Published in final edited form as:

Radiat Res. 2010 September ; 174(3): 297–312. doi:10.1667/RR2019.1.

Intraesophageal Manganese Superoxide Dismutase-Plasmid Liposomes Ameliorates Novel Total-Body and Thoracic Radiation Sensitivity of NOS1^{-/-} Mice

Malolan S. Rajagopalan^a, Brandon Stone^a, Jean-Claude Rwigema^a, Umar Salimi^a, Michael W. Epperly^a, Julie Goff^a, Darcy Franicola^a, Tracy Dixon^a, Shaonan Cao^a, Xichen Zhang^a, Bettina M. Buchholz^b, Anthony J. Bauer^b, Serah Choi^a, Christopher Bakkenist^a, Hong Wang^a, and Joel S. Greenberger^{a,1}

^a Department of Radiation Oncology, University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania

^b Department of Medicine, Division of Gastroenterology, University of Pittsburgh, Pittsburgh, Pennsylvania

Abstract

The effect of deletion of the nitric oxide synthase 1 gene (NOS1^{-/-}) on radiosensitivity was determined. *In vitro*, long-term cultures of bone marrow stromal cells derived from NOS1^{-/-} were more radioresistant than cells from C57BL/6NHsd (wild-type), NOS2^{-/-} or NOS3^{-/-} mice. Mice from each strain received 20 Gy thoracic irradiation or 9.5 Gy total-body irradiation (TBI), and NOS1^{-/-} mice were more sensitive to both. To determine the etiology of radiosensitivity, studies of histopathology, lower esophageal contractility, gastrointestinal transit, blood counts, electrolytes and inflammatory markers were performed; no significant differences between irradiated NOS1^{-/-} and control mice were found. Video camera surveillance revealed the cause of death in NOS1^{-/-} mice to be grand mal seizures; control mice died with fatigue and listlessness associated with low blood counts after TBI. NOS1^{-/-} mice were not sensitive to brain-only irradiation. MnSOD-PL therapy delivered to the esophagus of wild-type and NOS1^{-/-} mice resulted in equivalent biochemical levels in both; however, in NOS1^{-/-} mice, MnSOD-PL significantly increased survival after both thoracic and total-body irradiation. The mechanism of radiosensitivity of NOS1^{-/-} mice and its reversal by MnSOD-PL may be related to the developmental esophageal enteric neuronal innervation abnormalities described in these mice.

INTRODUCTION

Mitochondria play a critical role in ionizing radiation-induced apoptosis (1,2). Radiation induces both superoxide and nitric oxide, which combine to form the potent oxidant peroxynitrite (3,4). Peroxynitrite induces lipid peroxidation of the mitochondrial membrane, leading to release of cytochrome c into the cytoplasm and caspase-mediated apoptosis (2,5, 6). Mechanisms to reduce nitric oxide production in mitochondria after irradiation may potentially mitigate the effects of ionizing radiation.

The synthesis of nitric oxide from L-arginine and oxygen is catalyzed by the enzyme nitric oxide synthase (NOS). There are three isoforms of NOS: NOS1 (neuronal NOS), NOS2

¹Address for correspondence: Department of Radiation Oncology, University of Pittsburgh Cancer Institute, 5150 Centre Avenue, Rm. 533, Pittsburgh, PA 15232; greenbergerjs@upmc.edu.

(inducible NOS), and NOS3 (endothelial NOS). NOS1 and NOS3 are constitutively expressed, but NOS2 expression is modulated by inflammatory cytokines (7). The identity of the NOS isoform found in the mitochondria (mtNOS) has been a topic of controversy (8,9). In experiments using a probe to detect NO production by the mitochondria, NO was elicited from NOS2^{-/-} and NOS3^{-/-} but not from NOS1^{-/-} mouse-derived mitochondria (10). These results were corroborated in another study using mass spectrometry, amino acid analysis and analysis of PCR fragments to identify mtNOS as the α splice variant of NOS1 (11).

Mouse knockout strains for each of three isoforms of NOS (12–14) have been established. NOS2^{-/-} mice were indistinguishable from wild-type mice except for the inability of macrophages to generate NO after exposure to lipopolysaccharide (13). NOS3^{-/-} mice were also similar to wild-type mice but displayed significant blood pressure elevation and a lower heart rate, suggesting a role in cardiovascular homeostasis (14). NOS1^{-/-} mice, which display an α splice variant deficiency, showed no significant life shortening, infertility or locomotor abnormalities compared to wild-type mice but did have pyloric stenosis and esophageal dilation (15).

The diffuse enlargement of the esophagus and stomach with hypertrophy of the pyloric sphincter in NOS1^{-/-} mice resembled infantile hypertrophic pyloric stenosis, a condition in which NOS1 expression is reduced (12,16). However, NOS1^{-/-} mice demonstrated NOS deficiency only at the pylorus (17). NOS1^{-/-} mice also displayed diffuse gastric muscle thickening associated with functional delay in gastric emptying of both solids and liquids (18). The lower esophageal sphincter in NOS1^{-/-} mice demonstrated hypertension and impaired relaxation to swallowing and efferent vagal stimulation resembling achalasia. This abnormality was incomplete, and mice had residual relaxation potential for both swallowing and vagal stimulation (19). While selective NOS1 disruption depleted NO neurotransmission throughout the gastrointestinal system and manifested as delayed gastric emptying and also showed lower esophageal sphincter hypertension and dilation of both the esophagus and stomach, no significant morbidity related to these changes was reported.

Bone marrow stromal cells from NOS1^{-/-} mice were more radioresistant than those from wild-type mice, suggesting that the NOS1^{-/-} genotype confers resistance to oxidative stress (20). Furthermore, after pharmacological antagonism of NOS or disruption of the NOS1 gene, radiation resistance of the mouse bladder was demonstrated (21). Whether NOS2^{-/-} and NOS3^{-/-} mouse marrow cells or mice display alterations of radiosensitivity is not known.

In the present studies, we evaluated the response of NOS1^{-/-} mice to thoracic and total-body irradiation compared to NOS2^{-/-}, NOS3^{-/-} and wild-type mice. Absence of the NOS1 gene product was expected to reduce peroxynitrite formation and thereby protect mice from radiation-induced toxicity and death. Instead, we found increased radiosensitivity. This was related to the abnormal esophageal physiology of these mice, since the defect was ameliorated by local intraesophageal MnSOD-PL administration.

MATERIALS AND METHODS

Long-Term Bone Marrow Cultures

Long-term bone marrow cultures were established from the femur and tibia of 6–8-week-old mice as described previously (eight cultures per mouse strain) (22). Measurement of confluence of the adherent layer, cobblestone islands, and hematopoietic cell production was as described previously (22). Quantification of 7- and 14-day colony-forming units-granulocyte, erythrocyte, macrophage, megakaryocyte (CFU-GEMM) was carried out as described previously (20).

Clonogenic Radiation Survival Curves

Bone marrow stromal cell lines were established from adherent cells harvested from long-term bone marrow cultures at week 20 as described previously (20). Cells from parent lines NOS1^{-/-}, NOS2^{-/-} and NOS3^{-/-} and wild-type (C57BL/6NHsd) mice were each irradiated with 0–8 Gy γ rays. Cells were placed in Linbro four-well tissue culture plates (Flow Laboratories, McLean, VA) at concentrations of 500, 1000 or 4000 cells per 4-ml well. Cells were incubated in a high-humidity incubator at 37°C with 95% air/5% CO₂ for 10 days, and colonies of greater than 50 cells were scored according to published methods (23).

Hematopoietic Progenitor Cell Assays

Radiation survival curves were performed for CFU-GEMM. Bone marrow was harvested from the tibias and femurs of NOS1^{-/-} and C57BL/6NHsd mice (20). Cells were counted with a cell counter (Beckman Coulter, Fullerton, CA) after lysis of red blood cells. Then 1×10^6 viable cells/ml were irradiated with 0–8 Gy and plated in methylcellulose-containing medium (MethoCult GF 3434, StemCell Technologies, Vancouver, BC, Canada) at cell concentrations ranging from 20,000–100,000 cells/well. Cultures were incubated at 37°C in a high-humidity incubator with 95% air/5% CO₂ for 14 days, and colonies of over 50 cells were scored.

