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Altered Responses to Bacterial Infection and Endotoxic Shock in Mice Lacking Inducible Nitric Oxide Synthase

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Summary

Mice deficient in inducible nitric oxide synthase (iNOS) were generated to test the idea that iNOS defends the host against infectious agents and tumor cells at the risk of contributing to tissue damage and shock. iNOS$^{-/-}$ mice failed to restrain the replication of Listeria monocytogenes in vivo or lymphoma cells in vitro. Bacterial endotoxic lipopolysaccharide (LPS) caused shock and death in anesthetized wild-type mice, but in iNOS$^{-/-}$ mice, the fall in central arterial blood pressure was markedly attenuated and early death averted. However, unanesthetized iNOS$^{-/-}$ mice suffered as much LPS-induced liver damage as wild type, and when primed with Propionobacterium acnes and challenged with LPS, they succumbed at the same rate as wild type. Thus, there exist both iNOS-dependent and iNOS-independent routes to LPS-induced hypotension and death.

Introduction

Nitric oxide (NO) participates in the physiologic and pathophysiologic regulation of every mammalian organ system (Moncada et al., 1991; Nathan and Xie, 1994b). Three known NO synthases (NOSs) encoded by distinct genes are activated by binding calmodulin, but one of them, NOS2, does so without requiring an agonist-induced elevation of the intracellular Ca^{2+} concentration (Nathan and Xie, 1994b). Instead, the chief means of activation of NOS2 is its transcriptional induction by cytokines and microbial products (Xie et al., 1992). The independence of NOS2 from reliance on elevated Ca^{2+} led to the designation iNOS (Xie et al., 1992) and confers the capacity for sustained production of NO. High-output NO production by macrophages, hepatocytes, chondrocytes, endothelial cells, fibroblasts, smooth myocytes, cardiac myocytes, and other cells appears to restrict the replication of helminths, protozoa, bacteria, viruses, and tumor cells at the potential cost of inflammatory damage to host cells and tissues (Hibbs et al., 1987; Stuehr and Nathan, 1989; Nathan and Hibbs, 1991; Karupiah et al., 1993; Nathan and Xie, 1994a, 1994b).

Functions unique to iNOS have not been adequately defined with pharmacologic inhibitors. To varying degrees, inhibitors of iNOS activity also affect NOS1 (constitutively expressed in neurons, skeletal muscle, renal mesangial cells, and pancreatic islets) and NOS3 (constitutive in endothelium, neurons, and epithelial cells) and, like all drugs, do or may have side effects. Further, the diversity of the molecular origins, targets, and actions of NO makes it difficult to anticipate the consequences of inhibiting its production in disease. Animals genetically deficient in NOSs bypass these issues; mice lacking NOS1 or NOS3 have been generated (Huang et al., 1993; P. Huang, personal communication). Herein are described mice (iNOS$^{-/-}$) whose *NOS2* gene has been inactivated through homologous recombination. These mice allowed us to test the concept that iNOS is a two-edged sword, an enzyme that defends the host against infection, but at substantial risk.

Results

Generation of iNOS-Deficient Mice

Overlapping cosmid clones were isolated comprising a 72 kb mouse genomic contig that contained ~36 kb of the *NOS2* locus plus 36 kb of upstream sequences. The gene replacement vector pINOS-RV1 was designed to delete the proximal 585 bases of the iNOS promoter, a region required for iNOS expression in macrophages (Xie et al., 1993), plus exons 1–4, including the ATG translational start site in exon 2 (Figure 1A). The targeting vector was electroporated into mouse embryonic stem (ES) cells. Two karyotypically normal targeted clones were isolated at frequencies of 1:937 with coelectroporation of herpes simplex virus thymidine kinase (HSV–*tk*) and ~1:4000 without HSV–*tk* (Figures 1B–1D). Only the latter clone gave germline transmission from coat color chimeric mice generated by blastocyst injection. From these we derived viable, homozygous knockout mice (Figure 1E).

Southern blot hybridization analysis of wild-type mice using iNOS cDNA fragments as probes, including the region described as duplicated in rat based on reverse transcription polymerase chain reaction (PCR) (Mohaupt et al., 1994), detected only single hybridizing genomic fragments in the mouse, and these mapped to the *NOS2* allele contained in the overlapping cosmids of Figure 1A. In iNOS$^{-/-}$ mice, Southern blot hybridization was negative with sequences from exons 1, 2, and 4 (data not shown). Thus, mice have only one *NOS2* allele, and in iNOS$^{-/-}$ mice its, 5′ sequences are deleted.

*These authors contributed equally to the work.

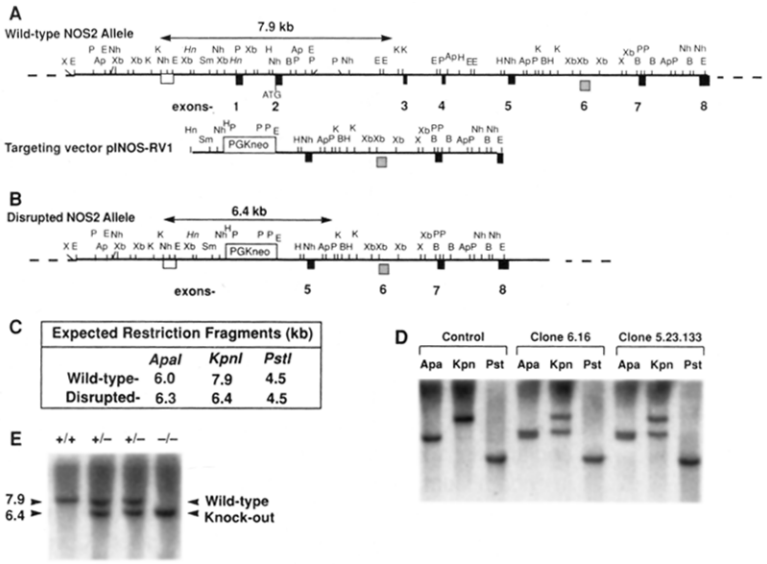

Figure 1. Targeted Disruption of the Murine *NOS2* Gene

(A) Map of the 5′ end of *NOS2* locus and the targeting vector pINOS-RV1. Closed boxes denote exons 1–8. Location of exon 6 (stippled box) is approximate. Exon 2 contains the translation start site ATG. Restriction enzyme abbreviations: Ap, ApaI; B, BamHI; E, EcoRI; H, HindIII; Hn, HincII; K, KpnI:, Nh, NheI; P, PstI; Xb, XbaI; X, XhoI. Open box indicates position of the 5′ KpnI–EcoRI probe used for Southern blot hybridization in (C)–(E); arrows mark diagnostic KpnI fragments.

