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Reduced Nitric Oxide Synthase 2 (NOS2) Promoter Activity in the Syrian Hamster Renders the Animal Functionally Deficient in NOS2 Activity and Unable to Control an Intracellular Pathogen[1]

Luis E. Perez,*† Bysani Chandrasekar,*† Omar A. Saldarriaga,†‡ Weiguo Zhao,*† Lourdes T. Arteaga,*† Bruno L. Travi,§ and Peter C. Melby[2]*†‡

Progressive disease in the hamster model of visceral leishmaniasis, caused by *Leishmania donovani*, in contrast to infection in mice, mimics the progressive disease observed in untreated humans. During progressive infection in hamsters, there was a vigorous type 1 cellular immune response, which is typically associated with control of infection, suggesting that there was ineffective IFN-γ-mediated macrophage activation. Indeed, at the site of infection, hamsters did not express NO synthase 2 (NOS2), which is the primary mechanism for control of infection in mice. Furthermore, in striking contrast to mouse macrophages, IFN-γ-activated hamster macrophages did not did not express NOS2 nor generate NO, and were unable to restrict the replication of intracellular *L. donovani*. The absent hamster NOS2 expression was not the result of *NOS2* gene deletion and the NOS2 cDNA had an intact open reading frame. Furthermore, the impaired transcription of NOS2 mRNA was selective and not due to global impairment of IFN-γ signaling (members of the IFN-γ-signaling pathway were expressed and functional and IFN-γ up-regulated several primary and secondary response genes). Strikingly, the proximal hamster NOS2 promoter, like the human ortholog, had >20-fold less basal and IFN-γ/LPS-inducible activity than the corresponding mouse promoter. Thus, reduced basal and IFN-γ-induced activity of the hamster NOS2 transcriptional unit, which is unique to this small animal and similar to the human counterpart, accompanies the inability of the animal to control an intracellular pathogen. ***The Journal of Immunology,* 2006, 176: 5519–5528.**

In humans, active visceral leishmaniasis (VL),[3] caused by the intracellular protozoan *Leishmania donovani*, is a progressive, potentially fatal infection characterized by chronic fever, hepatosplenomegaly, pancytopenia, and profound cachexia. Unfortunately, VL remains a significant cause of morbidity and mortality in the developing world; hundreds of thousands of people have died in recent years in epidemics in Sudan and India.

There is a major deficit in understanding of the molecular and cellular determinants underlying VL pathogenesis. Most studies aimed at dissecting VL pathogenesis have used the murine infection model, but the course of disease, and potentially its pathogenesis in mice, differ significantly from that in humans. Visceral *L. donovani* infection in the murine model is characterized by an early increase in parasite burden, but over the course of 4–8 wk, the infected mouse mounts a type 1 $CD4^+$ and $CD8^+$ T cell response that leads to control of the infection, primarily through the up-regulation of inducible NO synthase 2 (iNOS or NOS2) and generation of NO in the spleen and liver (1–3).

Because many of the clinicopathological features of the Syrian hamster (*Mesocricetus auratus*) model of VL mimic active human disease and differ significantly from the mouse model, we have used this unique model to dissect the immunopathogenic mechanisms underlying VL. In a recent report (4), we demonstrated that despite progressive disease, the hamster mounts a vigorous type 1 cellular immune response, an immunological event that is typically associated with disease control and resolution. This paradoxical finding, which was reminiscent of findings in humans with active VL (5, 6), suggested that the inability to control parasite replication could be related to ineffective IFN-γ-mediated induction of macrophage effector function. Indeed, the expression of iNOS (NOS2), which is the primary mechanism by which mice control *Leishmania* infection (1, 7–13), was absent during the progressive course of disease in hamster VL (4). This is in agreement with a prior report of an absence of elevated serum nitrate in hamsters with VL (14), but contrasts with a brief report that demonstrated elevated nitrite production by spleen cells and PBMCs isolated from hamsters infected with *L. donovani* (15). Although there has been substantial debate over the role of NOS2 expression and generation of NO in human macrophage antimicrobial function (16, 17), it is clear that activated human monocytes/macrophages express NOS2 and produce NO at much lower levels than is observed in activated mouse macrophages (18–20).

The contrast in NOS2 expression in human and mouse macrophages has prompted intense interest in elucidating the molecular determinants underlying NOS2 regulation. NOS2 is controlled primarily at the transcriptional level, and based on in vitro studies, the

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[3] Abbreviations used in this paper: VL, visceral leishmaniasis; iNOS, inducible NO synthase; NOS2, NO synthase 2; ISRE, IFN-stimulated response element; GAS, IFN-γ-activated site; HIFCS, heat-inactivated FCS; BHK, baby hamster kidney; MMLV, Moloney murine leukemia virus; IRF, IFN regulatory factor; HPRT, hypoxanthine phosphoribosyltransferase.

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prevailing paradigm is that differences in the *cis* elements between the human and mouse promoters account for the differences in expression. Most of the transcriptional regulatory elements of the mouse *NOS2* gene are found within 1.6 kb upstream of the start codon (21), which includes the basal promoter (region I) and enhancer (region II) regions (22). NF-κB elements in both the basal and enhancer regions, and the IFN-stimulated response element (ISRE) and IFN-γ-activated site (GAS) in the enhancer region are critical to the induction of murine NOS2 by IFN-γ/LPS (23–26). Differences in DNA sequence of the ISRE and GAS located in region II of the human promoter compared with the mouse sequence contribute to its unresponsiveness to LPS and IFN-γ stimulation (22).

Given the apparent similarities of active VL in humans and hamsters, and the undetectable NOS2 response to *L. donovani* infection in hamsters, we sought to use this model to understand the mechanisms behind the impaired IFN-γ-mediated NOS2 expression. In this present study, we show that, unlike mouse macrophages, hamster macrophages do not generate NO in response to stimulation with IFN-γ/LPS or IFN-γ/*Leishmania*, and are unable to control intracellular *Leishmania* replication. The lack of IFN-γ/LPS-induced NO production was due to a defect in the transcriptional activation of NOS2, and not due to a global defect in IFN-γ signaling. Luciferase reporter assays demonstrated that the hamster NOS2 promoter, like the human NOS2 promoter, has reduced basal and IFN-γ/LPS-induced activity compared with the mouse promoter. Thus, the Syrian hamster provides a unique small animal model for study the immunopathogenesis of intracellular pathogens in the context of low basal and inducible NOS2 expression.

Materials and Methods

Hamsters and mice

Six- to 8-wk-old outbred Syrian golden hamsters (*Mesocricetus auratus*) and 6-wk-old BALB/c mice were obtained from Charles River Laboratories and maintained in a specific pathogen-free facility. Animals were handled according to local and federal regulations and research protocols were approved by our Institutional Animal Care and Use Committee.

Macrophage isolation

Resident peritoneal macrophages were isolated from mice or hamsters by peritoneal lavage with DMEM containing 2% heat-inactivated FCS (HIFCS) and cultured in complete medium (DMEM containing 10% HIFCS, 2 mM glutamine, 50 μM 2-ME, 20 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin). Bone marrow cells were isolated by flushing the marrow cavity of both femurs with ice-cold DMEM containing 2% HIFCS. The bone marrow cells were cultured at 5×10^6 cells/ml for 5 days in complete medium containing 50% conditioned medium from LA/9 cells. Splenic macrophages were isolated by adherence to plastic culture dishes.

Parasites and macrophage infection

L. donovani (MHOM/SD/001S-2D) promastigotes were cultured as described previously (27). Stationary phase promastigotes obtained from 5- to 6-day-old cultures were opsonized with fresh, normal mouse or hamster serum (20% in DMEM) for 1 h at 37°C and 5% CO_2, washed, and used immediately for the macrophage infections.