Mice and Ionizing Radiation

All animal experiments and procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at University of Pittsburgh. Experiments were performed on NOS1^{-/-}, NOS2^{-/-}, NOS3^{-/-} and control background strain C57BL/6NHsd mice 6–10 weeks of age.

Thoracic irradiation was administered to female mice by a linear accelerator to a dose of 20 Gy using an established model of radiation-induced organizing alveolitis/fibrosis at around 120–150 days in the C57/B6NHsd mouse (24). Mice were followed for development of radiation lung damage and survival.

Total-body irradiation (TBI) to the LD_{50/30} of 9.5 Gy (25) was delivered to male mice by a ¹³⁷Cs irradiator (Mark MKI-68, J. L. Shepherd and Associates, San Fernando, CA) at a dose rate of 80 cGy per minute. Mice were followed for development of gastrointestinal and/or hematopoietic syndrome and survival.

Whole-brain irradiation was administered to mice using a linear accelerator to a dose of 9.5 Gy to the head and neck region only. Mice were followed for development of neurological signs or seizures.

Esophageal Delivery of Liposomes Containing DS-Red or MnSOD-PL

To assess potential radioprotective mechanisms, manganese superoxide dismutase in plasmid liposomes (MnSOD-PL) was given orally to C57BL/6NHsd and NOS1^{-/-} mice 24 h prior to thoracic irradiation. The formulation of MnSOD-PL has been described previously (24). Mice received intraesophageal administration of 100 μ l of water followed by 100 μ l of MnSOD-PL (100 μ g plasmid DNA) by placing a feeding tube attached to a 1-ml syringe at the proximal end of the esophagus. The volume was injected at the proximal esophagus and mice were allowed to swallow. In a separate experiment to ensure delivery of genetic material to the esophagus, liposomes containing the DS-red transgene plasmid were created as described previously (26) and injected using the same methodology as above. The mice were killed humanely 24 h later and each esophagus was excised, frozen in OCT, sectioned and examined for the expression of DS-red.

MnSOD Biochemical Activity

NOS1^{-/-} mice and C57BL/6NHsd mice were treated with MnSOD-PL as described above. The esophagus from each mouse was removed 24 h later, frozen on dry ice, and assayed for MnSOD biochemical activity as described previously (23,24). One unit of MnSOD biochemical activity resulted in a 50% inhibition of nitroblue tetrazolium (NBT) reduction at the maximal rate per milligram of protein.

Pharmacological Inhibition of NOS

Global pharmacological inhibition of NOS activity was achieved by systemic administration of L-NAME (N ω -nitro-L-arginine methyl ester) (Sigma-Aldrich, St. Louis, MO) as described previously (27). Osmotic mini-pumps (ALZET, Cupertino, CA) were implanted subcutaneously, providing a constant systemic supply (0.5 μ l/h) of L-NAME (125 mg/ml PBS, $n = 15$) or PBS ($n = 15$) for 14 days. On day 3 after implantation, 10 mice from the L-NAME group and 10 from the PBS group were treated with 9.5 Gy TBI, leaving five mice from each group as nonirradiated controls. Survival of these mice was then followed.

In a second experiment, two groups of mice were treated with either L-NAME ($n = 12$) or PBS ($n = 14$) via implanted osmotic pumps for 9 days. Then the pumps were removed, and nine mice from the L-NAME group and 10 mice from the PBS group were treated with 9.5 Gy TBI. Three mice from the L-NAME group and four mice from the PBS group served as nonirradiated controls. Survival of these mice was then followed.

Histopathological Analysis of Tissues

Mice were killed humanely and perfused with 10 ml of PBS prior to necropsy. Collected organs were fixed in 10% buffered formalin for 24 h, sectioned (5 mm), and subsequently stained with hematoxylin and eosin (H&E) for histological analysis. Tissue was collected from the esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, spleen, liver, heart and lung. For comparison of intestinal villus height, sections from each animal were obtained from identical anatomical positions in the duodenum. Between 50 and 100 longitudinal intestinal villi were measured in random order from the base of the crypt to the top of the villus.

Peripheral Blood and Serum Chemistry Analyses

Serum chemistry and whole blood analyses were performed in C57BL/6NHsd and NOS1^{-/-} mice 6 days after 9.5 Gy TBI and in nonirradiated control mice from both strains. Whole blood was collected by tail vein into EDTA-containing collection tubes (MiniCollect, Greiner Bio-ONE, Austria). Blood was evaluated using a Vet ABC-Diff Hematology Analyzer, which measured white blood cells (WBC), red blood cells (RBC), thrombocytes (plt), hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC).

Blood for serum chemistry analyses was collected by cardiac puncture immediately after killing. Approximately 1 ml of blood was collected in Microtainer tubes with no additives (Becton, Dickinson and Company, Franklin Lakes, NJ). Blood was allowed to coagulate for 30 min and was then centrifuged at 2000g for 3 min. Serum was collected and sent to Antech GLP (Antech Diagnostics Inc, Morrisville, NC) for analysis.

RT-PCR for Measurement of Inflammatory Cytokines and NOS Isoforms

RNA was extracted from mouse esophagus, stomach and intestine using the TRIzol reagent (Invitrogen Carlsbad, CA) following the manufacturer's instructions, quantified using a spectrophotometer, and stored at -80°C . Reverse transcription of 2 μ g of total RNA to complementary DNA (cDNA) was accomplished using the High Capacity cDNA Reverse

Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol.

In subsequent steps, expression of GUSB (GenBank: NM_010368.1), NF- κ B (GenBank: NM_199267.2), TNF- α (GenBank: NM_013693.2), IFN- γ (GenBank: NM_008337.3), NOS1 (GenBank: NM_008712.2), NOS2 (GenBank: NM_010927.3), and NOS3 (GenBank: NM_008713.4) was quantified by real-time polymerase chain reaction (RT-PCR). Ninety-six-well plates were prepared with 10 μ l of Taqman Gene Expression Master mix, 5 μ l RNase-free water, 1 μ l of the corresponding Taqman Gene Expression probe, and 4 μ l of cDNA (totaling 2 μ g cDNA) using the Eppendorf epMotion 5070 automated pipetting system (Eppendorf, Westbury, NY). The cDNA was amplified with 40 cycles of 95°C (denaturation) for 15 s and 60°C (annealing and elongation) for 1 min using the Eppendorf Realplex2 Mastercycler.

Data for each gene were normalized by calculating the differences (Δ Ct) from the CtGUSB and Cttarget genes. Subsequently, the relative increase or decrease in expression was calculated by comparing the reference gene with the target gene ($\Delta\Delta$ Ct) and using the formula for relative expression ($=2^{-\Delta\Delta$ Ct). Subsequently, ($\Delta\Delta$ Ct) levels were compared and *P* values were calculated using one-way ANOVA followed by Tukey's multiple comparison tests.

Gastrointestinal Transit Studies

Mouse gastrointestinal transit studies were performed in nonirradiated NOS1^{-/-} and C57BL/6NHsd mice or in irradiated mice 6 days after 9.5 Gy TBI, with at least four mice per group. The gastrointestinal distribution of orally administered FITC-labeled dextran (Molecular Probes, Eugene, OR) was evaluated in all groups, and the geometric center (GC) was calculated as described previously (28).

Electric-Field Stimulation Studies of the Lower Esophageal Sphincter

Mechanical *in vitro* contractile activity was evaluated by using muscle strips of the lower esophageal sphincter from NOS1^{-/-} and C57BL/6NHsd mice with and without exposure to 20 Gy thoracic irradiation. Muscle strips were incubated in a basal solution of L-arginine, and dose-response curves for muscle contractility were generated by electric-field stimulation (EFS) with frequencies ranging from 0.1 to 15 Hz as described previously. Dose-response curves were also generated after EFS after antagonization of NOS receptors (with L-nitro-arginine) and antagonization of muscarinic receptors (with atropine) (28).

Immunoprecipitation Analysis for ATM

Bone marrow stromal cell lines from NOS1^{-/-} or C57BL/6NHsd mice were grown to 80% confluence in 10-cm petri dishes. One dish from each mouse strain was irradiated to 2 Gy in a ¹³⁷Cs irradiator (Mark MKI-68, J. L. Shepherd and Associates) and incubated for 1 h. Cells were manually scraped, pelleted by centrifugation, and lysed in RIPA buffer supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma Aldrich) and 2 $\times$ Halt^{TM} phosphatase inhibitor cocktail (Pierce, Rockford, IL). Proteins were separated by SDS-polyacrylamide gel electrophoresis followed by transfer to BioTrace^{TM} PVDF membrane (Pall Life Sciences, East Hills, NY). After transfer, the membrane was blocked in TBS with 5% milk and 0.1% Tween-20 (Sigma) for 1 h at room temperature. Membranes were incubated with the following ATM antibody at 4°C overnight. Detection of bound antibodies was performed with ECL Advance^{TM} Western Blotting Detection kit (Amersham Biosciences, Piscataway NJ). Chemiluminescence signals were recorded using Hyperfilm^{TM} (Amersham Biosciences).

