis in mice. E. coli inoculation was 1×10⁷ CFU. (A) TUNEL staining of liver sections of Wt and NOS2−/− mice pre- and 3 days post-fibrin-clot implantation. Top row, Wt mice showed occasional scattered small clusters and independent TUNEL-positive cell nuclei (red), whereas NOS2−/− mice show many stained nuclei throughout. Bottom row, TUNEL merged with DAPI nuclear staining. (B) NOS2−/− mice showed greater cleavage of hepatic caspase-3 than Wt mice. (C) NOS2−/− mice have a higher 7-day mortality than Wt mice (P<0.05).
Fluorescence microscopy

Mitochondrial Hsp60 was localized by fluorescence microscopy of fixed liver sections in mitochondrial reporter mice that express GFP constitutively in the mitochondria. Control sections revealed a typical monotonic distribution of mitochondria and modest Hsp60 staining. At 24 h after *E. coli* administration, strong Hsp60 localization to mitochondria was present, as shown in Supplemental Figs. S1A and S1B (yellow). However, when 1400W was administered to inhibit NOS2 before *E. coli* challenge, the induction of Hsp60 was heterogeneous and the protein distribution did not effectively match that of mitochondria (Supplemental Figs. S1C and S1D, orange). These colocalization data provided evidence that NOS2 facilitates functional integration of Hsp60 into the hepatic mitochondrial system.

![Fig. 2. NOS2 effects on transcriptional control of mitochondrial biogenesis.](image)

(A–C) Real-time RT-PCR analysis of nuclear-encoded transcriptional activators of mitochondrial biogenesis, NRF1 and NRF2, and the PGC-1α coactivator indicated increases in all three mRNA levels after *E. coli* challenge in Wt and NOS2−/− mice. NRF1 and PGC-1α transcript responses were attenuated and PGC-1α response was late-shifted in NOS2−/− mice. (D, E) Transient elevation of nuclear-encoded transcript levels for Polγ and Tfam, downstream genes involved in mtDNA transcription and replication. In NOS2−/− mice, transcript levels for these mitochondrial proteins were attenuated compared with the Wt responses (n = 4 mice for all time points; *P < 0.05 vs baseline; †P < 0.05 Wt vs NOS2−/−).

![Fig. 3. Nuclear Western analysis of NRF1 and PGC-1α proteins.](image)

Fig. 3. Nuclear Western analysis of NRF1 and PGC-1α proteins. (A) Nuclear protein levels for upstream regulators of Tfam and Polγ gene expression by Western analysis at 0, 1, 2, and 3 days after *E. coli* administration in Wt and NOS2−/− mice. (B) Densitometry confirms delayed nuclear enrichment of NRF1 and PGC-1α in NOS2−/− compared with Wt mice (n = 4 samples at each time point; *P < 0.05 vs baseline; †P < 0.05 Wt vs NOS2−/− mice).
NOS2-dependent Hsp60 association with Tfam and Poly

Levels of several other antioxidant antiapoptotic proteins in mitochondria were checked, i.e., thioredoxin-2, thioredoxin reductase-2, and peroxiredoxin-3, which are also involved in mitochondrial NO transfer (Fig. 7A). Mitochondrial protein levels for all three proteins increased on day 1 in Wt mice, but similar to Tfam and Poly, did not increase in NOS2−/− mice.

Mitochondrial protein–protein interactions among NOS2, Hsp60 (or mtHsp70), Tfam, and Poly were evaluated by protein immunoprecipitation and Western analysis before and at 1 day after E. coli challenge. Anti-Hsp60 was used to precipitate mitochondrial proteins, and strong NOS2 co precipitation was identified postchallenge (Fig. 7B; top). Hsp60 also associated with Tfam and Poly in Wt mice, but this association was diminished in NOS2−/− mice (Fig. 7B; second and third rows). Analogously, Tfam and Poly interacted similarly with Hsp70 protein (data not shown). By contrast, Hsp60 associated only weakly with SOD2, but SOD2 and Tfam strongly coprecipitated before and 1 day after E. coli in the mitochondria of both strains (Fig. 7C).

Biotin-switch assays

The biotin switch demonstrated constitutive hepatic Hsp60 and mtHsp70 SNO protein formation, but after E. coli administration, SNO levels for both proteins increased only in Wt mice (Fig. 8A; left). In contrast, SNO levels were stable or actually declined in NOS2−/− mice after E. coli (Fig. 8A). In isolated whole liver mitochondria, the low-molecular-weight NO donor CSNO fully S-nitros(yl)ated Hsp60 at 15 μM and Hsp70 at 50 μM CSNO (Fig. 8A; middle). Fig. 8A (right) also demonstrates that mitochondria of Wt control and NOS2−/− mice generate ascorbate-dependent Hsp60 and Hsp70 SNO formation in vitro.