(B) Predicted map of the disrupted allele.

(C) Expected sizes for ApaI, KpnI, and PstI restriction fragments.

(D) Southern blot hybridization analysis of genomic DNA from control ES cells (129/Sv ES cell line D3) and *NOS2*-targeted clones *6.16* and *5.23.133*.

(E) Southern blot hybridization of KpnI-digested genomic DNA from tail biopsies of F1 intercross progeny.

Extent and Selectivity of iNOS Deficiency

The extent and selectivity of iNOS deficiency in vivo were evaluated by organ Northern blots. After wild-type mice were injected with endotoxic bacterial lipopolysaccharide (LPS), their hearts, kidneys, lungs, and spleens contained iNOS mRNA, but none was detected in iNOS$^{-/-}$ mice using a cDNA probe for exons 2–8 (Figure 2). In contrast, mRNAs for *NOS1* (in brain) and *NOS3* (in most organs) were neither diminished along with nor increased in compensation for iNOS deficiency (Figure 2). Expression of *NOS3* decreased in heart following injection of LPS, similar to observations with endothelial cells in vitro (Nishida et al., 1992; MacNaul and Hutchinson, 1993). *NOS3* mRNA in heart from untreated mice cross-hybridized faintly with iNOS probes (nucleotides 200–1017 and 1595–2468) (Figure 2; data not shown).

At the cellular level, the analysis focused on peritoneal macrophages, in which mammalian production of inorganic oxides of nitrogen was initially demonstrated in vitro (Stuehr and Marletta, 1985), in which a function of NO production was discerned (Hibbs et al., 1987) and from which iNOS was purified (Stuehr et al., 1991) and cloned (Lyons et al., 1992; Xie et al., 1992; Lowenstein et al., 1992). Peritoneal macrophages of iNOS$^{-/-}$ mice lacked iNOS mRNA (Figure 3A), protein (Figure 3B), and enzyme activity (Figure 3C) with every inductive regimen tested that led to iNOS expression in wild-type mice. Thus, macrophages from iNOS$^{-/-}$ mice were deficient in producing NO not only in response to the combination of interferon-γ (IFNγ) plus LPS, but also with an inductive regimen that was independent of IFNγ (namely, IFNα/β plus LPS), even though the primary IFNγ response element in the *NOS2* promoter, IRF-E (Kamijo et al., 1994; Martin et al., 1994), was not deleted. Likewise, these cells were deficient in producing NO in response to inductive regimens independent of LPS (namely, IFNγ alone or together with tumor necrosis factor α [TNFα]).

The respiratory burst of activated macrophages closely resembles the high-output NO pathway in that it is a major IFNγ-enhanced antimicrobial mechanism that consists of NADPH-, O_2-, FAD-, and cytochrome-dependent generation of a cytotoxic, inorganic radical gas (superoxide). Macrophages from iNOS$^{-/-}$ mice activated in vivo with IFNγ plus LPS preserved their respiratory burst capacity. This was assessed by release of the dismutation product of superoxide, H_2O_2, following triggering by phorbol myristate acetate, bacillus Calmette-Guérin, or Listeria monocytogenes (Figure 3D). The modest increase in respiratory burst of macrophages from iNOS$^{-/-}$ compared with wild-type mice is consistent with the enhancement in H_2O_2 release from wild-type macrophages seen with a NOS inhibitor (Ding et al., 1988) and suggests that NO may normally damage the respiratory burst oxidase (Clancy et al., 1992), compete for NADPH, or react with some of the superoxide,

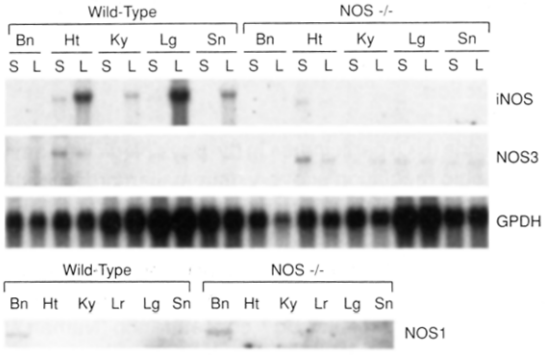

Figure 2. Comparative Expression of NOS Isoforms in Vivo

(Top) Total RNAs from brain (Bn), heart (Ht), kidney (Ky), lung (Lg), and spleen (Sn) from mice injected 5.5 hr earlier with saline (S) or LPS (L) were probed with an iNOS cDNA fragment (nucleotides 200–1017; exons 2–8) and additionally with a rat *NOS3* cDNA fragment. Blots were stripped and reprobed for glyceraldehyde 3-phosphate dehydrogenase (GPDH).

(Bottom) Total RNAs from the same organs as above plus liver (Lr) from LPS-treated mice were probed with a rat *NOS1* cDNA fragment.