To assess intracellular parasite killing, the macrophages were first cultured for 12–16 h with or without the addition of recombinant murine IFN-γ (100 U/ml) or purified recombinant hamster IFN-γ (100 U/ml). The macrophages were infected with opsonized promastigotes in suspension for 2 h and cultured in complete medium at 2×10^5/100 μl/well in chamber slides (Labtek Chamber Slide; Nalge Nunc International). After 2 h, the nonadherent cells and remnant-free parasites were removed by washing, and the adherent macrophages were cultured with fresh medium $\pm$ IFN-γ for 2, 24, 48, and 72 h. The parasite burden was determined by fluorescence microscopy by staining with serum derived from 56-day infected hamsters (diluted 1/200 in PBS + 5% FBS) and then with a Cy2-labeled rabbit anti-hamster IgG (diluted 1/50 in PBS + 5% FBS; The Jackson Laboratory). Macrophage nuclei were stained with propidium iodide (20 μg/ml in PBS) for 5 min. The amastigotes per 200 macrophages were counted by fluorescence microscopy.

Expression and purification of hamster IFN-γ

Recombinant hamster IFN-γ, engineered to remove the C-terminal 17 aa because we found this to significantly increase the biological activity (28) (W. Zhao and P. C. Melby, unpublished data), was stably expressed in CHO cells, and supernatants were collected after culture for 48 h. Recombinant hamster IFN-γ, lacking the signal sequence, was also expressed in *Escherichia coli* as a 6× histidine fusion protein in the pET-30b plasmid (Novagen) and purified from urea-solubilized inclusion bodies and metal affinity chromatography (Talon; BD Clontech). The specific activity of the purified rIFN-γ was determined by titration in a vesicular stomatitis virus assay with 1 U of activity defined as the amount of IFN-γ that gave 50% maximal antiviral activity.

NO production by activated macrophages

Resident peritoneal macrophages were cultured in compete medium with supernatants from Con A-stimulated spleen cells (50% v/v), or with 20–100 U/ml rIFN-γ. In some cultures, 2 μg/ml LPS or washed *L. donovani* promastigotes were added to IFN-γ-primed macrophages as a triggering stimulus. NO (nitrite/nitrate) production was determined after 24 h in cell culture medium by the Griess reaction (Nitrate/Nitrite Assay kit (Cayman Chemical). The limit of detection in the NO assay was 0.8 μM.

Determination of hamster IFN-γ-signaling activity

The capacity of hamster IFN-γ to transduce a signal through the IFN-γ receptor was determined using a GAS- and ISRE-luciferase reporter system (PathDetect GAS *cis*-Reporting System; Stratagene). The GAS_4-luciferase reporter plasmid was modified by insertion of a neomycin resistance gene. Baby hamster kidney (BHK) fibroblasts were stably transfected with Gas-Luc plasmid or transiently transfected with the ISRE-luciferase reporter plasmid. Following stimulation of the transfected cells with IFN-γ, the cells were washed with PBS and lysed, and the luciferase activity was measured.

Cloning and determination of expression of IFN-γ-inducible genes

BHK fibroblasts were stimulated with hamster IFN-γ and the total RNA isolated (Absolutely RNA; Stratagene). One microgram of RNA was reverse transcribed with Moloney murine leukemia virus (MMLV) reverse transcriptase and random hexamers. The cDNA was amplified with a mixture of *Taq* and Pfu polymerases (15/1) and the following primers: MHC class IIα forward, 5′-CTGGGTCAGCCCAACACC-3′, reverse, 5′-CAGTGCTCCACCTTGCAGTC-3′; STAT1 forward, 5′-ATGGAAATCAGACAGTACCTG-3′, reverse, 5′-CATGTTCATCACTTTTGTGTG-3′; IFN regulatory factor-1 (IRF-1) forward: 5′-CATGCCHATCACTCGRATGCG-3′, reverse, 5′-TARCTGCTGTGGTCATCAGG-3′. The amplified DNA was cloned into the pCR2.1 TOPO vector (Invitrogen Life Technologies) and identified by sequencing and search of the National Center for Biotechnology Information (NCBI) database using the basic local alignment search tool algorithm (29). These partial cDNAs were then used to isolate cDNAs containing the complete coding region from a Syrian hamster spleen cell cDNA library in pCMV (DNA Technologies). The hamster NOS2 cDNA was cloned from normal spleen tissue using multiple degenerate primers whose sequences were based on regions of homology in the NOS2 sequences of other species. The 3′ end of the cDNA was isolated by rapid amplification of cDNA ends (3′ RACE System; Invitrogen Life Technologies). Overlapping amplicons were aligned, and a single cDNA that included the full-length coding region was amplified using the following primers: NOS2 forward: 5′-AAACCTCTCAGCCACCCTGG-3′, reverse, 5′-GAGTCCGTCACTGAATTCTG-3′. The GenBank accession numbers for the cloned hamster cDNAs are as follows: STAT1 (DQ092343), IRF-1 (DQ092344), MHC class IIα (DQ092501), NOS2 (DQ355357).

The splenic expression of IFN-γ, IFN-γR1, STAT1, IRF-1, MHC class IIα, and NOS2 mRNA was determined by Northern blotting in uninfected and *L. donovani*-infected hamsters as described previously (4). Hybridization with the hypoxanthine phosphoribosyltransferase (HPRT) probe was used to assess loading equivalency and RNA integrity. In addition to the MHC class II, STAT-1, and IRF-1 cDNA probes described above, the hamster IFN-γ and NOS2 cDNAs (4) and the 1.7-kb human IFN-γR1 cDNA (ATCC 59872; American Type Culture Collection) were used. The expression of NOS2 and IFN-γ-inducible genes in resting and IFN-γ-stimulated hamster and mouse peritoneal macrophages (2×10^6 cells) was

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determined by Northern blotting or nuclear run-on assay as described previously (4, 30).

The expression of IRF-1 and STAT-1 in adherent spleen cells was determined in uninfected and 6-wk *L. donovani* infected female hamsters. Spleen cells were isolated by gentle teasing, the RBC lysed, and the remaining cells ($1–4 \times 10^7$/well; 6-well plate) allowed to attach for 4 h at 37°C/5% CO_2. The wells were washed three times with warmed PBS to remove nonadherent cells and the adherent monolayer lysed with 300 μl of RLT lysis buffer. RNA was isolated using the Qiagen RNeasy mini kit according to the manufacturer's directions. Two hundred nanograms of total RNA was then reverse transcribed with MMLV reverse transcriptase and random hexamers and 1/10 (uninfected) or 1/20 (infected) dilutions of the cDNA were amplified by PCR (35 cycles of 95°C/55°C/72°C) using the following primers: STAT1 forward, 5′-AAGACTGGGAGCACGCTGC-3′, reverse, 5′-TCTTCGCTTCCACTCCACTAACTC-3′; IRF-1 forward: 5′-ATGCCTATCACTCGGGTGC-3′, reverse, 5′-TCAGGCAGAGTGG AGCTGC-3′. Amplification of hamster γ-actin, used to assess equivalency of RNA concentration and integrity, was accomplished with the following primers: γ-actin forward, 5′-AGGAGCACCCGGTGCTTCTG-3′, reverse, 5′-TGGTGAAGCTGTAGCCTC-3′. In initial experiments, we used a range of cDNA dilutions to determine a dilution that fell within the exponential portion of the amplification curve. Amplification products were separated and visualized on an ethidium bromide-stained agarose gel.