Site-directed mutagenesis and mitochondrial localization of Hsp60

After the conversion of Cys to Ala in the Hsp60 KCE motif by site-directed mutagenesis, rat H4IIE hepatocytes were transfected with control or mutant Hsp60 (Fig. 8B). Mitochondrial immunoprecipitation studies demonstrated that native Hsp60 associates with mitochondria and binds Tfam after NOS2 induction by LPS/TNF-α administration. However, mut-Hsp60-expressing cells demonstrated neither tagged mitochondrial Hsp60 nor Tfam binding despite the presence of endogenous NOS2 (Fig. 8B). The inability of mut-Hsp60 to attach to mitochondria or bind to Tfam after NOS2 induction provided further evidence that the enzyme S-nitros(yl)ates Hsp60 and targets it to mitochondria.

Discussion

The role of NOS2 induction in the mammalian antibacterial defenses has been difficult to define since it became apparent that NO overproduction adversely affects outcome [7,10]. The systemic release of enteric bacteria activates the innate immune response primarily through TLR4 and classical NF-κB signaling, which generates early phase inflammatory cytokines and induces NOS2 [29,39,40]. Although NOS2 causes deleterious vasodilation and hypotension, in the liver, a sentinel organ of host defense, and particularly in the Kupffer cell, NOS2 is protective. Here, NOS2−/− mice demonstrated higher mortality in infective E. coli peritonitis compared with Wt mice as well as greater hepatic caspase-3 activation and apoptosis. Whether the mortality difference is related to mitochondrial damage is not clear, but the involvement of NOS2 in the prosurvival program of mitochondrial biogenesis is important because mtDNA depletion is associated with higher apoptosis rates in accordance with the known susceptibility to intrinsic apoptosis produced by a lack of NO [21].
NOS3 and NOS1 are known to participate in mitochondrial biogenesis, e.g., via cGMP-linked expression of the PGC-1α coactivator and its transcription factor partners; however, such a role for NOS2, apart from late transcriptional activation of the program in the heart in NOS2−/− mice, has not been established [41]. Here NOS2−/− mice displayed slower recovery of hepatic mtDNA copy number compared with Wt mice despite comparable early declines in copy number after E. coli challenge. Postchallenge mRNA levels for mitochondrial Cyt b and ND1 in NOS2−/− mice also did not respond, implying that the low mtDNA copy number interfered with mtDNA transcription. This could explain the restricted capacity of NOS2−/− mice to expand State 3 respiration rates compared with Wt mice, which has important implications for cell survival and organ failure, especially during continuing inflammatory stress.

The initial phase of this study described the effects of NOS2 deficiency on the major nuclear transcriptional regulatory factors for hepatic mitochondrial biogenesis. NOS2−/− mice did show modest induction of NRF1, NRF2 (GABPα), and PGC-1α, but the nuclear accumulation of NRF1 and PGC-1α protein was impaired. Any resulting NOS2-dependent differences in gene expression should contribute to a delay in recovery from organ damage; for instance, the delayed expression of Tfam and Pol γ mRNA probably contributed to the lack of accumulation of these proteins in the mitochondria. In combination, these events would have a braking effect on the mtDNA transcription and replication necessary for mitochondrial biogenesis.

The disparity between Wt and NOS2−/− mice in the mitochondrial content of the Tfam and Pol γ mtDNA-binding proteins, despite the comparable early mtDNA depletion, implicated NOS2 primarily in mtDNA recovery rather than in mtDNA damage in this model. Moreover, mitochondrial thioredoxin-2 and peroxiredoxin-3 increased relative to porin in Wt but not NOS2−/− mice, reflecting selective NO influences over mitochondrial protein composition [42,43]. Mitochondrial SOD2 levels, however, were not appreciably affected, presumably indicating that superoxide leak rates and retrograde signaling for angiogenesis [44] and mitochondrial biogenesis were comparable in the two strains [45].

Functional linkage of NOS2 to the mitochondrial transcriptome is also implicit in the different ratios of the transcriptome proteins to porin, a reasonably stable outer membrane protein [46]. The strong imbalance in mitochondrial Tfam/Pol γ protein levels between strains after E. coli is telling, as these proteins tripled in Wt mice but failed to increase in NOS2−/− mice. A general interpretation of this observation is that mitochondrial protein importation for maintenance of mtDNA copy number is governed by NO availability. Mitochondrial proteins are imported in several ways, but matrix proteins usually require transit peptides that target preproteins to a translocase system spanning the outer and inner mitochondrial membranes [47]. This highly ordered system also incorporates the outer membrane sorting and assembly machinery and uses energy from ATP or from the transmembrane proton gradient. The translocase guides unfolded proteins to the inner mitochondrial membrane.
Fig. 6. Mitochondrial Hsp Tfam and Poly proteins. (A) Tfam and Poly proteins in Wt and NOS2 \textsuperscript{−/−} mouse liver mitochondrial extract by Western blot relative to porin. These mitochondrial proteins increased significantly in Wt but not in NOS2 \textsuperscript{−/−} mice. Mitochondrial Hsp60 and Hsp70 content showed a small increase in Hsp60 relative to porin after \textit{E. coli} in Wt, but a minimal difference compared with NOS2 \textsuperscript{−/−} mice. Hsp70 levels were stable in both types of mice. (B) SOD2 mRNA increased on day 1 after \textit{E. coli} by 50%, but then returned to baseline in both lines. Mitochondrial SOD2 protein responded with less than a twofold increase in NOS2 \textsuperscript{−/−} and Wt mice. (C) Total hepatic mRNA and mitochondrial protein levels for Hsp60 increased comparably experiment-wide in both types of mice. (D) Total hepatic mRNA and mitochondrial protein levels for Hsp70 as in (C) (densitometry for A-D is \(n=4\) samples per time point; \(*P<0.05\) vs baseline; \(\dagger P<0.05\) vs baseline and vs NOS2 \textsuperscript{−/−}).
cytosolic preproteins inward via matrix chaperones, most importantly Hsp60 and mtHsp70, which are part of a poorly understood molecular motor [48]. Nonetheless, these chaperones clearly facilitate macromolecular assembly, including the initial folding as well as the refolding of polypeptides denatured by matrix stress.