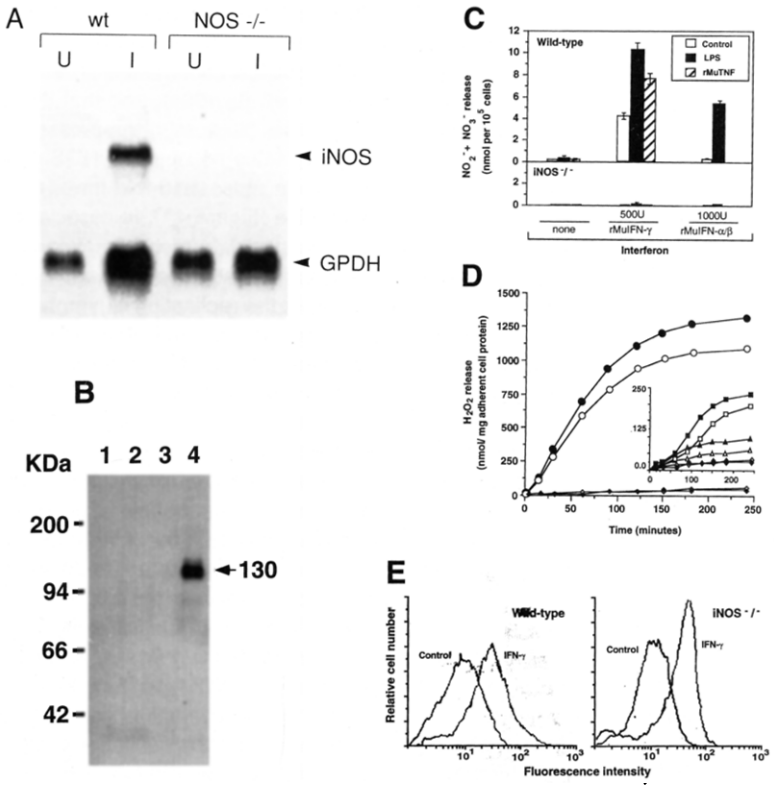

Figure 3. Selective Inability of iNOS$^{-/-}$ Macrophages to Express iNOS mRNA, Protein, or Enzyme Activity

(A) Northern blot analysis. Total RNA from thioglycollate broth–elicited peritoneal exudate macrophages unstimulated (U) or activated (I) in vitro for 20 hr with LPS and rMuIFNγ was probed for iNOS and GPDH. wt, wild type.

(B) Western blot analysis. Peritoneal exudate macrophages from iNOS$^{-/-}$ (lanes 1 and 2) or wild-type (lanes 3 and 4) mice were activated in vitro without (lanes 1 and 3) or with (lanes 2 and 4) LPS and rMuIFNγ and their lysates immunoblotted with rabbit anti-holo-iNOS IgG.

(C) NO generation. Thioglycollate broth–elicited macrophages were exposed in vitro for 48 hr to buffer alone, LPS, rMuIFNγ, rMuTNFα, natural mouse IFNα/β, or some combination of these.

(D) Respiratory burst. H_2O_2 release from peritoneal macrophages from iNOS$^{-/-}$ (closed symbols) or wild-type (open symbols) mice activated in vivo by intraperitoneal injection of LPS plus rMuIFNγ 24 hr before harvest. In vitro, cells were untreated (open diamonds) or triggered with phorbol myristate acetate (open circles), bacille Calmette-Guérin (open squares), or human serum-coated L. monocytogenes (open triangles). Values are means for triplicates; SEM fall within the symbols.

(E) IFNγ-induced expression of MHC class II antigen. Thioglycollate broth–elicited peritoneal macrophages were incubated 48 hr with or without rMuIFNγ and then stained with rat anti-mouse MHC class II MAb followed by fluorescein isothiocyanate–conjugated goat F(ab')$_2$ anti-rat IgG for analysis by flow microfluorimetry.

diverting it to peroxynitrite (Carreras et al., 1994). That iNOS$^{-/-}$ macrophages remained directly responsive to IFNγ was demonstrated by the in vitro induction of major histocompatibility complex (MHC) class II antigen expression (Figure 3E).

Phenotype of Uninfected iNOS-Deficient Mice

Under specific pathogen-free conditions, iNOS$^{-/-}$ mice survived weaning at the expected Mendelian ratio: genotyping of 353 F2 mice, as shown in Figure 1E, revealed 26% knockout, 26% wild-type, and 48% heterozygous animals. From 4–11 weeks of life, iNOS$^{-/-}$ mice weighed the same as wild type and gained weight at the same rate (data not shown). Mature iNOS$^{-/-}$ mice produced litters at a normal frequency and size.

The following tissues were examined grossly and microscopically in six iNOS$^{-/-}$ mice (three males) and four wild-type mice (two males) aged 9–10 weeks in the F2 generation (129/SvEv × C57BL/6) and were found to be histologically normal: brain, pituitary, spinal cord, peripheral nerve, eye, Harderian gland, nose, tongue, salivary gland, thyroid, parathyroid, heart, aorta, lung, thymus, lymph node, skeletal muscle, skin, mammary gland, stomach, small and large intestine, liver, gall bladder, pancreas, spleen, adrenal, kidney, urinary bladder, uterus, ovaries, vagina, brown fat, bone/joints, and bone marrow. In the same mice, the following were normal: erythrocyte count, hemoglobin, hematocrit, mean corpuscular volume, platelet count, leukocyte count, proportion and morphology of neutrophils, eosinophils, monocytes and lymphocytes, blood glucose, urea nitrogen, creatinine, total protein, albumin, globulin, total bilirubin, alkaline phosphatase, alanine aminotransferase (ALT), aspartate aminotransferase (AST), cholesterol, triglycerides, sodium, potassium, chloride, bicarbonate, calcium, phosphorus, magnesium, and osmolality.

Suspensions of thymus and spleen cells from iNOS$^{-/-}$ and wild-type mice were indistinguishable when analyzed by flow microfluorimetry to enumerate lymphocytes positive for immunoglobulin, CD3, CD4, CD8, or MHC class II antigens. Moreover, inflammatory responses were normal in iNOS$^{-/-}$ mice, in that the same types and numbers of leukocytes accumulated in the peritoneal cavity as in wild-type mice in response to intraperitoneal injection of thioglycollate broth, sodium periodate, or IFNγ plus LPS (data not shown).