Continuous Videography

To detect any changes in feeding, behavior or function in $\text{NOS1}^{-/-}$ mice after TBI, continuous videography was performed. $\text{NOS1}^{-/-}$ mice and wild-type were irradiated to 9.5 Gy as described previously and were housed three per cage. An electronic surveillance system (Logitech Wilife Indoor Camera Master System, Logitech Inc., Fremont, CA) was used for continuous digital recording to a dedicated computer. All recorded data were reviewed visually for evidence of changes in feeding, energy or behavior.

Statistics

Data from radiation survival curves were analyzed using the linear-quadratic and single-hit, multitarget models (23). Results are presented as means $\pm$ SEM from at least three measurements. Student's *t* test and two-way ANOVA were used to compare means of different groups where appropriate. Electrical-field stimulation data were analyzed using ANOVA with Tukey's post hoc group comparisons using InerSTAT-a v1.3. All analyses were performed on 10 Hz values.

RESULTS

Long-Term Bone Marrow Cultures Derived from $\text{NOS1}^{-/-}$ Mice Demonstrate Increased Longevity of Production of CFU-GEMM Indicative of Stem Cell Resistance to Oxidative Stress Compared to Those from $\text{NOS2}^{-/-}$ or $\text{NOS3}^{-/-}$ Mice

The longevity of hematopoiesis in long-term bone marrow cultures has been shown to be associated with resistance to the oxidative stress of aging *in vitro* as reflected by persistence of hematopoietic stem cell islands in the adherent layer, termed cobblestone islands. Continuous bone marrow cultures were established from $\text{NOS1}^{-/-}$, $\text{NOS2}^{-/-}$, $\text{NOS3}^{-/-}$ and C57BL/6NHsd (wild-type) mice. All culture groups had stromal cells covering over 75% of the flask surface by 5 weeks and maintained stable adherent layers covering over 95% of the flask surface at 35 weeks (Fig. 1A).

Weekly numbers of cobblestone islands (indicative of adherent hematopoietic foci) (Fig. 1B) were counted, and the cumulative numbers were calculated (Fig. 1C) for all culture groups. The cumulative number of cobblestone islands was approximately the same for all groups until week 10, when $\text{NOS1}^{-/-}$ and $\text{NOS2}^{-/-}$ cultures demonstrated increased numbers. $\text{NOS1}^{-/-}$ and $\text{NOS2}^{-/-}$ cultures both had more nonadherent cells than did $\text{NOS3}^{-/-}$ and wild-type cultures (Fig. 1D, E). This reflects a better stability of hematopoiesis in the adherent layer of $\text{NOS1}^{-/-}$ and $\text{NOS2}^{-/-}$ compared to $\text{NOS3}^{-/-}$ and wild-type bone marrow cultures. However, $\text{NOS1}^{-/-}$ cultures produced significantly more hematopoietic cells forming colonies after 7 (Fig. 1F) and 14 days (Fig. 1G) in semisolid CFU-GEMM culture than did other groups.

$\text{NOS1}^{-/-}$ Marrow Stromal Cells Demonstrate Relative Radiation Resistance

Representative radiation survival curves for all four cell lines are shown in Fig. 2. $\text{NOS1}^{-/-}$ mouse-derived bone marrow stromal cells demonstrated significant radiation resistance ($\bar{n}$ of 20.8 ± 5.6 and D_q of 5.2 ± 1.3 Gy) compared to control C57BL/6NHsd mouse stromal cells ($\bar{n}$ of 8.3 ± 2.4 and D_q of 1.8 ± 1.5 Gy). There was no significant difference in radiosensitivity between C57BL/6NHsd, $\text{NOS2}^{-/-}$ or $\text{NOS3}^{-/-}$ marrow stromal cell lines compared to $\text{NOS1}^{-/-}$ cells ($D_0 = 1.4 \pm 0.2$ compared to 1.6 ± 0.1 , respectively).

$\text{NOS1}^{-/-}$ Mice Display Thoracic Radiation Sensitivity

To determine whether the resistance to oxidative stress reflected as increased persistence of hematopoiesis in long-term bone marrow cultures and increased radiation resistance of $\text{NOS1}^{-/-}$ mouse-derived bone marrow stromal cells translated to increased radiation resistance

in vivo, NOS1^{-/-}, NOS2^{-/-}, NOS3^{-/-} and C57BL/6NHsd mice were irradiated with 20 Gy to the thorax (Fig. 3A). In two separate experiments over 2 years ($n = 20/\text{group}$ each time), NOS1^{-/-} mice were significantly more sensitive to thoracic irradiation, with 50% of the mice dead at 41 days compared to 156, 148 and 159 days for C57BL/6NHsd, NOS2^{-/-} and NOS3^{-/-} mice, respectively ($P < 0.0001$).

NOS1^{-/-} Mice are Sensitive to TBI

NOS1^{-/-}, NOS2^{-/-}, NOS3^{-/-} and C57BL/6NHsd mice ($n = 6/\text{group}$) were irradiated with 9.5 Gy and followed for survival (Fig. 3B). This dose is the LD_{50/30} for C57BL/6NHsd mice at our facility (27). Fifty percent of the NOS1^{-/-} mice showed an LD₅₀ of 9 days compared to 18.5 and 25 for C57BL/6NHsd, NOS2 or NOS3^{-/-} mice ($P = 0.006$).

The dose-modifying effect of NOS1 gene deletion was measured. C57BL/6NHsd mice were irradiated with total-body doses ranging from 9.5 Gy to 18 Gy, followed for death from the hematopoietic syndrome, and compared to NOS1^{-/-} mice receiving 9.5 Gy TBI. C57BL/6NHsd mice irradiated with 12 Gy demonstrated similar survival times to these of NOS1^{-/-} mice irradiated with 9.5 Gy (Fig. 3C). Therefore, the dose-modifying factor for the absence of the NOS1 gene was 0.79.

Gross Anatomic Pathological Demonstration of Dilatation of Esophagus and Stomach in Irradiated and Nonirradiated NOS1^{-/-} Mice

Based upon the rapid death within 7–10 days in NOS1^{-/-} mice after 9.5 Gy TBI, complete necropsies were carried out in a separate group of NOS1^{-/-} mice. Unchanged dilated esophagus and stomach in NOS1^{-/-} mice (Fig. 4) was the only finding.

Histopathological Analysis of the GI Tract Revealed no Significant Structural Changes at 6 Days in TBI NOS1^{-/-} Mice

Histopathology of all major organs including esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, spleen, liver, lung and heart was carried out on fixed sections stained with H&E ($n = 3/\text{group}$). Review of multiple sections of each organ from all strains of mice revealed no significant structural differences after irradiation between the organs from NOS1^{-/-} and C57BL/6NHsd mice (see the Appendix). Results for the intestine are shown in Fig. 5.

Morphology of the esophagus, stomach, intestine and colon on H&E-stained sections appeared intact and similar between irradiated NOS1^{-/-} and nonirradiated NOS1^{-/-} mice and wild-type mice. Specifically, small bowel villi and crypt cell numbers and length were intact and crypt cells were intact and showed no signs of radiation damage (Fig. 5). Villus + crypt height was tabulated for NOS1^{-/-} and C57BL/6NHsd mice after exposure to 9.5 Gy TBI; NOS1^{-/-} mice had significantly longer intestinal villi than wild-type C57BL/6NHsd mice at day 6 (1.3 ± 0.2 compared to 1.1 ± 0.3 mm). Results were the same for jejunum and ileum.