Determination of GAS- and ISRE-binding activities

GAS- and ISRE-binding activities were measured by EMSA as described previously (31) using adherent spleen cells from 6-wk *L. donovani*-infected female hamsters. Spleen cells were isolated as described above and either left untreated or stimulated with hamster IFN-γ (50 U/ml) and LPS (100 ng/ml) and the nuclear proteins were extracted. The nuclear protein (5 μg) was incubated with end-radiolabeled ([γ-^{32}P]ATP) GAS and ISRE double-stranded consensus oligonucleotides (GAS sense 5′-AGTTTCATATTA CTCTAAATC-3′ (Santa Cruz Biotechnology) and ISRE sense 5′-CTAGTTTCACTTTCCCTAG-3′ (Stratagene)). Controls included absence of nuclear protein extract, competition with 100-fold molar excess unlabeled consensus oligonucleotide, or competition with 100-fold molar excess unlabeled mutant oligonucleotide ($ISRE_m$-sense, 5′-CTAGATCTACTTTCCCTAG-3′/ GAS_m-sense, 5′- AGTGGTCTATTACTCTAAATC-3′; mutant sequence underlined). Following incubation of the nuclear protein and oligonucleotides the labeled complexes were separated by 4% PAGE, the gels dried, and the signals were visualized by autoradiography.

Isolation of the hamster proximal NOS2 promoter

A 199-bp fragment from the hamster NOS2 5′ UTR was PCR amplified from hamster genomic DNA using the following primers: forward, 5′-GCAGAATGTGACCATCATGG-3′ and reverse, 5′-CTCKAYCTGRAG TAGTAGAA-3′. The amplified fragment, which showed 90% identity with the sequence of the mouse NOS2 5′ UTR, was used to isolate a 20-kb DNA fragment from a genomic Syrian golden hamster library (λ DASH II phage library; Stratagene). A 2-kb fragment from the 3′ end (proximal NOS2 promoter) was sequenced, which can be found under GenBank accession number DQ355358.

Construction of the luciferase reporter vectors with the hamster, mouse, and human proximal NOS2 promoters

The proximal promoter and 5′-flanking regions of the hamster, mouse, (GenBank accession number L23806; plasmid provided by Dr. W. Murphy, University of Kansas Medical Center, Kansas City, KS) (21), and human (GenBank accession number L36031; plasmid provided by Dr. V. Laubach, University of Virginia, Charlottesville, VA) (22) *NOS2* genes were amplified and cloned into the pGL3-basic luciferase reporter vector (Promega). The constructs included a 1213-bp fragment of hamster DNA (see Fig. 4, construct A, nt −1146 to +67), the 1207-bp fragment of mouse DNA (see Fig. 4, construct B, nt −1140 to +67), and the 1407-bp fragment from human DNA (nt −1340 to +67). Each cDNA included the regions corresponding to the basal promoter (region I) and enhancer region (region II) reported for the mouse NOS2 promoter (21).

To further define the region responsible for differences in activity of the mouse and hamster NOS2 promoters, a number of constructs were created by truncation and/or swapping of DNA fragments between the mouse and hamster promoters. The correct sequence of the various constructs was confirmed and the promoter activity was determined in the pGL3-basic luciferase reporter vector. A schematic of the different constructs is included in Fig. 4.

Transient transfection and luciferase reporter assay

Mouse macrophages (RAW 264.7 cell line) suspended at 2×10^7/ml in complete medium were electroporated with 10 μg of endotoxin-free plasmid DNA in a 0.4-cm electroporation cuvette at 300 V, 960 μF using a Bio-Rad Gene Pulser. Each cell sample was cotransfected with 1 μg of endotoxin-free *Renilla* luciferase vector (pRL-null; Promega) to normalize any difference in transfection efficiency. Transfected cells from two separate electroporations were pooled and cultured for 72 h. Cells were then stimulated for 8 h with recombinant murine IFN-γ (100 U/ml), LPS (10 ng/ml), or both and the cells were harvested for the dual-luciferase assay (Promega).

Statistical analysis

Statistical significance of differences between means of groups was determined by two-sample *t* test assuming unequal variance.

Results

IFN-γ-activated hamster macrophages have impaired capacity to kill intracellular L. donovani

Murine macrophages, when activated by IFN-γ, are able to control intracellular replication of *Leishmania* amastigotes, primarily through a NOS2-dependent mechanism (9–11). We compared the capacity of hamster and murine resident peritoneal macrophages to control intracellular *L. donovani* when activated with IFN-γ (Fig. 1). For all experiments, the biological activity of the recombinant hamster IFN-γ was confirmed by its capacity to induce signaling in a GAS-luciferase reporter assay (for example see Fig. 3*B*) and in an antiviral assay (data not shown). Infection of IFN-γ-primed mouse resident peritoneal macrophages with complement-opsonized *L. donovani* promastigotes resulted in a reduction in the number of parasites after 24, 48, or 72 h ($p \leq 0.012$). In contrast,

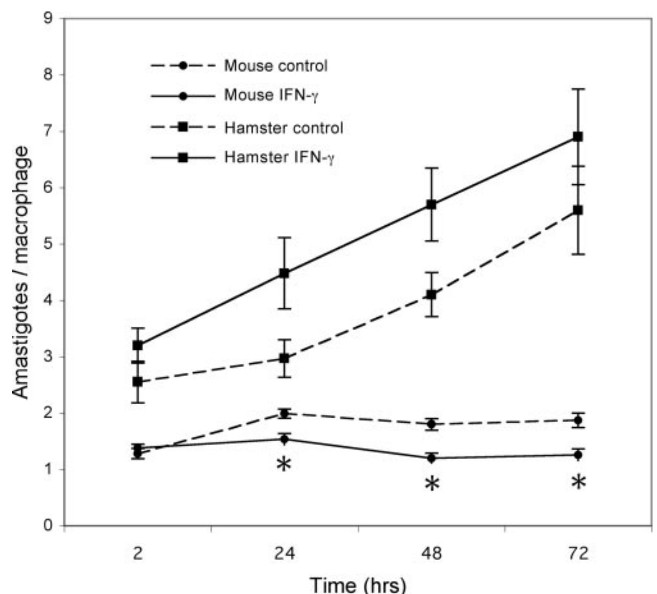

FIGURE 1. Impaired killing of *L. donovani* by IFN-γ-activated hamster macrophages. Resident peritoneal macrophages were isolated from mice and hamsters ($n = 6$/group) and cultured for 12–16 h with or without the addition of purified recombinant mouse or hamster IFN-γ (100 U/ml). The macrophages were then infected for 2 h with *L. donovani* at a ratio of two promastigotes per cell and then washed to remove free parasites. The infected macrophages were cultured in the presence or absence of rIFN-γ and the parasite burden was assessed by immunofluorescence after 2, 24, 48, and 72 h. The data (mean ± SEM amastigotes per macrophage) are from a single experiment representative of six independent experiments. *, Significant differences ($p \leq 0.012$) in parasite burden between control and IFN-γ-activated mouse macrophages.

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there was no reduction in parasite load when IFN-γ-primed hamster resident peritoneal macrophages were similarly infected (Fig. 1). In fact, in six independent experiments, the permissiveness of hamster macrophages to infection with *L. donovani* was evident by 1) the consistently higher parasite burden at 2 h postinfection in hamster (IFN-γ primed or unprimed) compared with similarly treated mouse macrophages ($p \leq 0.01$), and 2) the significant increase in parasite burden in IFN-γ-primed or unprimed hamster macrophages over time ($p \leq 0.015$ for 48 and 72 h vs 2 h).