Host Defense Functions of iNOS

The iNOS$^{-/-}$ mice were used to test the importance of iNOS in host defense against bacteria and tumor cells. L. monocytogenes, a gram-positive opportunistic pathogen, enters Kupffer cells, hepatocytes, and splenic macrophages, eliciting a neutrophilic followed by a monocytic infiltrate (Conlan and North, 1994). Each of these host

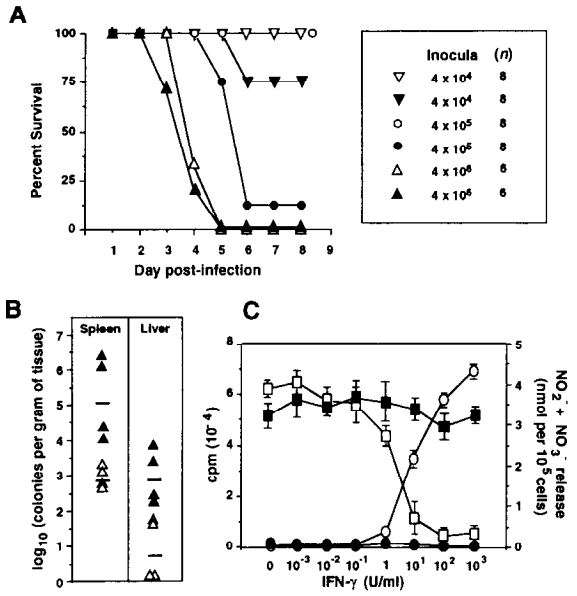

Figure 4. Role of iNOS in Host Defense

(A) Survival after intravenous infection of iNOS$^{-/-}$ (closed symbols) or wild-type (open symbols) mice with the indicated inocula of L. monocytogenes.

(B) Bacterial burden in liver and spleen. iNOS$^{-/-}$ (closed symbols) or wild-type (open symbols) mice were inoculated intraperitoneally with 4 x 10^5 cfu of Listeria and sacrificed 3 days later. Values are means for individual organs; SEM fall within the symbols. Horizontal bars show group means.

(C) Restriction of tumor cell growth. Thioglycollate broth–elicited peritoneal exudate macrophages from iNOS$^{-/-}$ (closed symbols) or wild-type (open symbols) mice were exposed to LPS (10 ng/ml) and the indicated concentrations of rMuIFNγ at the same time as they were admixed with lymphoma cells. Tumor cell proliferation (open squares) and NO_3^- plus NO_2^- (open circles) were measured. Values are means ± SEM for triplicate experiments.

cells is capable of expressing iNOS (Xie et al., 1992; Geller et al., 1993; Nathan and Xie, 1994a), often under the influence of IFNγ produced by natural killer cells and antigen-specific T cells, and IFNγ confers antilisterial activity (Farrar and Schreiber, 1993). Studies with NOS inhibitors have been conflicting, suggesting both that iNOS is protective in listeriosis, perhaps because it is bacteriostatic (Beckerman et al., 1993; Boockvar et al., 1994), and that iNOS is counterproductive, possibly because it suppresses T cells (Gregory et al., 1993). Mice deficient in iNOS succumbed to inocula of Listeria at least 10-fold lower than those lethal to wild-type mice (Figure 4A), in association with ~100-fold greater bacterial burdens in liver and spleen (Figure 4B). Likewise, activated macrophages from iNOS$^{-/-}$ mice failed to restrict the replication of lymphoma cells in vitro (Figure 4C), confirming the interpretation of pharmacologic evidence for the importance of NO in the same system (Stuehr and Nathan, 1989).

Role of iNOS in Septic Shock

The role of iNOS in septic shock is controversial. Accordingly, we wished to measure both arterial blood pressure and survival in iNOS$^{-/-}$ and wild-type mice. Given the increased susceptibility of iNOS$^{-/-}$ mice to some bacterial infections, demonstrated above, we used models of septic shock that did not involve injection of live bacteria.

To measure blood pressure, mice were anesthetized and a carotid artery cannulated. Wild-type mice injected with LPS (1 mg/kg) experienced a progressive, severe drop in arterial pressure. By 3.5 hr, the decline averaged 64% (Figure 5); shortly thereafter, all wild-type mice had expired. When mice were given normal saline in place of LPS, arterial pressure remained stable for the 5 hr period of observation. In contrast, LPS-treated iNOS$^{-/-}$ mice all survived the experiment; their blood pressure fell only 15% by 2 hr and thereafter was stable. The LPS-induced hypotensive response of heterozygotes (iNOS$^{+/-}$) was intermediate between that of wild-type and iNOS$^{-/-}$ mice. All survived; by 5 hr, however, their blood pressure had declined 30% (Figure 5). Heart rate, monitored from the electrocardiogram, was not altered in any treatment group.

Unanesthetized wild-type mice injected with LPS (10 mg/kg) accumulated high serum levels of nitrate plus ni-