NOS1^{-/-} and Wild-Type Mice Show Similar Hematological Responses to TBI

We next determined whether a rapid decrease in blood count reflective of the hematopoietic syndrome may have been responsible for the rapid death of NOS1^{-/-} mice after TBI. Complete blood counts were performed in nonirradiated mice or in mice 6 days after TBI (Table 1). White blood cells and platelets were reduced in all irradiated groups (Table 1). Blood counts for C57BL/6NHsd mice were more sensitive to radiation, with significant decreases in red blood cells (RBC), and hemoglobin and hematocrit. NOS1^{-/-}, NOS2^{-/-} and NOS3^{-/-} mice demonstrated no significant relative differences in changes in WBC, RBC, hemoglobin or hematocrit. These findings indicate that the rapid death of NOS1^{-/-} mice was not associated

with a rapid drop in complete blood counts and that the rapid death after TBI in NOS1^{-/-} mice could not be attributed to the hematopoietic syndrome.

NOS1^{-/-} and C57BL/6NHsd Mouse Marrow Show Similar Marrow CFU-GM, BFU-E and CFU-GEMM Radiosensitivity

We next tested for relative radiosensitivity of marrow stem cells. CFU-GEMM radiation survival curves were constructed for bone marrow from NOS1^{-/-} and C57BL/6NHsd mice. Colonies counts were performed for CFU-GM, BFU-E (indicative of more differentiation of granulocytes-macrophage colony-forming or burst-forming unit erythroid colony-forming cells, respectively), and CFU-GEMM in both strains of mice. In C57BL/6NHsd mouse marrow, the D_0 's for CFU-GM, BFU-E and CFU-GEMM colonies were 1.51, 1.03 and 3.04, respectively (Fig. 6A). In NOS1^{-/-} mice, D_0 values were 2.00, 1.73 and 2.56, respectively (Fig. 6B). The survival curves for NOS1^{-/-} mice and C57BL/6NHsd mice were not significantly different ($P > 0.5$).

Serum Chemistry in Irradiated NOS1^{-/-} Mice Showed no Abnormalities Compared to Irradiated C57BL/6NHsd Mice

We next tested whether NOS1^{-/-} mice died of an atypical metabolic response to 9.5 Gy TBI. Basic serum electrolytes and renal, liver and pancreatic function were examined 6 days after TBI in C57BL/6NHsd and NOS1^{-/-} mice. Prior to irradiation NOS1^{-/-} mice showed relative increases in chloride and decreases in albumin (Table 2). Both nonirradiated and irradiated C57BL/6NHsd mice demonstrated no significant differences in any of the parameters. NOS1^{-/-} mice receiving 9.5 Gy TBI demonstrated a significant drop in potassium and BUN compared to nonirradiated mice but not into a range known to cause renal failure.

NOS1^{-/-} and C57BL/6NHsd mice demonstrated no significant preirradiation differences in electrolytes or measures of renal or liver/pancreatic function. After 9.5 Gy TBI, NOS1^{-/-} mice demonstrated no differences from control mice. The data provide evidence that electrolyte disturbances, glucose levels and abnormalities in renal, liver or pancreatic function did not cause the rapid death of NOS1^{-/-} mice after 9.5 Gy TBI. Taken together with the complete blood count findings and the absence of neutrophils or inflammatory cell infiltrates in histopathologically examined tissues, the data indicate that septicemia or shock could not explain the rapid death of irradiated NOS1^{-/-} mice.

RT-PCR-Based Detection of Cytokine and NOS Isoform Expression in Irradiated Mice

We next sought to determine whether TBI NOS1^{-/-} mice demonstrated exacerbation of an inflammatory response. Inflammatory markers and NOS isoform expression were compared in the NOS1^{-/-} and C57BL/6NHsd wild-type mice before (day 0) and 1 and 6 days after 9.5 Gy TBI. The esophagus, stomach and intestine were evaluated (Table 3).

NF- κ B expression was significantly higher in the wild-type C57BL/6NHsd mouse esophagus than in NOS1^{-/-} mice 1 and 6 days after irradiation ($P < 0.01$). NOS3 was also expressed at a significantly higher level ($P < 0.0001$) in NOS1^{-/-} mice compared to wild-type mice at these times. In the stomach, there were no significant differences in expression of any gene transcripts measured. In the intestine, TNF- α ($P < 0.0001$) and NOS2 ($P < 0.001$) were significantly increased over baseline preirradiation levels in wild-type compared to NOS1^{-/-} mice. Intestinal TNF- α ($P < 0.0001$) and IFN- γ ($P < 0.001$) were expressed at significantly higher levels 1 day after TBI in wild-type compared to NOS1^{-/-} mice. Intestinal NF- κ B was expressed at a significantly higher level ($P < 0.0001$) in wild-type compared to NOS1^{-/-} mice at 6 days after irradiation. While significant differences were detected between mouse strains in some assays, there was no up-regulation of a specific inflammatory cytokine or NOS isoform to a level that could explain the rapid death of NOS1^{-/-} mice after 9.5 Gy TBI.

ATM Kinase Levels are not Altered in NOS1^{-/-} Mice

To measure DNA repair capacity, immunoprecipitation for ATM was conducted in bone marrow stromal cells from NOS1^{-/-} and C57BL/6NHsd mice that were either nonirradiated or exposed to 2 Gy (Fig. 7). A typical rise in the level of phospho-ATM, the active form of the protein, was seen in irradiated C57BL/6NHsd mouse cells. A similar rise was seen in irradiated NOS1^{-/-} mouse cells. Therefore, ATM-dependent DNA damage repair was intact in NOS1^{-/-} cells.

Electric-Field Stimulation (EFS) Studies of the Lower Esophageal Sphincter after Thoracic Irradiation Revealed no Significant Differences in NOS1^{-/-} Mice

The known abnormality in the NOS1^{-/-} mouse is in the neuromuscular system. Tissue from the lower esophageal sphincter of C57BL/6NHsd and NOS1^{-/-} mice was excised and stimulated by an electric field across a range of frequencies, and the degree of contraction was measured (Fig. 8). Studies using L-arginine as the basal substrate were conducted in nonirradiated mice and in mice 9 days after exposure to 20 Gy thoracic radiation, the time at which mice began to die (Fig. 3A). Contraction after EFS was measured after antagonizing NOS receptors (with the addition of L-nitro-arginine) or antagonizing both NOS and muscarinic receptors (with L-nitro-arginine and atropine).

In the wild-type C57BL/6NHsd mouse tissue, increased contractility with increasing EFS frequency was seen. Inhibition of NOS or muscarinic receptors did not produce significant differences before or after irradiation. In NOS1^{-/-} mice, contractility after antagonization of both muscarinic and NOS receptors despite high-frequency EFS stimulation was seen and persisted after thoracic irradiation. This loss of contractility after antagonization of both receptors could be caused by a deficiency in tachykinin nerve responses in NOS1^{-/-} mice and/or the loss of membrane rebound-induced contractions, which are normally produced by transient nitrergic membrane hyperpolarization of the ICC/smooth muscle cells. The data did not reveal a cause of the rapid death of irradiated NOS1^{-/-} mice.

NOS1^{-/-} Mice Show no Significant Differences in Postirradiation Gastrointestinal Motility

We next evaluated whether the functional defect in intestinal muscle contractibility might explain the *in vivo* thoracic and total-body radiosensitivity of NOS1^{-/-} mice. NOS1^{-/-} and C57BL/6NHsd were evaluated 6 days after 9.5 Gy TBI and compared to nonirradiated mice. Fluorescein isothiocyanate (FITC)-labeled dextran was administered orally, and after 75 min the mice were killed and the geometric center of fluorescence in the gastrointestinal system was determined. Higher numbers for the geometric center indicate faster transit through the gastrointestinal system. No significant difference was seen in either group after irradiation. The expected slower transit in nonirradiated NOS1^{-/-} mice compared to wild-type mice was observed; however, there was no evidence of radiation-induced exacerbation of gastroparesis or delayed intestinal motility. These data provide evidence that an abnormal gastrointestinal motility was not responsible for the radiation sensitivity of NOS1^{-/-} mice.