L. donovani-*infected hamsters and IFN-γ-activated hamster macrophages do not express NOS2 mRNA or produce NO*

The results of the aforementioned macrophage infections mimicked our in vivo findings (4) that demonstrated that despite abundant IFN-γ expression (Fig. 2*A*), infected hamsters developed progressive disease. Furthermore, in new experiments, we confirmed that despite prominent expression of IFN-γ, no NOS2 mRNA expression was detectable by Northern blot (Fig. 2*A*). In light of these observations, we determined the capacity of isolated hamster macrophages to transcribe NOS2 mRNA and generate NO in response to cytokines plus LPS or *Leishmania*. When mouse peritoneal macrophages were incubated in the presence of Con A-activated mouse spleen cell supernatant (which have a high concentration of IFN-γ) plus LPS, a 9-fold increase in NO production was observed ($p < 0.0001$). Stimulation of mouse macrophages with supernatants from cells transfected with mouse IFN-γ cDNA plus LPS, or purified mouse rIFN-γ (100U/ml) plus LPS, resulted in a 15- and 17-fold increase in NO production, respectively ($p < 0.0001$; Fig. 2*B*). In striking contrast to mouse macrophages, NO production was not detected in hamster macrophages stimulated with Con A-activated hamster spleen cell supernatant plus LPS, or supernatants from cells transfected with hamster IFN-γ cDNA plus LPS, or purified hamster rIFN-γ (100 U/ml) plus LPS ($p > 0.2$; Fig. 2*B*). LPS by itself did not induce significant NO production, but LPS-induced signaling, measured by TNF-α production and IL-1β expression, was intact in hamster

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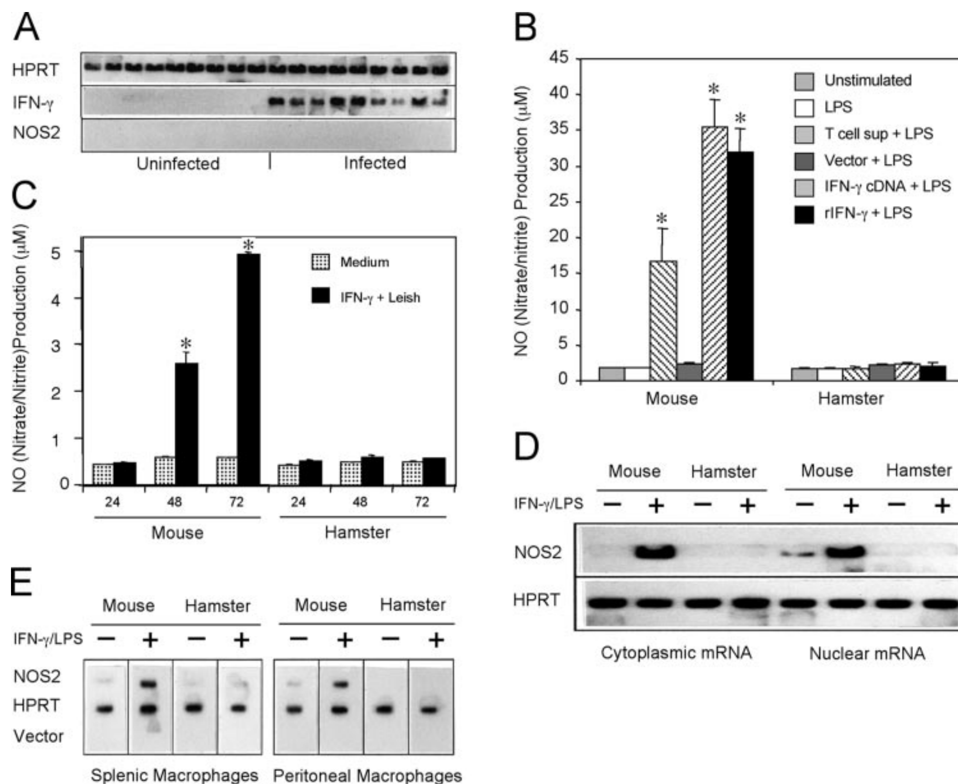

FIGURE 2. Impaired IFN-γ-induced NOS2 mRNA and NO in hamster macrophages. *A*, Absent splenic expression of NOS2 mRNA despite prominent expression of IFN-γ. IFN-γ and NOS2 expression in uninfected or day-56 *L. donovani*-infected hamsters ($n = 9$/group) was determined by Northern blotting of 30 μg of hamster spleen RNA. Loading equivalency and RNA integrity was assessed by hybridization with the HPRT cDNA. Each lane represents mRNA isolated from a single animal. *B*, Absent IFN-γ/LPS-induced production of NO by hamster macrophages. Mouse and hamster peritoneal macrophages were cultured with medium alone (unstimulated), LPS alone, 25% syngeneic Con A-activated T cell supernatant + LPS, supernatants from cells transfected with the syngeneic hamster or mouse IFN-γ cDNA in pcDNA3.1 (or empty vector control) + LPS, or syngeneic purified rIFN-γ (100 U/ml) + LPS. The release of NO into the culture medium was measured by the Griess reaction. The supernatants from IFN-γ-transfected cells were added to the macrophages at a 30% v/v concentration. The data shown (mean ± SEM of the total nitrate/nitrite concentration in micromoles) are from a single experiment representative of six independent experiments. *, Significant differences ($p < 0.0001$) between control and IFN-γ/LPS-induced NO production. *C*, Absent IFN-γ/*Leishmania*-induced production of NO by hamster macrophages. NO production from mouse and hamster peritoneal macrophages that were cultured in the presence of medium alone, or rIFN-γ (100 U/ml) + viable *Leishmania* promastigotes (five parasites per macrophage) for 24, 48, and 72 h was determined as described above. The data shown are representative of two independent experiments. *, Significant differences ($p \leq 0.003$) between control and IFN-γ/LPS-induced NO production. *D* and *E*, Absent IFN-γ/LPS-induced expression of NOS2 mRNA in hamster macrophages. Mouse and hamster peritoneal macrophages were cultured as described above in the presence of medium alone, or rIFN-γ + LPS for 4 h. *D*, Northern blot analysis of expression of cytoplasmic or nuclear NOS2 mRNA in hamster and mouse macrophages in response to IFN-γ + LPS. The data shown are from a single experiment representative of two independent experiments. *E*, Nuclear run-on assay analysis of NOS2 transcription in mouse and hamster macrophages in response to IFN-γ + LPS. Hybridization of [^{32}P]dCTP-labeled nuclear RNA to the hamster NOS2 and HPRT cDNAs (in TOPO 2.1 plasmid), or the empty plasmid (vector) was detected by autoradiography. The data shown are from a single experiment representative of two independent experiments.

peritoneal macrophages (data not shown). Furthermore, *Leishmania* promastigotes triggered NO production from IFN-γ-primed mouse macrophages (an 8-fold increase over unstimulated macrophages, $p \leq 0.003$ at 48 and 72 h), but there was no increase in NO production from IFN-γ-primed, *Leishmania*-triggered hamster macrophages (Fig. 2*C*).

Impaired NOS2 expression and NO production are due to a defect in transcription

We next sought to determine whether the failure to detect NOS2 mRNA in vivo or NO production in IFN-γ/LPS-activated hamster macrophages was due to impairment at the level of transcription, posttranscription, or translation. Because NOS2 knockout mice develop progressive disease following *Leishmania* infection (1, 7), we first considered the possibility that hamsters have natural deletion/mutation of the coding segment of NOS2 (we had previously demonstrated the presence of the *NOS2* gene by Southern hybridization (4)). We cloned overlapping PCR amplicons that collectively comprised a 4089-bp hamster NOS2 cDNA. This cDNA included a 3450-bp intact open reading frame whose deduced amino acid sequence (1150 aa) showed 90% identity to the mouse NOS2 protein sequence (data not shown; see GenBank Accession number DQ355357). Thus, we rejected the hypothesis that the failure to detect NOS2 mRNA in hamsters with VL and the failure to detect NO from activated hamster macrophages was due to deletion of the *NOS2* gene or a defect in the coding sequence.