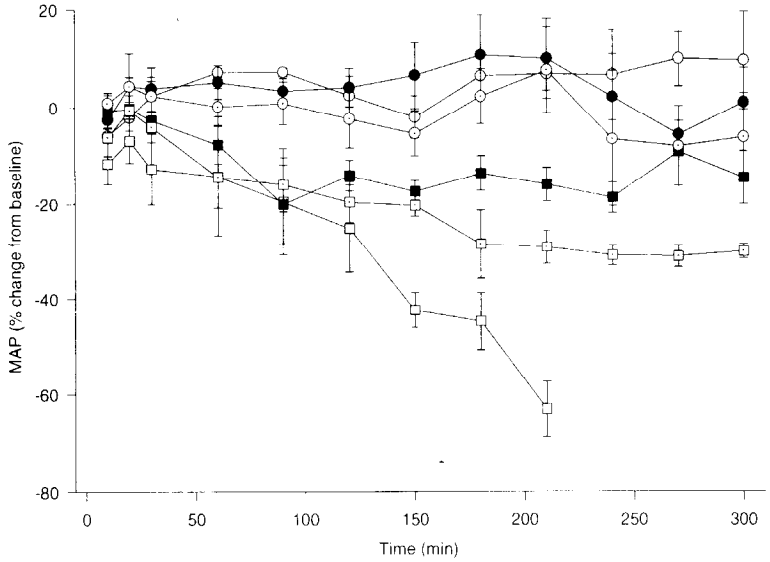

Figure 5. Role of iNOS in Endotoxic Shock

iNOS$^{-/-}$ mice (closed symbols), wild-type mice (open symbols), or iNOS$^{+/-}$ heterozygotes (symbols with central dot) were matched in each experiment for gender and age (8–12 weeks). Changes in mean arterial blood pressure (MAP) (means ± SEM) were monitored for 5 hr or until death following the intraperitoneal injection of saline (circles) or LPS (squares). Baseline blood pressure averaged 61 ± 3, 70 ± 4, and 53 ± 4 mm Hg and basal heart rate 431 ± 35, 526 ± 42, and 320 ± 20 beats/min in seven iNOS$^{-/-}$ mice (three given saline; four given LPS), seven iNOS$^{+/-}$ heterozygotes (three given saline; four given LPS), and six wild-type mice (two given saline; four given LPS), respectively.

trite (Figure 6A) and lactate dehydrogenase (LDH), ALT, and AST (Figure 6B). Elevation in the serum enzymes indicated damage to hepatocytes. In iNOS$^{-/-}$ mice, LPS caused no increase in serum nitrate (Figure 6A). Nonetheless, levels of LDH, ALT, and AST were elevated (Figure 6B), as in LPS-injected wild-type mice treated with a NOS inhibitor (Harbrecht et al., 1994). Serum cytokines were measured in LPS challenged mice both without (data not shown) and with (Figure 6C) an ongoing inflammatory response engendered by prior injection of heat-killed Propionobacterium acnes, a granuloma-forming bacterium. Levels of interleukin-1α (IL-1α) (data not shown), IL-1β, TNFα, and IL-6 were similar in both genotypes (Figure 6C).

Unanesthetized wild-type mice of the strain in which most knockouts have been generated (129/SvEv × C57BL/6) are relatively resistant to the lethal action of LPS as a sole agent; their LPS responses are usually tested after sensitization by prior injection of bacteria, carrageenan, or the hepatotoxin D-galactosamine (Pfeffer et al., 1993; Rothe et al., 1993; Kamijo et al., 1993; Xu et al., 1994). We tested survival of unanesthetized, uninstrumented mice both without (Figure 7A) and with (Figure 7B) prior injection with heat-killed P. acnes. In untreated mice (Figure 7A), iNOS deficiency conferred partial resistance to lethality induced by high-dose LPS (30 mg/kg), although not at the lower doses tested. In contrast, in the context of P. acnes–induced inflammation, LPS-treated iNOS$^{-/-}$ mice died at the same rate as wild type (Figure 7B), without the increase of the latter in serum nitrate (see Figure 6A).

Necropsies of three iNOS$^{-/-}$ and two wild-type mice that died after LPS administration revealed pyogranulomata infiltrating liver, lymph nodes, and mesentery, consistent with injection of heat-killed P. acnes 6 days earlier. The acute changes attributable to endotoxemia were marked margination of leukocytes, primarily polymorphonuclear cells, along the endothelia of arteries, veins, arterioles, venules, and capillaries, especially in lung and liver; multifocal hepatocellular necrosis and hepatic endothelial cell injury; and disseminated intravascular coagulation. The histopathology in wild-type and iNOS$^{-/-}$ mice was indistinguishable (data not shown).

Discussion

Protean roles ascribed to iNOS include participation in the dilatation of the gravid uterus, with rapid disappearance of the enzyme marking the onset of parturition (Sladek et al., 1993; Yallampalli et al., 1994). Thus, it was doubtful whether mice lacking iNOS could bring pregnancies to term, just as the role of NOS1 in penile erection (Rajfer et al., 1992; Burnett et al., 1992) raised the possibility that NOS1$^{-/-}$ mice would fail to reproduce. However, as for NOS1 (Huang et al., 1993), the generation of enzyme-

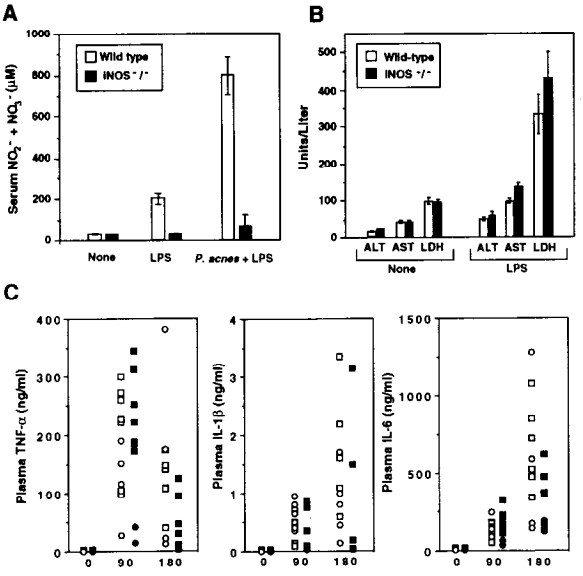

Figure 6. Serum Markers in LPS-Challenged Mice

(A) NO_3^- plus NO_2^- in sera of 11 mice per group injected with saline or LPS and sacrificed 5.5 hr later or in six mice per group injected with heat-killed P. acnes followed 6 days later by LPS. Sera were collected from the latter mice after they died naturally 9–10.5 hr after administration of LPS.

(B) ALT, AST, and LDH (see text) activities in the sera of the mice in (A) not given P. acnes.

(C) Levels of IL-1β, TNFα, and IL-6 determined by ELISA in plasma of iNOS$^{-/-}$ mice (closed symbols) and wild-type mice (open symbols) (circles for females; squares for males) injected with heat-killed P. acnes (1 mg) followed 6 days later by LPS (100 μg). Blood was collected 0, 90, or 180 min after injection of LPS. Results for IL-1α closely resembled those for IL-1β.