Pharmacological Inhibition of NOS in Wild-Type Mice Produces Radioresistance

To determine whether pharmacological inhibition of NOS1 in wild-type mice replicated the radiosensitivity observed in NOS1^{-/-} mice, we next tested the effects of a broad NOS inhibitor that reduces NOS1 as well as NOS2 and NOS3. Groups of C57BL/6NHsd mice were implanted subcutaneously with osmotic pumps containing either PBS or the NOS inhibitor L-NAME (which inhibits all three isoforms of NOS). In the first set of experiments, mice were implanted, irradiated 3 days later, and followed for survival (Fig. 9A). The L-NAME-treated mice survived significantly longer than the PBS-treated mice ($P = 0.0012$). In a second set of experiments, mice were implanted with osmotic mini-pumps that were removed 9 days later, NOS levels

were allowed to recover, and then the mice were irradiated (Fig. 9B). There was no survival advantage under these conditions. The data indicated that continuous NOS inhibition during and after irradiation confers a significant survival advantage in wild-type mice. The data did not support a direct role of systemic NOS1 inhibition in the radiation sensitivity of NOS1^{-/-} mice.

Intraesophageal MnSOD-PL Ameliorates NOS1^{-/-} Mouse Radiosensitivity and Increases Survival after Thoracic and Total-Body Irradiation

Since the developmental abnormality in the esophagus was the only known gastrointestinal abnormality in NOS1^{-/-} mice, we next tested whether locally delivered antioxidant transgene therapy ameliorated the increased radiosensitivity. MnSOD-PL intraesophageal gene therapy was tested for protection from thoracic irradiation (Fig. 10A) and TBI (Fig. 10B). NOS1^{-/-} mice either were injected intraesophageally with MnSOD-PL 24 h prior to irradiation or received no treatment. Subsequently mice received 20 Gy to the thorax or 9.5 Gy TBI and were then followed for survival. In the thoracic irradiation model, NOS1^{-/-} mice treated with MnSOD-PL prior to irradiation demonstrated significantly increased survival. All mice survived past 30 days ($n = 6$). In contrast NOS1^{-/-} mice receiving no treatment had a mean survival time of 11 days ($P = 0.031$). With TBI, mice treated with MnSOD-PL also lived significantly longer than untreated NOS1^{-/-} mice ($P = 0.043$).

MnSOD-PL DS-Red PI Uptake is Restricted to the Esophagus of both NOS1^{-/-} and Wild-Type Mice

To ensure that the delivery method resulted in transgene delivery only to the esophagus, experiments were conducted in which DS-red plasmid in plasmid liposomes was injected at the proximal end of the esophagus in NOS1^{-/-} mice. Twenty-four hours later, mice were killed, and their esophaguses were fixed, sectioned and analyzed. Examination of slides revealed evidence of uptake of transgene material in the esophagus of mice receiving plasmid liposomes. The stomach demonstrated no fluorescence in treated mice. In untreated mice, no fluorescence activity was detected. The data provide evidence that swallowed plasmid liposomes delivered the plasmid-liposome-mediated transgene to the esophagus.

Intraesophageal MnSOD-PL Administration Increases MnSOD Biochemical Activity Equally in NOS1^{-/-} and Wild-Type Mouse Esophagus

To confirm that MnSOD activity was increased after delivery of MnSOD-PL in NOS1^{-/-} and C57BL/6NHsd mice, enzyme activity was measured (Fig. 10C). MnSOD activity in C57BL/6NHsd mice was significantly increased 24 h after administration of MnSOD-PL. Similarly, in NOS1^{-/-} mice, MnSOD activity was significantly increased 24 h after administration of MnSOD-PL. There was no difference in MnSOD activity between NOS1^{-/-} and C57BL/6NHsd mice after administration of MnSOD-PL.

Continuous Videography of NOS1^{-/-} Mice after 9.5 Gy TBI Reveals Death from Grand Mal Seizures

Groups of female NOS1^{-/-} mice were irradiated to 9.5 Gy TBI and were continuously monitored using a digital video surveillance system. All mice were found to have normal oral intake, activity, bowel movements and urination after irradiation. There was no significant weight loss and no observable change in behavior. Normal activity and feeding were observed until approximately 4–6 h prior to death. At this time, mice were found to be profoundly lethargic, demonstrated gait instability, and showed minimal responsiveness to noxious stimuli. All mice died after a generalized tonic-clonic seizure that lasted 30–60 s. Two mice died 9 days after TBI and one died after 10 days. In contrast, monitored C57BL/6J mice died significantly later with evidence of lethargy and listlessness and no evidence of Grand Mal seizures.

Isolated Brain Irradiation of NOS1^{-/-} Mice does not Produce Early Death

We next determined whether the seizures that were observed in NOS1^{-/-} mice after 9.5 Gy TBI were attributable to brain irradiation. NOS1^{-/-} and C57BL/6NHsd mice were irradiated with 9.5 Gy to the head and neck only. One cohort in each strain received intraoral MnSOD-PL 24 h prior to irradiation. No early deaths secondary to brain irradiation were observed in NOS1^{-/-} mice (Fig. 3D).

DISCUSSION

The present studies report a significant radiosensitivity of homozygous deletion recombinant negative NOS1^{-/-} mice to both thoracic and total-body irradiation and amelioration of this effect with intraesophageal MnSOD-PL. The data establishing radiosensitivity are in marked contrast to the increased longevity of hematopoiesis in long-term bone marrow cultures reflecting the resistance to oxidative stress, the radiation resistance of NOS1^{-/-} bone marrow stromal cells *in vitro*, and the radioresistance of bladder *in vivo* (10). NOS1 has been shown to localize to the mitochondria and is known to be critical in the formation of peroxynitrite, a potent oxidant that leads to apoptosis of irradiated cells (2,5,6).

The mechanism of the radiosensitivity of NOS1^{-/-} mice was not obvious. Histopathology of all major organs including the gastrointestinal system revealed no evidence of inflammation, intestinal villus shortening, gross pathology or other radiation toxicity. Gastrointestinal transit studies and electric-field stimulation experiments to assess dysmotility, gastroparesis and general enteric nervous dysfunction showed no changes in irradiated NOS1^{-/-} mice compared to wild-type mice. Complete blood counts, serum chemistry, liver function and renal function studies were carried out to find evidence of a rapid hematopoietic syndrome or electrolyte disturbance and revealed no cause of rapid death in irradiated NOS1^{-/-} mice compared to wild-type mice. Studies of mRNA expression in multiple organs were conducted to determine whether irradiated NOS1^{-/-} mice up-regulated either inflammatory cytokines (TNF- α , NF- κ B, IFN- γ) or other NOS isoforms (NOS2, NOS3). While subtle changes were detected, including an increase in NOS3 expression in irradiated NOS1^{-/-} mice, these differences could not explain the rapid mortality after irradiation. Mice exhibited normal behavior, feeding and elimination until the final day of life, during which a 4–6-h period of marked lethargy was observed followed by a generalized tonic-clonic seizure. However, brain-only irradiation of NOS1^{-/-} mice with 9.5 Gy, the dose that precipitated seizures with TBI, failed to elicit neurological deficit or seizures. Thus, while seizures were the apparent cause of death, they were not reproduced with brain-only irradiation.

Several different genetic determinants of radiation sensitivity have been described in the literature. Mouse models of ataxia telangiectasia (ATM^{-/-}) (29,30), Fanconi anemia (FANCG^{-/-}) (31), and other disorders (32) show sensitivity to radiation *in vitro* and *in vivo*. In these models, radiosensitivity is explained by defective capacity to repair damaged DNA. ATM in irradiated and nonirradiated NOS1^{-/-} bone marrow stromal cells showed no difference in ATM-mediated DNA damage repair compared to wild-type mice.

Clinical correlations of abnormal NOS1 expression in humans include infantile pyloric stenosis and psychotic disorders. Previous studies have suggested that genetic variation of NOS1 may be implicated in the pathogenesis of schizophrenia and bipolar disorder (33,34). This notion may be analogous to aggressive and depressive-like behaviors that have been reported in NOS1 knockout mice (35). The mechanism of genetic susceptibility to psychotic disorders in humans is believed to be related to the chromosomal location of the NOS1 gene, which has been shown to be a susceptibility locus in linkage studies (36–38). Moreover, the role of nitric oxide as a second messenger of the N-methyl-D-aspartate (NMDA) receptor (39) as well as the close interaction of nitric oxide pathway with the dopaminergic and serotonergic pathways may

explain the involvement of NO in these disorders. Also, a recent study suggested that certain NOS1 polymorphisms may be associated with an increased risk to cerebral malaria (40).

Pharmacological broad NOS inhibition of all isoforms in wild-type mice (using L-NAME) showed that postirradiation but not preirradiation treatment increased survival after TBI. The increased survival after TBI with continuous pharmacological NOS inhibition was consistent with our *in vitro* experiments showing radioresistance in NOS1^{-/-} bone marrow stromal cells and could not explain the radiosensitivity of NOS1^{-/-} mice to thoracic or total-body irradiation.