To determine whether the defect in NO production from IFN-γ/LPS-activated hamster macrophages was due to impaired transcription, we compared NOS2 mRNA expression in activated hamster and mouse macrophages. NOS2 mRNA was readily detected by Northern blot in both the cytoplasmic and nuclear fractions of IFN-γ/LPS-activated mouse macrophages, but no transcripts could be detected in either fraction of activated hamster macrophages (Fig. 2*D*). We also performed NOS2 nuclear run-on assays, a more rigorous test of transcriptional activity, on IFN-γ/LPS-activated mouse and hamster macrophages. Although there appeared to be minimal basal NOS2 expression in splenic (but not peritoneal) macrophages, once again there was no evidence of IFN-γ/LPS-induced NOS2 transcriptional activity in the hamster macrophages, whereas transcription of NOS2 was abundant in the mouse macrophages (Fig. 2*E*). Collectively, these data demonstrated that the lack of NO production by hamster macrophages is due to a defect at the level of transcription.

The IFN-γ-signaling pathway is intact in hamsters

NOS2 induction by IFN-γ is dependent on several intermediary signaling/transcriptional factors (32). Having narrowed down the defect in NOS2 mRNA expression to the transcriptional level, we next hypothesized that the hamster could be genetically and/or functionally deficient in one or more of the factors involved in the IFN-γ-NOS2-signaling pathway. The basis for this hypothesis was the observation that mice genetically inactivated for various members of this signaling cascade are not only highly susceptible to *Leishmania* infection, but also have impaired NOS2 expression (Refs. 33 and 34; and M. Quinones and S. S. Ahuja, unpublished observations). To test this hypothesis, splenic expression of IFN-γR1, STAT1, and IRF-1 was examined at 56 days postinfection (Fig. 3*A*), a time point at which hamsters have significant disease and parasite burden (4). By densitometry analysis, there was no difference in splenic IFN-γR1 expression in the infected and uninfected spleen tissue ($p = 0.78$), but there was a 1.4-fold ($p = 0.00006$) and 1.5-fold ($p = 0.024$) increase in STAT1 and IRF-1 mRNA levels, respectively, in infected compared with uninfected animals. Furthermore, there was no difference in the expression of STAT1 and IRF-1 mRNAs in splenic macrophages isolated by adherence from uninfected and infected hamsters (Fig. 3*B*).

In addition to the indirect evidence demonstrated in the in vivo infection model, several lines of evidence indicated that the impaired capacity of hamster macrophages to produce NO was not related to a global defect in IFN-γ receptor-mediated signal transduction. First, the open reading frames of the cloned hamster STAT1 and IRF-1 cDNAs were intact and 97 and 90% identical to the murine homologs, respectively (see GenBank accession numbers DQ092343 (STAT1) and DQ092344 (IRF-1)).

Second, a Syrian hamster fibroblast (BHK) cell line transfected with a GAS-luciferase reporter vector showed a 10- to 40-fold increase in luciferase activity when stimulated with supernatants from Con A-activated hamster spleen cells ($p = 0.003$ for IFN-γ-stimulated vs unstimulated cells), or supernatants from CHO fibroblasts transfected with the hamster IFN-γ cDNA in pcDNA3.1 ($p = 0.0009$ for supernatants from IFN-γ-transfected vs vector-transfected cells) (Fig. 3*C*). Purified recombinant hamster IFN-γ (*E. coli*-derived) had similar biological activity (data not shown). These findings indicated that the JAK-STAT signaling pathway was intact from the IFN-γ receptor to the GAS transcription element (to which the translocated STAT-1 homodimer binds).

Third, BHK cells transfected with an ISRE-luciferase reporter vector showed a significant increase in luciferase activity when stimulated with supernatants from Con A-activated hamster spleen cells ($p = 0.002$ for IFN-γ-stimulated vs unstimulated cells), or supernatants from CHO fibroblasts transfected with the hamster IFN-γ cDNA in pcDNA3.1 ($p = 0.0003$ for supernatants from IFN-γ-transfected vs vector-transfected cells) (Fig. 3*D*). This demonstrated that the IFN-γ-signaling pathway, at least through IRF-1 translocation and binding to the ISRE element, was intact in hamster fibroblasts. To extend these findings to macrophages, because we were unable to use the reporter constructs in primary hamster macrophages and there are no hamster macrophage cell lines available for study, we used EMSAs to demonstrate that GAS and ISRE binding was present in splenic macrophages isolated from infected hamsters. Indeed, the binding pattern typical for GAS/STAT-1 (single band) and ISRE/IRF-1 (complex of multiple bands) (26, 35) was evident and specific to these consensus sequences (blocked by unlabeled consensus probe but not by unlabeled mutant probe) (Fig. 3*E*). IFN-γ/LPS stimulation of the splenic macrophages did not increase the binding activity over that of the infection alone. Hamster-specific Abs for STAT-1 and IRF-1 are not available so we were unable to confirm the identity of the binding proteins by supershift assay.

Finally, appropriate up-regulation of several IFN-γ-responsive genes (STAT1, IRF-1, and MHC class IIα) was detected in Syrian hamster resident peritoneal macrophages following stimulation with IFN-γ (Fig. 3*F*). There was basal expression of STAT1 in unstimulated macrophages that increased 2.2- and 3.5-fold in splenic and peritoneal macrophages, respectively. IRF-1 was not expressed in unstimulated cells, but increased dramatically between 0.5 and 4 h of IFN-γ stimulation in both splenic and peritoneal macrophages. Thus, these primary IFN-γ response genes, which are also part of the IFN-γ-NOS2-signaling pathway, were appropriately up-regulated by IFN-γ in hamster macrophages. Furthermore, mRNA of hamster MHC class IIα, a secondary response gene, was up-regulated 2.3- and 2.4-fold in splenic and peritoneal macrophages in response to IFN-γ stimulation. Collectively, these experiments demonstrated appropriate expression and function of members of the IFN-γ-signaling pathway and that the failure to detect NOS2 mRNA was due to selective impairment of the NOS2 transcriptional unit.

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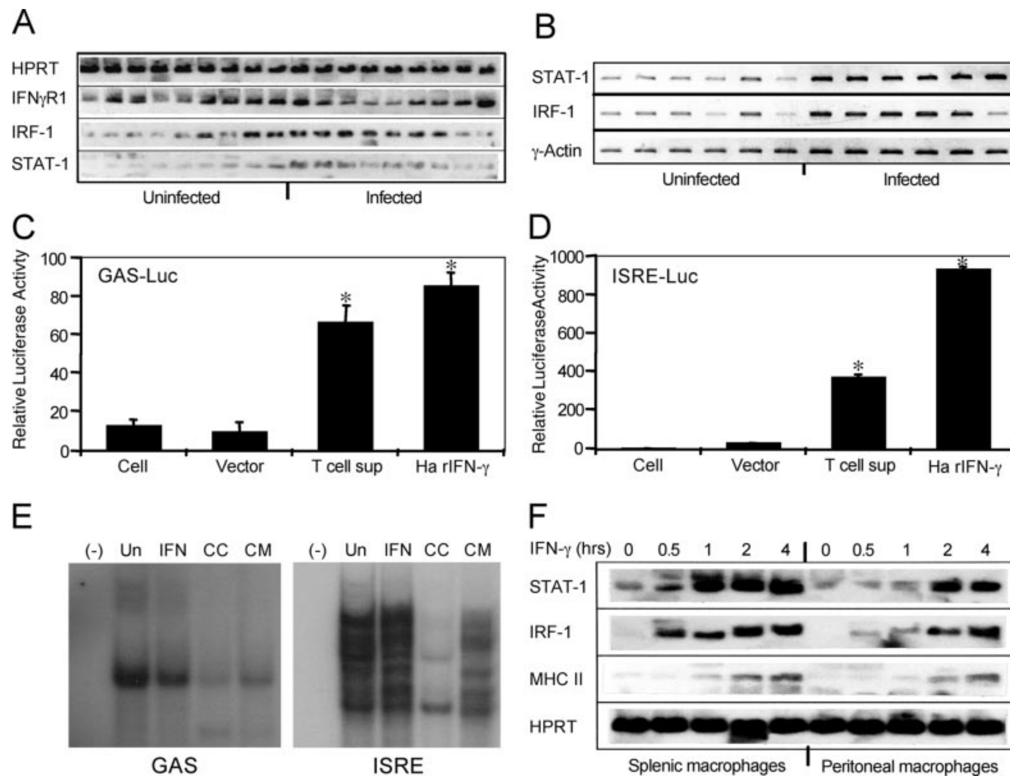