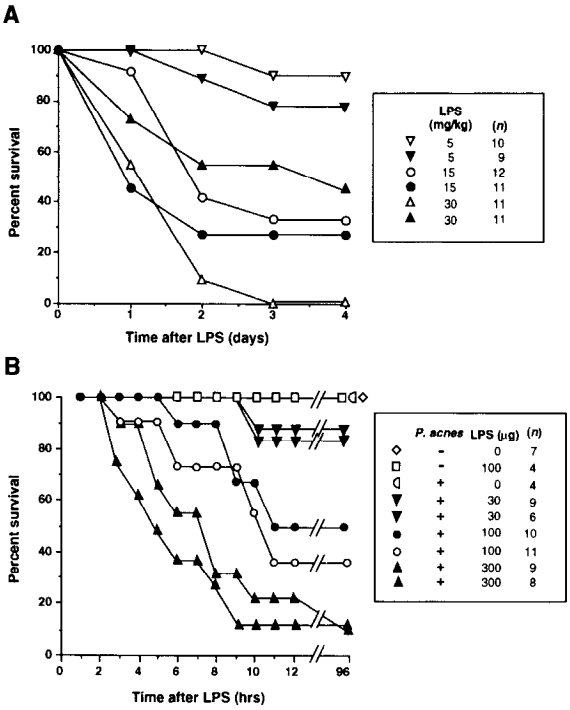

Figure 7. Survival of Unanesthetized Mice Challenged with LPS

(A) Mice were injected with the indicated doses of LPS. (B) Mice were injected with saline or heat-killed P. acnes followed 6 days later by vehicle alone or vehicle containing the indicated doses of LPS. In both panels, open symbols denote wild-type mice and closed symbols, iNOS$^{-/-}$ mice.

deficient mice by homologous recombination has proved that iNOS is dispensable in the development and reproduction of the mouse.

The normality of uninfected iNOS$^{-/-}$ mice extended to the accumulation of leukocytes in the irritated peritoneal cavity; the formation of granulomas in the bacteria-laden liver, spleen, lymph nodes, and mesentery; and the margination of neutrophils in the LPS-challenged lung. These normal inflammatory responses were surprising, given evidence implicating iNOS in leukocyte adhesion, migration, and accumulation (Mulligan et al., 1991; Belenky et al., 1993). Nor did iNOS appear to block the production of inflammatory cytokines in endotoxin-treated mice, as it was inferred to do in mice provoked with enterotoxin (Florquin et al., 1994).

Studies in iNOS$^{-/-}$ mice strongly supported the role of iNOS in host defense against Listeria in vivo and lymphoma in vitro. Macrophages from NF-IL6-deficient mice resembled those from iNOS$^{-/-}$ mice in their poor antibacterial and antitumor activity (Tanaka et al., 1995). NF-IL6$^{-/-}$ macrophages expressed iNOS normally, yet the cytotoxic activities of wild-type macrophages in the same assays were NO dependent. Tanaka et al. (1995) concluded that macrophage-mediated cytotoxicity requires two different mechanisms, one unidentified but depending on NF-IL6, the other involving iNOS. Our results are consistent with this formulation. Many other models of infectious and malignant disease deserve to be tested in iNOS deficiency. Given the redundancy of host defense systems and the diverse countermechanisms of pathogens, it can be anticipated that the relative role of iNOS will range from minimal to critical.

The most controversial issue addressed through the use of iNOS$^{-/-}$ mice is the pathogenesis of septic shock. This complex syndrome, the leading cause of death in intensive care units, claims >100,000 lives per year in the United States alone (Parrillo, 1993; Natanson et al., 1994). Septic shock is a systemic inflammatory process that erupts when LPS or other microbial products trigger production of TNF, IL-1, IL-12, IFNγ, leukemia inhibitory factor, migration inhibitory factor, platelet-activating factor, eicosanoids, and products of the complement and clotting cascades (Beutler et al., 1985; Taylor et al., 1987; Ohlsson et al., 1990; Bone, 1992; Kohler et al., 1993; Block et al., 1993; Creasey et al., 1993; Pfeffer et al., 1993; Rothe et al., 1993; Kamijo et al., 1993; Bernhagen et al., 1993; Xu et al., 1994). On the presumed final common path to death, central blood pressure falls and becomes refractory to pressors, blood flow maldistributes, the myocardium is depressed, tissues extract oxygen poorly, and organs fail.

NO can lower blood pressure (Moncada et al., 1991), block response to pressors (Thiemermann, 1994), influence the distribution of the circulation (Moncada et al., 1991), suppress cardiac myocytes (Roberts et al., 1992; Finkel et al., 1992; Balligand et al., 1993), and interfere with cellular respiration (Stuehr and Nathan, 1989; Hibbs et al., 1990). NO is produced in large amounts during infection (Stuehr and Marletta, 1987; Granger et al., 1991; Ochoa et al., 1991; Lai and Komarov, 1994). Infection and LPS induce iNOS (Xie et al., 1992; Lowenstein et al., 1992; Evans et al., 1993; Thiemermann, 1994), but LPS can also activate constitutively expressed NOSs (Salvemini et al., 1990; Finkel et al., 1992; Thiemermann, 1994), and it is not known which NOS(s) generates the excess NO. NOS inhibitors reversed hypotension in septic patients (Petros et al., 1991, 1994; Schilling et al., 1993; Lorente et al., 1993) and in dogs and rats given LPS or TNF (Kilbourn et al., 1990a, 1990b; Thiemermann and Vane, 1990) and improved survival in infected or LPS-treated mice (Teale and Atkinson, 1992; Szabó et al., 1994). However, in other studies, such drugs worsened outcome (Nava et al., 1991; Harbrecht et al., 1992; Cobb et al., 1992; Minnard et al., 1994; Florquin et al., 1994; Pastor et al., 1994), perhaps by blocking the ability of endothelium to generate NO needed to forestall thrombosis and dilate small vessels perfusing vital organs (Harbrecht et al., 1992). Thus, it has been proposed that iNOS sits astride the final common path to death in septic shock. The implication for therapy is that the septic host might be helped by an inhibitor that targets iNOS but spares NOS3 in endothelium. The advent of iNOS$^{-/-}$ mice afforded the opportunity to test the validity of this concept.