Intraesophageal MnSOD-PL gene therapy reversed both the thoracic and total-body radiosensitivity of NOS1^{-/-} mice. The MnSOD-PL gene product is a potent antioxidant that has been shown to increase survival of mice exposed to thoracic radiation and to facilitate tissue repair after irradiation (26,41). That this therapy restored survival in NOS1^{-/-} mice after thoracic or total-body irradiation indicated that the mechanism of death was at the level of the esophagus. The data suggest that defects in embryogenesis and development of NOS1^{-/-} mice, perhaps in the enteric nervous system, may have a critical role in establishing the thoracic or TBI radiosensitivity. NOS1^{-/-} mice have baseline deficits in neurotransmission that may have been exacerbated by thoracic or total-body irradiation (42).

While NOS1 has clear a function in the neurological system (43–45), supported by the present studies, the mitochondrial localization of NOS1 and its role in non-neuronal cell types are controversial (9,46). The present results establish two additional non-neuronal effects of NOS1 deletion: radioresistance of marrow stromal cells and an increase in longevity of hematopoiesis in long-term marrow cultures. Since NOS1^{-/-} mouse hematopoietic progenitor cells were not radioresistant *in vivo* or *in vitro*, the data indicate that the effects are probably attributable to marrow stromal cells, which have been reported to display some neural cellular characteristics (47,48). Further studies will be required to explain the effects of NOS1 deletion on marrow stromal cells.

The mechanism of the observed sensitivity of NOS1^{-/-} mice to thoracic and total-body irradiation and its correction by intraesophageal MnSOD-PL is unknown, but it may be attributable to their known abnormal enteric neuronal innervation, leading to pyloric stenosis and esophageal dilation (12,19,42,49). Since wild-type mice were protected from higher radiation dose-induced esophageal death by local administration of MnSOD-PL (26), we pursued this target organ as potentially being involved in the mechanism of thoracic radiosensitivity of NOS1^{-/-} mice. We discovered that localized intraesophageal delivery by swallowed plasmid liposomes of the radioprotective MnSOD transgene product prior to thoracic or total-body irradiation of NOS1^{-/-} mice restored their level of radioresponsiveness to that of wild-type mice. More extensive characterization of neuroenteric and esophageal function in irradiated NOS1^{-/-} mice may reveal the control mechanism (50). Further studies will be required to explain the mechanism of the intrinsic thoracic and total-body radiosensitivity of NOS1^{-/-} mice and its reversal by intraesophageal MnSOD-PL radioprotective gene therapy.

Acknowledgments

This project was supported by award number U19AI068021 from the National Institute of Allergy and Infectious Diseases and by T32AG21885, T35HL074708-04 and R01CA11927 from the National Institutes of Health. The authors wish to thank Drs. Timothy Billiar and Bruce Freeman for helpful discussions.

References

1. Daniak NN, Korsmeyer SJ. Cell death: critical control points. Cell 2004;116:205–219. [PubMed: 14744432]

2. Green DR, Reed JC. Mitochondria and apoptosis. *Science* 1998;281:1309–1312. [PubMed: 9721092]
3. Robinson VK, Sato E, Nelson DK, Camhi SL, Robbins RA, Hoyt JC. Peroxynitrite inhibits inducible (type 2) nitric oxide synthase in murine lung epithelial cells *in vitro*. *Free Radic Biol Med* 2001;30:986–991. [PubMed: 11316578]
4. Beckman JS, Chen J, Ischiropoulos H, Crow JP. Oxidative chemistry of peroxynitrite. *Methods Enzymol* 1994;233:229–240. [PubMed: 8015460]
5. Liu X, Kim CN, Yang J, Jemmerson R, Wang X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* 1996;86:147–157. [PubMed: 8689682]
6. Wang ZB, Liu YQ, Cui YF. Pathways to caspase activation. *Cell Biol Int* 2005;29:489–496. [PubMed: 15939633]
7. Carreras MC, Poderoso JJ. Mitochondrial nitric oxide in the signaling of cell integrated responses. *Am J Physiol Cell Physiol* 2007;292:C1569–C1580. [PubMed: 17496232]
8. Ghafourifar P, Cadenas E. Mitochondrial nitric oxide synthase. *Trends Pharmacol Sci* 2005;26:190–195. [PubMed: 15808343]
9. Venkatakrishnan P, Nakayasu ES, Almeida IC, Miller RT. Absence of nitric oxide synthase in sequentially purified rat liver mitochondria. *J Biol Chem* 2009;16:16.
10. Kanai AJ, Pearce LL, Clemens PR, Birder LA, VanBibber MM, Choi SY, de Groat WC, Peterson J. Identification of a neuronal nitric oxide synthase in isolated cardiac mitochondria using electrochemical detection. *Proc Natl Acad Sci USA* 2001;98:14126–14131. [PubMed: 11717466]
11. Elfering SL, Sarkela TM, Giulivi C. Biochemistry of mitochondrial nitric-oxide synthase. *J Biol Chem* 2002;277:38079–38086. [PubMed: 12154090]
12. Huang PL, Dawson TM, Brecht DS, Snyder SH, Fishman MC. Targeted disruption of the neuronal nitric oxide synthase gene. *Cell* 1993;75:1273–1286. [PubMed: 7505721]
13. Laubach VE, Shesely EG, Smithies O, Sherman PA. Mice lacking inducible nitric oxide synthase are not resistant to lipopolysaccharide-induced death. *Proc Natl Acad Sci USA* 1995;92:10688–10692. [PubMed: 7479866]
14. Shesely EG, Maeda N, Kim HS, Desai KM, Krege JH, Laubach VE, Sherman PA, Sessa WC, Smithies O. Elevated blood pressures in mice lacking endothelial nitric oxide synthase. *Proc Natl Acad Sci USA* 1996;93:13176–13181. [PubMed: 8917564]
15. Ghafourifar P, Richter C. Nitric oxide synthase activity in mitochondria. *FEBS Lett* 1997;418:291–296. [PubMed: 9428730]
16. Saur D, Vanderwinden JM, Seidler B, Schmid RM, De Laet MH, Allescher HD. Single-nucleotide promoter polymorphism alters transcription of neuronal nitric oxide synthase exon 1c in infantile hypertrophic pyloric stenosis. *Proc Natl Acad Sci USA* 2004;101:1662–1667. [PubMed: 14757827]
17. Vanderwinden JM, Mailleux P, Schiffmann SN, Vanderhaeghen JJ, De Laet MH. Nitric oxide synthase activity in infantile hypertrophic pyloric stenosis. *N Engl J Med* 1992;327:511–515. [PubMed: 1378938]
18. Mashimo H, Kjellin A, Goyal RK. Gastric stasis in neuronal nitric oxide synthase-deficient knockout mice. *Gastroenterology* 2000;119:766–773. [PubMed: 10982771]
19. Sivarao DV, Mashimo HL, Thatte HS, Goyal RK. Lower esophageal sphincter is achalasic in nNOS (–/–) and hypotensive in W/W(v) mutant mice. *Gastroenterology* 2001;121:34–42. [PubMed: 11438492]
20. Epperly MW, Cao S, Zhang X, Franicola D, Shen H, Greenberger EE, Epperly LD, Greenberger JS. Increased longevity of hematopoiesis in continuous bone marrow cultures derived from NOS1 (nNOS, mtNOS) homozygous recombinant negative mice correlates with radioresistance of hematopoietic and marrow stromal cells. *Exp Hematol* 2007;35:137–145. [PubMed: 17198882]
21. Kanai A, Epperly M, Pearce L, Birder L, Zeidel M, Meyers S, Greenberger J, de Groat W, Apodaca G, Peterson J. Differing roles of mitochondrial nitric oxide synthase in cardiomyocytes and urothelial cells. *Am J Physiol Heart Circ Physiol* 2004;286:H13–H21. [PubMed: 14684357]
22. Sakakeeny MA, Greenberger JS. Granulopoiesis longevity in continuous bone marrow cultures and factor-dependent cell line generation: significant variation among 28 inbred mouse strains and outbred stocks. *J Natl Cancer Inst* 1982;68:305–317. [PubMed: 6950163]