FIGURE 3. IFN-γ-stimulated signaling is intact in hamsters. *A*, Members of the IFN-γ-signaling pathway are expressed in vivo at the site of infection. The expression IFN-γR1, IRF-1, and STAT-1 in spleens of uninfected or infected hamsters was determined by Northern blotting as described in Fig. 2. Each lane represents mRNA isolated from a single animal. *B*, Expression of STAT-1 and IRF-1 in splenic macrophages. Adherent splenic macrophages were isolated from uninfected and 6-wk *L. donovani* infected hamsters ($n = 6$/group). cDNA from reverse-transcribed RNA was amplified by PCR with primers specific for hamster STAT-1, IRF-1, and γ-actin (used to assess equivalency of RNA concentration and integrity). Amplification products were separated and visualized on an ethidium bromide-stained agarose gel. The data shown are from a single experiment representative of two independent experiments. *C*, IFN-γ-induced STAT1-GAS signaling is intact in hamster fibroblasts. BHK cells were stably transfected with the GAS-luciferase reporter plasmid and stimulated with supernatants (20% v/v) from CHO cells transfected with the hamster IFN-γ cDNA in pcDNA 3.1 (rIFN-γ) or empty vector, or with supernatants (25% v/v) from Con A-activated spleen cells for 5 h. The luciferase activity in cellular extracts was determined and the data are presented as the mean ± SEM luciferase activity of triplicate cultures. ∗, Significant differences ($p \leq 0.003$) between control and IFN-γ-induced signaling. The data shown are from a single experiment representative of five independent experiments. *D*, IFN-γ-induced IRF1-ISRE (IFN-stimulated response element) signaling is intact in hamster fibroblasts. BHK cells were transfected transiently with the ISRE-Luc reporter plasmid, stimulated with hamster IFN-γ, and the luciferase activity in cellular extracts was determined as described above. ∗, Significant differences ($p \leq 0.002$) between control and IFN-γ-induced signaling. The data shown are from a single experiment representative of three independent experiments. *E*, GAS- and ISRE-binding activity in macrophages from infected hamsters. Adherent splenic macrophages from 6-wk *L. donovani* infected hamsters were left untreated (Un) or stimulated with hamster IFN-γ (50 U/ml) and LPS (100 ng/ml) (IFN) and the nuclear proteins were extracted. The proteins were incubated with end-labeled ([γ-^{32}P]ATP) consensus GAS and ISRE double-stranded oligonucleotides and the complexes were separated by PAGE and visualized by autoradiography. Controls included absence of nuclear protein (−), competition with 100-fold molar excess cold (unlabeled) consensus oligonucleotide (CC), or competition with 100-fold molar excess cold mutant oligonucleotide (CM). The data shown are from a single experiment representative of two independent experiments. *F*, IFN-γ-inducible genes are up-regulated in hamster macrophages in response to IFN-γ. Total RNA was isolated from hamster peritoneal macrophages that were unstimulated or stimulated with hamster rIFN-γ (100 U/ml) for 0.5–4 h. The expression of MHC class II, STAT1, and IRF-1 mRNA was determined by Northern blotting using cloned hamster cDNA probes and a single blot that was stripped and reprobed. Hybridization with the HPRT cDNA was used to assess loading equivalency and RNA integrity. The data shown are from a single experiment representative of two independent experiments.

The proximal hamster NOS2 promoter has reduced basal and IFN-γ-stimulated activity

To test the hypothesis that the hamster NOS2 promoter was unresponsive to activating stimuli, we isolated a 20-kb fragment of DNA that included the promoter from a hamster genomic DNA library. We sequenced (see GenBank accession number DQ355358) the proximal 2 kb of the promoter (immediately upstream of the NOS2-coding region) that included the transcriptional start site, TATA sequence, and the sequence homologous to the mouse basal promoter (region I; 85% sequence identity) and the IFN-γ/LPS-responsive enhancer (region II; 78% sequence identity) regions of the mouse promoter (21, 22). The sequence and location of the TATA box with respect to the transcriptional start site were identical between the hamster and mouse (data not shown).

Previous work demonstrated that the hyporesponsiveness of the human compared with the mouse NOS2 promoter to IFN-γ + LPS stimulation was due to nucleotide differences in the proximal promoter (22). We reasoned similarly that the sequence differences between the hamster and mouse proximal promoter might account for the impaired IFN-γ-mediated NO production in hamster macrophages. To test this possibility, we cloned ~1200-bp fragments of the hamster, mouse, and human NOS2 promoters that corresponded to the basal and IFN-γ/LPS-inducible regions of the mouse promoter (21) into a luciferase reporter vector. Mouse macrophages (RAW267.4 cell line) were transfected with each of these

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constructs and the luciferase activity measured in the basal state or following stimulation with recombinant murine IFN-γ± LPS. This cell line was chosen because it is known to express NOS2 in response to IFN-γ + LPS, and it has been used extensively to investigate the function of the mouse and human NOS2 promoters (21, 22, 25, 36).

We initially directly compared the basal and inducible activity of the hamster, mouse, and human proximal NOS2 promoters (Fig. 4*A*). As described previously (22), the mouse promoter demonstrated basal activity, which increased significantly upon stimulation with IFN-γ or IFN-γ + LPS (Fig. 4*A*, $p = 0.003$ for both). In contrast, the human (22) and hamster promoters had very low basal activity and a blunted response to IFN-γ + LPS (Fig. 4*A*). Despite the dramatically reduced basal and inducible activity of the hamster and human NOS2 promoters, the IFN-γ/LPS-induced response of the hamster promoter was still significantly increased over its basal activity ($p \leq 0.01$).

Following these initial experiments, we focused our comparison on the hamster and mouse NOS2 promoters (Fig. 4*B*). The basal activity of the hamster promoter (construct B) was ~23-fold less than the basal activity of the mouse promoter (construct A) (mean of >30 independent experiments, $p = 8.2 \times 10^{-8}$) and the IFN-γ/LPS-stimulated response of the hamster promoter was ~27-fold less than the response of the mouse construct (mean of >30 independent experiments, $p = 1.6 \times 10^{-7}$). Thus, compared with the mouse proximal promoter, the hamster proximal promoter had dramatically reduced activity, both in the basal state and after stimulation with IFN-γ/LPS.