A major finding of this study was that anesthetized mice deficient in iNOS were substantially protected from cardiovascular collapse induced by LPS, a cardinal feature of septic shock, and survived for the duration of the experiment. In contrast, wild-type mice became profoundly hypotensive and died. Heterozygotes (iNOS$^{+/-}$) had an intermediate degree of LPS-induced hypotension, and, like iNOS$^{-/-}$ mice, they survived. The gene-dosage effect implies that pharmacologic inhibitors specific for iNOS might provide a clinically significant degree of protection against LPS-induced hypotension even if they did not inhibit iNOS completely. At the same time, iNOS$^{-/-}$ mice provided proof not based on pharmacologic agents that there exist other, quantitatively less important but still significant contributor(s) to LPS-induced hypotension. The possible relevance of these findings to septic shock in humans is not readily dismissed. Blood nitrate was high in septic patients (Ochoa et al., 1991), and at least 15 types of human cells can express iNOS, including vascular smooth muscle cells, cardiac myocytes, hepatocytes, and macrophages (Nathan and Xie, 1994b).

Like anesthetized mice, conscious mice that were otherwise untreated appear to be protected by iNOS deficiency from death associated with high-dose LPS. Nonetheless, iNOS$^{-/-}$ mice injected with LPS had the same biochemical evidence of liver damage as wild type, and iNOS$^{-/-}$ mice primed with P. acnes and then challenged with LPS died as quickly as wild type. The latter model may approach clinical relevance insofar as it mimics localized, indolent, or partially controlled infections from which microbes or their products spill abruptly into the blood. The vulnerability of iNOS$^{-/-}$ mice in this setting suggests that there may be redundant routes to endotoxic death.

In animals, numerous candidate mediators of septic shock have been neutralized prophylactically by injection of inhibitors or generation of mice deficient in the mediators or their receptors (Beutler et al., 1985; Taylor et al., 1987; Ohlsson et al., 1990; Bone, 1992; Kohler et al., 1993;

Block et al., 1993; Creasey et al., 1993; Pfeffer et al., 1993; Rothe et al., 1993; Kamijo et al., 1993; Bernhagen et al., 1993; Xu et al., 1994). Leaving aside differences in experimental models, the efficacy of all these interventions tested singly suggests that early on, each such factor is necessary and none sufficient for endotoxic or septic death. These findings led to extensive clinical testing of single agents designed to neutralize either LPS itself or one of its mediators (TNFα or IL-1). Such trials have not yet been successful. Their outcome was foretold by studies in experimental animals in which, once shock had begun, no such intervention consistently reversed it. Indeed, pharmacologic inhibition of NOS has been one of the very few interventions to restore blood pressure when endotoxic or septic shock was underway. The lethality of LPS in P. acnes–primed iNOS$^{-/-}$ mice underscores that salvage from ongoing septic shock may require simultaneous interruption of more than one final common path, each being sufficient and none necessary for death of the host. In this view, the paradigm shifts as the syndrome unfolds.

How did LPS kill iNOS$^{-/-}$ mice, if not by making them severely hypotensive? Necropsy evidence directs attention to iNOS-independent mechanisms of hepatotoxicity and neutrophil-mediated endothelial injury. iNOS-independent causes of LPS-induced lethality may be amenable to analysis in iNOS$^{-/-}$ mice and in mice deficient in other candidate mediator systems with which iNOS$^{-/-}$ mice are interbred. Likewise, backcrossing of iNOS deficiency to disease-susceptible backgrounds will allow investigation of the role of iNOS in other inflammatory states.

Experimental Procedures

Studies at each institution are performed according to its guidelines for animal use and care.

Generation of iNOS-Deficient Mice

A cosmid library was constructed using J1 ES cell genomic DNA as described elsewhere (Mudgett and MacInnes, 1990), from which overlapping cosmid clones were isolated using a probe from iNOS cDNA (HincII–EcoRI) containing exons 2–8. Only one genomic locus was isolated. This genomic contig contained the 5′ 36 kb of mouse *NOS2* (~75% of the coding region) and contributed to construction of pINOS-RV1 as follows. A 5′ 1034 bp arm was subcloned from a murine iNOS promoter fragment (Xie et al., 1993) isolated from the same ES cell library. A *neo*′ marker gene driven by the PGK promoter (PGK–*neo*) was inserted in the antisense orientation, and a 3′ 6.7 kb arm containing exon 5 and downstream sequences was subcloned from a cosmid and inserted. AB2.1 ES cells (4 × 10^6) maintained as described elsewhere (Mudgett and Livelli, 1995) were electroporated in buffer (Specialty Media) with 5 μg of linearized pINOS-RV1 with or without a 6-fold molar excess of nonlinearized vector containing the HSV–*tk* gene driven by the PGK promoter. Transformants were selected in 200 μg/ml geneticin (GIBCO BRL) and 0.2 μM FIAU as single clones in 100 mm diameter plates or in pools of 15–25 clones in 13 mm diameter wells. Cotransfection with PGK–*tk* yielded an ~3-fold enrichment. Genomic DNAs from clones or pools were isolated and screened by PCR using a 5′ primer flanking the *NOS2* gene (5′-GAGCAATGTGACAAAGCTCCTTCAGACTAGG-3′) and a 3′ primer specific to the PGK–*neo* marker (5′-GCCTGAAGAACGAGATCAGCAGCCTCTG-3′). PCR-positive clones (*6.16*, isolated as a single clone; *5.23.133*, isolated from a pool) were shown by Southern blot hybridization to have a targeted iNOS allele.