Mitochondrial chaperones are also distinctly probiogenesis and antiapoptotic, but if Hsp60 accumulates in the cytosol, it can promote apoptosis by interacting with caspase-3 [49]. Here, Hsp60 and Hsp70 mRNA levels increased after E. coli, but the protein levels remained stable or increased slightly in both strains, implying that lack of translation of these chaperones did not explain why NOS2−/− mice failed to maintain mitochondrial transcriptome protein levels.

NOS2 lacks a canonical mitochondrial leader sequence, but still associates periodically with mitochondria [50] and here, after E. coli exposure, was found loosely attached to mitochondria in amounts commensurate with those in total liver homogenate. NOS2 is active at the mitochondrial interface will require better resolution. It is reiterated that this does not require a matrix NOS [51,52], the presence of which is controversial [53], but simply an association of NOS2 with the outer mitochondrial membrane.

The comparative stability of Hsp60 and 70 mitochondrial protein levels suggested that NOS2 has a distributive role in the chaperone function. Like the other NOS isoforms, NOS2 is capable of S-nitros(yl)ation of appropriate proteins, although by biotin switch, constitutive S-nitrosyl-Hsp60 and -Hsp70 was detected that was quantitatively indistinguishable from Wt controls in NOS2−/− mice. After E. coli, however, mitochondrial Hsp60 S-nitros(yl)ation increased only in Wt mice. This finding may reflect a basal compensatory mechanism in NOS2−/− mice that does not respond to activation of the LPS receptor, as in Wt mice. The fall-off in Hsp60–Tfam binding in stressed mitochondria in NOS2−/− mice may simply indicate that Tfam protein is lost by degradation or mitophagy at a faster rate than it can be imported.

Although both Hsp60 and Hsp70 showed greater S-nitros(yl)ation and association with mitochondrial Tfam in Wt than in NOS2−/− mice after E. coli, we focused on Hsp60 because the protein in intact mitochondria could be S-nitrosylated at low micromolar concentrations of CSNO. Hsp60 contains three cysteines (UniProt Knowledgebase; Swiss-Prot/TrEMBL), but only 237Cys is found in a motif (KCE) amenable to SNO formation [13]. MtHsp70 has four cysteines, one in a SNO motif at 317Cys, whereas all four cysteines in Tfam are in a distant site-directed mutagenesis for Hsp60 237Cys, by which this residue, lost by degradation or mitophagy at a faster rate than it can be imported.

Fig. 7. Mitochondrial Hsp60 interactions with Tfam and Poly protein. (A) Mitochondrial antioxidant, antiapoptotic proteins thioredoxin-2 (Trx2), thioredoxin reductase-2 (TrxR2), and peroxiredoxin-3 (PrX3) were increased as shown by Western analysis in Wt mice but failed to respond in NOS2−/− mice. (B) NOS2 protein was not detectable in mitochondria prechallenge, but coprecipitated with mitochondrial Hsp60 at day 1 post-E. coli (top gel). Hsp60 coprecipitated with Tfam and Poly before, but more strongly at 1 day postchallenge in Wt mice. In NOS2−/− mice, the Hsp60 association was unchanged or declined after challenge (second and third gels). (C) In contrast, in both lines of mice, mitochondrial Hsp60 coprecipitated weakly with SOD2 (left four lanes), whereas SOD2 coprecipitated strongly with Tfam before and after challenge (right four lanes).
activator, which among other genes regulate the nuclear-encoded expression of mitochondrial transcriptome proteins. Although the impact of NO regulation of mitochondrial biogenesis on organ function is not yet resolved, NOS2 clearly enhances S-nitrosylation of Hsp60, which improves the mitochondrial targeting of the Tfam protein. Conversely, failure to S-nitrosylation of Hsp60 interferes with intramitochondrial trafficking of the critical mtDNA binding proteins required for mtDNA maintenance. Therefore, we surmise that NO2 induction supports mitochondrial function by ensuring mtDNA transcription and replication, which maintains and expands cellular respiratory capacity. By implication, interference with NOS2 after innate immune activation would limit the cell’s ability to adjust its capacity for oxidative phosphorylation and predispose to apoptosis and organ dysfunction during progressive inflammation.

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Appendix A. Supplementary data