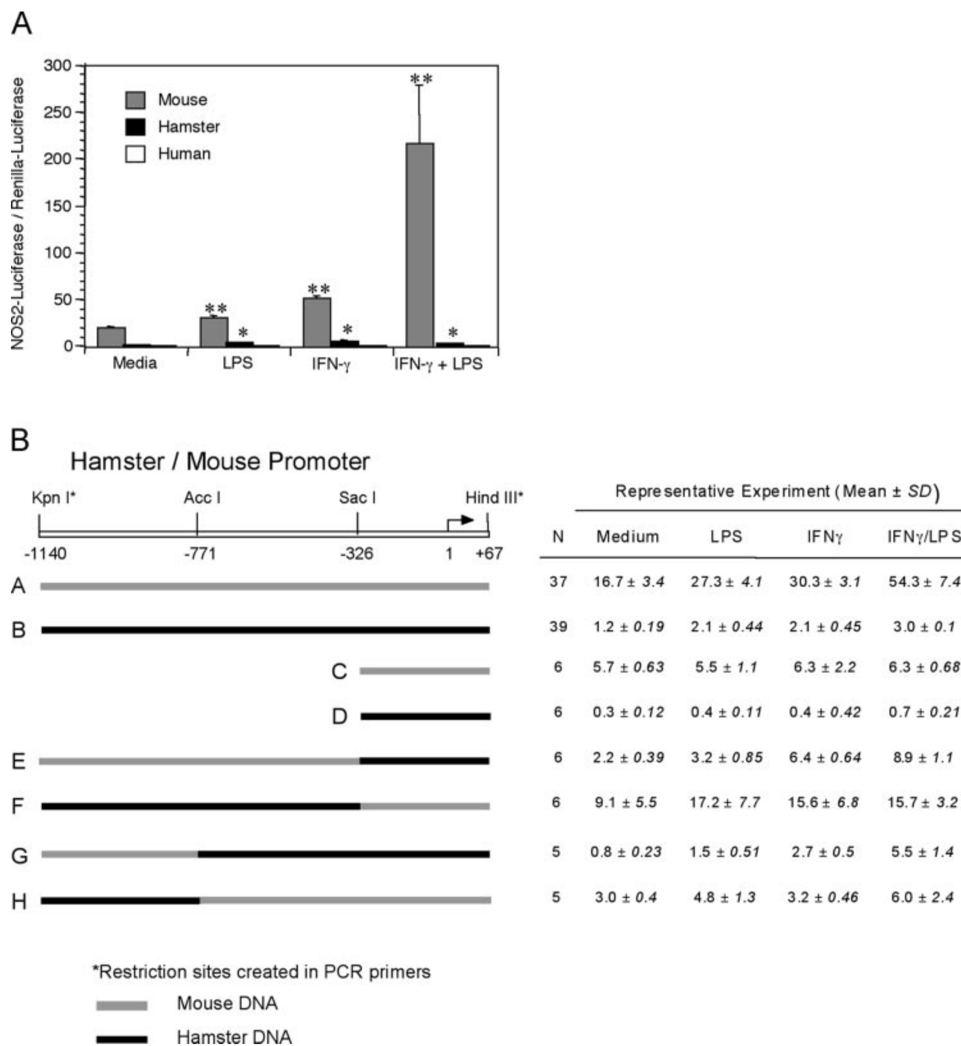

FIGURE 4. Activity of the hamster, mouse, and human proximal NOS2 promoters. *A*, Direct comparison of the basal and inducible activity of the hamster, mouse, and human proximal NOS2 promoters. The pGL3-basic luciferase reporter vector containing the proximal promoter and 5′-flanking regions of the hamster (nt −1146 to +67), mouse (nt −1140 to +67; GenBank accession number L23806) (21), and human (nt −1340 to +67; GenBank accession number, L36031) (22) *NOS2* genes were cotransfected by electroporation with the *Renilla* luciferase vector into the mouse macrophage (RAW 264.7) cell line. Transfected cells from two separate electroporations were pooled and cultured in complete medium at 2×10^7 cells/ml for 72 h, followed by stimulation for 8 h with recombinant murine IFN-γ (100 U/ml), LPS (10 ng/ml), or both. Luciferase activity was determined and the data are expressed as the mean ± SD of the ratio of NOS2-luc to *Renilla*-luc. *, Significant differences between control and induced promoter activity ($p \leq 0.04$); **, $p \leq 0.01$. The data shown are from a single experiment representative of six independent experiments. *B*, Defects in basal and inducible activity of the hamster promoter map to distinct regions of the proximal promoter. Truncated and hamster-mouse hybrid promoter constructs were studied by luciferase reporter assay as described above. The restriction sites used to create the constructs and the nucleotide number (based on the mouse sequence) are shown. The transcription start site is indicated by the arrow. ▨, Mouse DNA; ■, hamster DNA. Shown is the mean ± SD of the ratio of NOS2-luc to *Renilla*-luc in unstimulated (medium) or stimulated (murine IFN-γ (100 U/ml), LPS (10 ng/ml), or both) cells from an experiment representative of the number (*n*) of experiments designated.

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Distinct regions of the proximal hamster NOS2 promoter account for impaired basal and IFN-γ/LPS-induced activity

Previous studies of the mouse promoter indicated that region I was primarily responsible for basal promoter activity, and region II contained IFN-γ/LPS-responsive enhancer elements. Comparison of the human and mouse proximal NOS2 promoters suggested that the nucleotide differences in the *cis* elements in region II contributed to the unresponsiveness of the human promoter to IFN-γ/LPS stimulation (22). We postulated that the nucleotide differences in region I or region II of the hamster compared with the mouse DNA would contribute to the low basal activity and impaired induction by IFN-γ/LPS in the hamster.

We first investigated the reasons for the low basal activity of the hamster promoter by truncating and exchanging regions of the hamster and mouse promoters and testing their activity in a luciferase reporter system. When the mouse promoter was truncated to remove the DNA upstream of region I, this ~400-bp fragment, which has a NF-κB core-binding sequence, retained significant basal promoter activity (Fig. 4*B*, construct C). However, this activity was at least 2-fold less than the ~1200-bp proximal promoter that contained both regions I and II ($p = 0.0004$), indicating that the DNA upstream of region I enhanced basal promoter activity. In striking contrast, the ~400-bp hamster region I counterpart (Fig. 4*B*, construct D), which contained a NF-κB core-binding sequence that was identical to the mouse NF-κB sequence, had very low basal promoter activity. It had an average of 25-fold less activity than the mouse region I promoter over five independent experiments ($p = 0.018$), and was several-fold lower than the already low basal activity of the ~1200-bp proximal hamster promoter (construct B) ($p = 0.03$).

The capacity of region II of the mouse, and the corresponding region of the hamster promoter, to enhance basal activity was also tested in a heterologous promoter system. A ~369-bp *Kpn*I-*Acc*I mouse or hamster DNA fragment containing region II was cloned upstream of a SV40 promoter. The construct containing region II of the mouse had significantly increased basal activity over the construct containing only the SV40 promoter and the construct containing the SV40 promoter with the hamster region II (13.9 ± 1.8, 4.2 ± 0.15, and 3.2 ± 0.25 for the mouse region II-SV40, hamster region II-SV40, and SV40 control constructs, respectively; mean ± SD of three independent experiments; $p = 0.01$).

To confirm that the impaired basal activity of the hamster promoter was related to region I sequences, we exchanged a 396-bp *Sac*I-*Hind*III fragment corresponding to region I between the mouse and hamster proximal promoters. Replacement of the mouse region I with the hamster region I (Fig. 4*B*, construct E) resulted in a hybrid construct whose basal promoter activity was on average 4.3-fold less than that of the mouse promoter ($p \leq 0.02$ for each of five independent experiments). However, the induction of activity (fold-increase over basal activity) of this hybrid promoter by IFN-γ/LPS stimulation was not significantly different from the parent mouse promoter construct. Conversely, when the hamster region I was replaced by the mouse region I (Fig. 4*B*, construct F), the hybrid construct had on average an 11-fold increase in basal promoter activity over the parent hamster construct ($p \leq 0.03$ for each of five independent experiments), but recovery of basal activity did not enhance the IFN-γ/LPS-induced activity. Collectively, these data, along with results from an additional six hybrid or truncated constructs (data not shown), indicate that sequences in region I of the hamster proximal promoter (and not repressor sequences upstream of this region) are largely responsible for its blunted basal activity.