Northern and Western Blot Hybridizations

Total RNA was prepared from organs of two or three mice of each genotype, matched for age and gender, 5.5 hr following injection of saline or LPS (10 mg/kg), and from thioglycollate broth–elicited peritoneal exudate macrophages that had been left unstimulated or had been activated in vitro for 20 hr with LPS (100 ng/ml; from Escherichia coli 011:B4; Sigma) and recombinant murine INFγ (rMuIFNγ) (50 U/ml; Genzyme). The RNA (5 μg) was separated on formaldehyde agarose gels, bound to Duralon membranes (Stratagene), and probed with a ^{32}P-labeled cDNA fragment (nucleotides 200–1017) from mouse iNOS (Xie et al., 1992). For organ blots, filters were additionally probed with a rat *NOS3* cDNA fragment from T. Michel (aligns with nucleotides 2071–2394 of bovine *NOS3* cDNA; Genbank accession number U02534) and with a rat *NOS1* cDNA fragment (nucleotides 1546–1942) from D. Bredt (Bredt et al., 1991).

For immunoblots, thioglycollate broth–elicited peritoneal exudate macrophages were activated in vitro for 42 hr as described elsewhere (Ding et al., 1988) with or without LPS (100 ng/ml) and rMuIFNγ (50 U/ml). Lysates (60 μg of protein per lane) were subjected to SDS–PAGE, electrophoretic transfer to nitrocellulose, and blotting with rabbit anti-holo-iNOS immunoglobulin G (IgG) as detailed (Xie et al., 1992).

Macrophage NO Generation, Respiratory Burst, and MHC Class II Expression

NOS activity was assessed in macrophages elicited as above and exposed in vitro for 48 hr to buffer alone, LPS (100 ng/ml), rMuIFNγ (500 U/ml; Genentech), rMuTNFα (1000 U/ml; Genzyme), natural mouse IFNα/β (1000 U/ml; Lee Biomolecular), or some combination of these. NO_3^- in the medium was >90% converted to NO_2^- with aspergillus nitrate reductase (0.05 U/ml; Boehringer) and NO_3^- plus NO_2^- in triplicate cultures quantified by Griess's assay as detailed elsewhere (Ding et al., 1988).

The respiratory burst was measured by the H_2O_2-dependent oxidation of fluorescent scopoletin as in Ding et al. (1988) using peritoneal macrophages activated in vivo by intraperitoneal injection of LPS (5 μg) plus rMuIFNγ (10^4 U) 24 hr before harvest. Cells were cultured 3.5 hr to allow demise of the <4% contaminating neutrophils and then either left untreated or triggered with phorbol myristate acetate (100 ng/ml; Sigma), bacille Calmette-Guérin (6 × 10^6 cfu/ml; Pasteur strain; Trudeau Institute), or human serum-coated L. monocytogenes (2 × 10^7 cfu/ml; clinical isolate; New York Hospital).

Thioglycollate broth–elicited peritoneal macrophages were incubated 48 hr with or without rMuIFNγ (50 U/ml), then stained with rat anti-mouse IA^{b,d} monoclonal antibody (MAb) B21.2 (1 μg/ml; Steinman et al., 1980) followed by fluorescein isothiocyanate–conjugated goat F(ab′)$_2$ anti-rat IgG (Chemicon International). Cells (10^4 in each group) were analyzed by flow microfluorimetry (FACScan; Becton Dickinson).

Listeria Infection and Tumor Cytostasis

Mice matched for gender and age (8–10 weeks) were inoculated intraperitoneally with L. monocytogenes (4 × 10^5 cfu; EGD strain; Trudeau Institute) and sacrificed 3 days later, and the number of colony-forming units was determined in triplicate 10-fold serial dilutions of homogenates of liver and spleen (Conlan and North, 1994).

Thioglycollate broth–elicited peritoneal exudate macrophages (1 × 10^5 per well) were exposed to LPS (10 ng/ml) and the indicated concentrations of rMuIFNγ at the same time as they were admixed with L1210 mouse lymphoma cells (5 × 10^4 per well). Tumor cell proliferation was measured over 12 hr by the incorporation of [^{3}H]thymidine as described elsewhere (Stuehr and Nathan, 1989), and NO_3^- plus NO_2^- was measured as above.

Blood Pressure, Serum Factors, and Survival in Endotoxic Shock

Mice matched in each experiment for gender and age (8–12 weeks) were anesthetized with pentobarbital (60 mg/kg). Cannulas (30 gauge) were placed in the right carotid artery and connected to a pressure transducer (Gould). Blood pressure was monitored on a TA4000 Gould polygraph for 5 hr or until death following the intraperitoneal injection of saline (0.2 ml) or LPS (1 mg/kg in 0.2 ml of saline). The experimenter was not blinded, but administered no therapy.

Levels of NO_3^- plus NO_2^- were measured in triplicate by the method described above in sera of 11 mice per group injected intravenously with 0.2 ml of saline or LPS (10 mg/kg in 0.2 ml of saline) and sacrificed 5.5 hr later or in six mice per group injected intraperitoneally with autoclaved P. acnes (1 mg) followed 6 days later by LPS injection

intraperitoneally (100 μg). The autoclaved bacteria were shown by culture to be nonviable. Sera were collected from the latter mice after they died naturally 9–10.5 hr after administration of LPS.

Levels of IL-1α and IL-1β, TNFα, and IL-6 were determined by enzyme-linked immunosorbent assay (ELISA) (Fletcher et al., 1995). Serum ALT, AST, and LDH activities were measured spectrophotometrically on a Hitachi 911 automated clinical chemistry analyzer.

Survival studies in unanesthetized mice followed two protocols. First, previously untreated mice were injected intraperitoneally with 0.1 ml of saline or 0.1 ml of saline containing the indicated amounts of LPS. Second, 0.1 ml of saline or heat-killed P. acnes (1 mg in 0.1 ml of saline) was injected intraperitoneally. Mice were injected 6 days later with 0.1 ml of vehicle alone (5% normal mouse serum in saline) or 0.1 ml of vehicle containing the indicated amounts of LPS. Survival was monitored every 2–4 hr over 4 days and daily thereafter. There were no deaths after 4 days.

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