23. Epperly MW, Gretton JE, Sikora CA, Jefferson M, Bernarding M, Nie S, Greenberger JS. Mitochondrial localization of superoxide dismutase is required for decreasing radiation-induced cellular damage. *Radiat Res* 2003;160:568–578. [PubMed: 14565825]
24. Epperly MW, Bray JA, Krager S, Berry LM, Gooding W, Engelhardt JF, Zwacka R, Travis EL, Greenberger JS. Intratracheal injection of adenovirus containing the human MnSOD transgene protects athymic nude mice from irradiation-induced organizing alveolitis. *Int J Radiat Oncol Biol Phys* 1999;43:169–181. [PubMed: 9989524]
25. Epperly MW, Dixon T, Wang H, Schlesselman J, Franicola D, Greenberger JS. Modulation of radiation-induced life shortening by systemic intravenous MnSOD-plasmid liposome gene therapy. *Radiat Res* 2008;170:437–443. [PubMed: 19024650]
26. Zhang X, Epperly MW, Kay MA, Chen ZY, Dixon T, Franicola D, Greenberger BA, Komanduri P, Greenberger JS. Radioprotection *in vitro* and *in vivo* by minicircle plasmid carrying the human manganese superoxide dismutase transgene. *Hum Gene Ther* 2008;19:820–826. [PubMed: 18699723]
27. Jadeski LC, Lala PK. Nitric oxide synthase inhibition by N(G)-nitro-L-arginine methyl ester inhibits tumor-induced angiogenesis in mammary tumors. *Am J Pathol* 1999;155:1381–1390. [PubMed: 10514420]
28. Kalf JC, Buchholz BM, Eskandari MK, Hierholzer C, Schraut WH, Simmons RL, Bauer AJ. Biphasic response to gut manipulation and temporal correlation of cellular infiltrates and muscle dysfunction in rat. *Surgery* 1999;126:498–509. [PubMed: 10486602]
29. Lavin MF, Khanna KK. ATM: the protein encoded by the gene mutated in the radiosensitive syndrome ataxiatelangiectasia. *Int J Radiat Biol* 1999;75:1201–1214. [PubMed: 10549596]
30. Kuhne M, Riballo E, Rief N, Rothkamm K, Jeggo PA, Lobrich M. A double-strand break repair defect in ATM-deficient cells contributes to radiosensitivity. *Cancer Res* 2004;64:500–508. [PubMed: 14744762]
31. Yang Y, Kuang Y, Montes De Oca R, Hays T, Moreau L, Lu N, Seed B, D'Andrea AD. Targeted disruption of the murine Fanconi anemia gene, *Fancg/Xrcc9*. *Blood* 2001;98:3435–3440. [PubMed: 11719385]
32. Andrews AD, Barrett SF, Robbins JH. Xeroderma pigmentosum neurological abnormalities correlate with colony-forming ability after ultraviolet radiation. *Proc Natl Acad Sci USA* 1978;75:1984–1988. [PubMed: 273925]
33. Reif A, Herterich S, Strobel A, Ehlis AC, Saur D, Jacob CP, Wienker T, Topner T, Fritzen S, Lesch KP. A neuronal nitric oxide synthase (NOS-I) haplotype associated with schizophrenia modifies prefrontal cortex function. *Mol Psychiatry* 2006;11:286–300. [PubMed: 16389274]
34. Fallin MD, Lasserter VK, Avramopoulos D, Nicodemus KK, Wolynec PS, McGrath JA, Steel G, Nestadt G, Liang KY, Pulver AE. Bipolar I disorder and schizophrenia: a 440-single-nucleotide polymorphism screen of 64 candidate genes among Ashkenazi Jewish case-parent trios. *Am J Hum Genet* 2005;77:918–936. [PubMed: 16380905]
35. Nelson RJ, Trainor BC, Chiavegatto S, Demas GE. Pleiotropic contributions of nitric oxide to aggressive behavior. *Neurosci Biobehav Rev* 2006;30:346–355. [PubMed: 16483891]
36. Bailer U, Leisch F, Meszaros K, Lenzinger E, Willinger U, Strobl R, Gebhardt C, Gerhard E, Fuchs K, Aschauer HN. Genome scan for susceptibility loci for schizophrenia. *Neuropsychobiology* 2000;42:175–182. [PubMed: 11096332]
37. Bailer U, Leisch F, Meszaros K, Lenzinger E, Willinger U, Strobl R, Heiden A, Gebhardt C, Doge E, Aschauer HN. Genome scan for susceptibility loci for schizophrenia and bipolar disorder. *Biol Psychiatry* 2002;52:40–52. [PubMed: 12079729]
38. DeLisi LE, Shaw SH, Crow TJ, Shields G, Smith AB, Larach VW, Wellman N, Loftus J, Nanthakumar B, Sherrington R. A genome-wide scan for linkage to chromosomal regions in 382 sibling pairs with schizophrenia or schizoaffective disorder. *Am J Psychiatry* 2002;159:803–812. [PubMed: 11986135]
39. Garthwaite J, Garthwaite G, Palmer RM, Moncada S. NMDA receptor activation induces nitric oxide synthesis from arginine in rat brain slices. *Eur J Pharmacol* 1989;172:413–416. [PubMed: 2555211]
40. Dhangadamajhi G, Mohapatra BN, Kar SK, Ranjit M. Genetic variation in neuronal nitric oxide synthase (nNOS) gene and susceptibility to cerebral malaria in Indian adults. *Infect Genet Evol* 2009;9:908–911. [PubMed: 19540363]

41. Niu Y, Epperly MW, Shen H, Smith T, Wang H, Greenberger JS. Intraesophageal MnSOD-plasmid liposome enhances engraftment and self-renewal of bone marrow derived progenitors of esophageal squamous epithelium. *Gene Ther* 2008;15:347–356. [PubMed: 18097469]
42. Takahashi T. Pathophysiological significance of neuronal nitric oxide synthase in the gastrointestinal tract. *J Gastroenterol* 2003;38:421–430. [PubMed: 12768383]
43. Kano T, Shimizu-Sasamata M, Huang PL, Moskowitz MA, Lo EH. Effects of nitric oxide synthase gene knockout on neurotransmitter release in vivo. *Neuroscience* 1998;86:695–699. [PubMed: 9692709]
44. Kovacs R, Rabanus A, Otahal J, Patzak A, Kardos J, Albus K, Heinemann U, Kann O. Endogenous nitric oxide is a key promoting factor for initiation of seizure-like events in hippocampal and entorhinal cortex slices. *J Neurosci* 2009;29:8565–8577. [PubMed: 19571147]
45. Kim MJ, Chung YH, Joo KM, Oh GT, Kim J, Lee B, Cha CI. Immunohistochemical study of the distribution of neuronal voltage-gated calcium channels in the nNOS knock-out mouse cerebellum. *Neurosci Lett* 2004;369:39–43. [PubMed: 15380304]
46. Mungrue IN, Bredt DS, Stewart DJ, Husain M. From molecules to mammals: what's NOS got to do with it? *Acta Physiol Scand* 2003;179:123–135. [PubMed: 14510775]
47. Sauerzweig S, Munsch T, Lessmann V, Reymann KG, Braun H. A population of serum deprivation-induced bone marrow stem cells (SD-BMSC) expresses marker typical for embryonic and neural stem cells. *Exp Cell Res* 2009;315:50–66. [PubMed: 18992240]
48. Sanchez-Ramos J, Song S, Cardozo-Pelaez F, Hazzi C, Stedeford T, Willing A, Freeman TB, Saporta S, Janssen W, Sanberg PR. Adult bone marrow stromal cells differentiate into neural cells *in vitro*. *Exp Neurol* 2000;164:247–256. [PubMed: 10915564]
49. Sivarao DV, Mashimo H, Goyal RK. Pyloric sphincter dysfunction in nNOS^{-/-} and W/W^v mutant mice: animal models of gastroparesis and duodenogastric reflux. *Gastroenterology* 2008;135:1258–1266. [PubMed: 18640116]
50. Daigle JL, Hong JH, Chiang CS, McBride WH. The role of tumor necrosis factor signaling pathways in the response of murine brain to irradiation. *Cancer Res* 2001;61:8859–8865. [PubMed: 11751409]

FIG. 1.