Region I does not bear sole responsibility for the impaired responsiveness of the proximal promoter to IFN-γ/LPS stimulation. Because region I of both the mouse and hamster was unresponsive to LPS, IFN-γ, or IFN-γ + LPS stimulation (Fig. 4*B*, constructs C and D), and hybrid promoter experiments demonstrated that the DNA between regions I and II did not influence responsiveness (data not shown), we next focused our attention on region II. Nucleotide differences were found throughout region II, including within the ISRE and GAS response elements, in the hamster compared with the mouse sequence (data not shown). When the hamster region II was replaced by mouse region II (Fig. 4*B*, construct G) the basal promoter activity did not change compared with the wild-type hamster promoter (construct B), but the responsiveness to IFN-γ/LPS was significantly enhanced (a 6.5-fold increase over basal promoter activity, $p = 0.008$). However, despite this significant increase in promoter activity following IFN-γ/LPS stimulation, the overall promoter activity was low compared with the IFN-γ/LPS response obtained from the wild-type mouse proximal promoter (construct A). In contrast, when mouse region II was replaced by the hamster region II (construct H), there was reduction in both the basal activity ($p = 0.0009$) and IFN-γ/LPS-induced activity ($p = 0.0003$) compared with mouse wild-type proximal promoter. Collectively, these data indicate that region II of the mouse promoter is primarily responsible for IFN-γ/LPS responsiveness, and to a lesser extent enhances basal promoter activity (in agreement with the comparison of constructs A and C, and the heterologous promoter experiments described above), and these functions are impaired in the hamster region II through alteration of critical enhancer elements or the presence of repressor sequences, or both.

Discussion

In this study, we demonstrate through in vivo and in vitro experiments that in the Syrian hamster model of VL, which mimics active human disease, the inability to control the infection is accompanied by impaired macrophage effector function. We found that the clinicopathological similarities between active VL in hamsters and humans was paralleled by similarities in hamster and human macrophage function. IFN-γ-LPS-induced NOS2 expression and NO production in hamster macrophages was undetectable, just as has been described for activated human macrophages (22, 37), and basal and IFN-γ-LPS-induced activity was low in both the hamster and human NOS2 proximal promoters. These findings are all the more striking when viewed in the context of the more commonly studied murine model of *L. donovani* infection, in which the infection is controlled at a subclinical level because of dominant NOS2-dependent antimicrobial activity. Thus, the hamster is a unique small animal model whose NOS2-related macrophage microbicidal system has phenotypic and genotypic similarities to human macrophages, thereby providing opportunity to dissect relevant mechanisms of host defense and NOS2 regulation.

The human and rodent *NOS2* genes are regulated primarily at the transcriptional level (21, 25, 38, 39). In hamster macrophages, like human macrophages (22, 37), we found that the blunted IFN-γ/LPS- or IFN-γ/*Leishmania*-mediated NOS2 response was due to a lack of IFN-γ/LPS-induced transcription. The unresponsiveness of the hamster NOS2 transcriptional unit was accompanied by impaired macrophage killing of intracellular parasites and the inability of the animal to control systemic infection with *L. donovani*, which is characteristic of active VL in humans. These findings are in striking contrast to the outcome of infection in mice where expression of NOS2 and generation of NO by macrophages leads to control of intracellular parasite replication (9–11) and escape from

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progressive VL (1, 2). Although it is well-documented that *L. donovani* infection of macrophages leads to altered cell signaling and down-regulation of multiple functions (reviewed in Ref. 40), and such mechanisms may be playing a role in the progression of VL in the hamster, this possibility does not explain the reduced NOS2 transcription in naive, uninfected hamster macrophages.

The impaired transcription of NOS2 in the hamster led us to consider that either there was a global defect in IFN-γ signaling or that there was specific intrinsic unresponsiveness of the hamster *NOS2* gene to IFN-γ-mediated activation. Although the progressive disease in the hamster model was reminiscent of the uncontrolled *Leishmania* infection observed in mice that have a defect in the IFN-γ-signaling pathway (34, 41–43), we found that IFN-γ-mediated JAK-STAT-IRF-1 activation (reviewed in Ref. 32), and up-regulation of primary and secondary (other than NOS2) response genes was intact in hamster cells. Because of these findings, we focused our study on the NOS promoter. Indeed there was precedent for giving attention to the NOS2 promoter because of the observations that the low or absent production of NO from IFN-γ/LPS-stimulated human macrophages was due to impaired responsiveness of the human NOS2 promoter (22, 44). In cells that were fully capable of transcribing NOS2 and producing NO, we found that the hamster proximal NOS2 promoter, like the human homolog, was dramatically less active under both basal conditions and after stimulation with IFN-γ/LPS compared with the mouse promoter.

Both basal (constitutive) promoter activity and responsiveness of a promoter to a particular stimulus contribute to the expression of a gene on the whole. The low basal activity of the hamster NOS2 promoter is similar to what has been reported for the human promoter. This has been an underappreciated cause of the low level of NO production by human macrophages (22, 44); most attention has been given to the reduced responsiveness of the promoter to IFN-γ/LPS compared with the mouse promoter. The low basal activity of the hamster proximal promoter compared with the mouse was related to sequences that are intrinsic to region I of the hamster promoter, and as has been described for the human NOS2 promoter (22), the blunted iNOS2 promoter activity in the hamster was localized, at least in part, to region II. Unique nucleotide sequence(s) in the hamster promoter DNA may lead to the blunted gene activation directly through the inability to bind a critical transactivating factor, or indirectly through epigenetic mechanisms. Alternatively, these sequences may bind a protein that represses or silences constitutive promoter activity.

Although the hamster proximal NOS2 promoter (including regions I and II) clearly has impaired basal and inducible activity relative to the mouse proximal promoter, it must be recognized that these studies did not address the potential role of the large upstream region of the promoter in regulating transcriptional activity of the hamster *NOS2* gene. Cytokine responsive elements in the human NOS2 promoter, which distinguish it from the mouse promoter, have been shown to be located as far as 16 kb upstream of the transcriptional start site (19, 44, 45). Furthermore, the absence of detectable NOS2 expression by Northern blot and NO production by the Griess reaction does not eliminate the possibility of a contribution to host defense. Indeed, provocative work by Gantt et al. (20) demonstrated that inhibition of NOS2 impaired the leishmanicidal activity of human macrophages, despite the fact that NO production could not be detected. Lastly, although we have referred to hamster and human NOS2 transcription as being impaired (relative to the mouse), in fact it may be that the "hyperexpression" of the mouse (and rat) *NOS2* gene is aberrant. Because high or low NOS2 expression can be pathologic (8, 46), in vivo studies of other disease models will be needed to address the relative advantages and disadvantages of NOS2 "hypoexpression" in the hamster.

In conclusion, we have demonstrated that IFN-γ/LPS-induced activation of NOS2 is blunted in Syrian hamster macrophages. This reduced NOS2 transcriptional activity, which is unique to the *NOS2* gene and not the result of global impairment of IFN-γ signaling, is accompanied by a striking clinicopathologic phenotype of severe, progressive disease caused by infection with an intracellular parasite. This particular aspect of macrophage effector function in the hamster is similar to the function of human macrophages, so this small animal model can be used as a tool to dissect the effect of this unique *NOS2* gene regulation on disease phenotype. However, despite the relative IFN-γ unresponsiveness of the NOS2 transcriptional unit in this animal, protection against systemic parasite challenge by prior skin infection or vaccination can be achieved (47–51). Most notably, in hamsters vaccinated with DNA encoding the KMP-11 Ag, protection against parasite challenge was associated with expression of NOS2 mRNA and NO production (47). Collectively, these findings indicate that NOS2 can be transcriptionally activated under some circumstances, perhaps involving alternative mechanisms, and/or that NOS2-independent macrophage effector mechanisms can be activated to control the infection.

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Disclosures

The authors have no financial conflict of interest.

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