Biology of long-term bone marrow cultures derived from NOS1^{-/-}, NOS2^{-/-}, NOS3^{-/-} or wild-type C57BL/6J mice. The percentage surface area that was confluent was similar for all cell lines (panel A). The number of cobblestone islands (panel B) for each long-term bone marrow culture was counted each week and the cumulative number of cobblestone islands (panel C) was calculated. NOS1^{-/-} and NOS2^{-/-} long-term bone marrow cultures had significantly increased numbers of cobblestone islands ($P < 0.0001$). The number of nonadherent cells produced each week (panel D) and the cumulative number of nonadherent cells (panel E) showed that NOS2^{-/-} long-term bone marrow cultures produced more cumulative nonadherent cells than the other three groups ($P < 0.0001$). Nonadherent cells were plated in semisolid methylcellulose culture and the number of colonies of greater than 50 cells

was determined at 7 (panel F) and 14 days (panel G). NOS1^{-/-} long-term bone marrow cultures had increases in day 7 and 14 colonies compared with the other three groups ($P < 0.0001$).

FIG. 2. Survival curves for marrow stromal cell lines derived from NOS1 $^{-/-}$, NOS2 $^{-/-}$, NOS3 $^{-/-}$ and C57BL/6NHsd (wild-type) long-term bone marrow cultures fitted using the linear-quadratic model.

FIG. 3.

NOS1^{-/-} mice are sensitive to thoracic and total-body irradiation but not to brain-only irradiation. Panel A: Survival time after thoracic irradiation with 20 Gy. Panel B: Survival time after TBI with 9.5 Gy. Panel C: Survival times of C57BL/6NHsd mice after doses of 9.5 Gy–18 Gy and of C57BL/6NHsd mice NOS1^{-/-} mice irradiated with 9.5 Gy. Panel D: Head and neck irradiation with 9.5 Gy with and without administration of MnSOD-PL intraesophageally did not result in early deaths of the mice.

FIG. 4. Unchanged dilated esophagus and stomach in TBI compared to nonirradiated NOS1^{-/-} mice. C57BL/6NHsd and NOS1^{-/-} mice were autopsied without any treatment (panels A, C, respectively) and 6 days after 9.5 Gy TBI (panels B, D, respectively). Autopsy revealed an enlarged stomach and dilated esophagus in nonirradiated control NOS1^{-/-} mice (panel C) that was unchanged by irradiation (panel D).

FIG. 5. H&E-stained duodenum of C57BL/6NHsd and NOS1^{-/-} mice. Duodenum from control C57BL/6NHsd and NOS1^{-/-} mice (panels A, C, respectively) and mice 6 days after 9.5 Gy TBI (panels B, D, respectively) were sectioned and stained with H&E.

FIG. 6. NOS1^{-/-} mouse marrow CFU-GM, BFU-E and CFU-GEMM show radiosensitivities similar to those of cells from wild-type C57BL/6NHsd mouse marrow. C57BL/6NHsd mice (panel A) and NOS1^{-/-} mice (panel B).

FIG. 7.

ATM kinase activation is unchanged in irradiated NOS1^{-/-} mouse bone marrow stromal cells compared to wild-type mouse bone marrow stromal cells. Bone marrow stromal cells were irradiated with 2 Gy. Whole cell extracts were prepared after 1 h. ATM phosphorylated on serine 1987 (1981 in humans) was immunoprecipitated using a rabbit phosphospecific monoclonal antibody, resolved by SDS-PAGE, and immunoblotted using a generic ATM mouse monoclonal antibody.

FIG. 8.

Electric-field stimulation (EFS) studies of the lower esophageal sphincter and gastrointestinal transit after 20 Gy thoracic irradiation of NOS1^{-/-} mice compared to wild-type mice. In wild-type mice, there was increased contractility with increasing EFS frequency in muscle strips in basal substrate (L-arginine, circles). Inhibition of NOS (with addition of L-nitro arginine, +) or muscarinic receptors (with atropine, Δ) did not produce significant differences (panel A). There were no significant differences after irradiation (panel B). NOS1^{-/-} mice demonstrated contractility after antagonization of both muscarinic and NOS receptors despite high-frequency EFS stimulation (panel C) ($P < 0.05$). This persisted after thoracic irradiation with no other significant differences (panel D). Panel E: NOS1^{-/-} and C57BL/6NHsd mice (four per group) that were either nonirradiated or received 9.5 Gy TBI were given oral FITC-labeled dextran and the geometric center of the fluorescence signal through the gastrointestinal system was calculated. Mice receiving 9.5 Gy TBI showed no significant differences in GI transit ($P > 0.05$).

FIG. 9.

Continuous NOS inhibition (pre- and postirradiation) in C57BL/6NHsd mice confers protection from TBI. Panel A: C57BL/6NHsd mice were implanted with osmotic mini-pumps containing either L-NAME or PBS and irradiated with 9.5 Gy TBI 3 days later. Pumps were removed on day 11 after irradiation. Mice treated with L-NAME had a significantly increased survival compared to mice treated with PBS ($P = 0.0012$) that was reversed when pumps were removed. Panel B: C57BL/6NHsd mice were implanted with mini pumps containing either L-NAME or PBS on day 9. On day 0, the pumps were removed and the mice were irradiated with 9.5 Gy TBI. There was no significant difference in survival ($P = 0.15$).

FIG. 10.

Intraesophageal MnSOD-PL delivered 24 h prior to thoracic or total-body irradiation ameliorates the radiosensitivity of NOS1^{-/-} mice. NOS1^{-/-} mice received intraoral MnSOD-PL (100 μg plasmid DNA) at the top of the esophagus and the mice were allowed to swallow the solution. Twenty-four hours later, mice were irradiated. Panel A: With 20 Gy thoracic irradiation, NOS1^{-/-} mice treated with MnSOD-PL demonstrated increased survival times compared to untreated NOS1^{-/-} mice ($P = 0.031$). Panel B: With 9.5 Gy total-body irradiation, increased survival times in NOS1^{-/-} mice with MnSOD-PL administration were also seen compared to untreated NOS1^{-/-} mice ($P = 0.043$). Panel C: To demonstrate that the MnSOD-PL administration resulted in increased MnSOD biochemical activity, NOS1^{-/-} and C57BL/6NHsd mice were administered MnSOD-PL and killed 24 h later, each esophagus was removed and immediately frozen, and MnSOD biochemical activity was assayed. There was no significant difference in NOS1^{-/-} and C57BL/6NHsd mice in either baseline biochemical MnSOD activity or the elevation above baseline after administration of MnSOD-PL.

TABLE 1

NOS1^{-/-} Mice do not Display Evidence of a Rapid Hematopoietic Syndrome after 9.5 Gy TBI

	White blood cells		Red blood cells		Hemoglobin		Hematocrit		Platelets	
	0 Gy	9.5 Gy	0 Gy	9.5 Gy	0 Gy	9.5 Gy	0 Gy	9.5 Gy	0 Gy	9.5 Gy
NOS1 ^{-/-}	7.4 ± 0.6	0.4 ± 0.1*	11.0 ± 0.4	9.4 ± 1.1	17.1 ± 0.7	15.1 ± 1.7	59.8 ± 1.9	50.9 ± 1.7	929 ± 155	98 ± 6.1*
NOS2 ^{-/-}	10.4 ± 0.7	0.2 ± 0.0*	12.1 ± 0.2	11.8 ± 0.1	17.6 ± 0.2	17.4 ± 0.1	60.8 ± 1.1	60.2 ± 0.5	1283 ± 117	296 ± 67*
NOS3 ^{-/-}	10.5 ± 0.7	0.5 ± 0.2*	12.6 ± 0.2	10.6 ± 0.4	18.0 ± 0.1	15.2 ± 0.6	62.4 ± 1.1	52.1 ± 2.2	1352 ± 439	507 ± 52
C57BL/6NHsd	7.3 ± 0.2	0.6 ± 0.1*	11.2 ± 0.4	10.2 ± 0.1*	16.1 ± 0.5	15.3 ± 0.2*	57.3 ± 2.1	49.6 ± 0.5*	1199 ± 63	519 ± 24*

Notes. Whole blood was collected for analysis 6 days after irradiation.

* $P < 0.05$.

TABLE 2

Changes in Glucose, Serum Electrolytes and Liver, Pancreatic and Renal Function Tests in Nonirradiated Compared to 9.5 Gy Total-Body Irradiated NOS1^{-/-} Mice do not Explain Rapid Death

	Na (Sodium)		K (Potassium)		Cl (Chloride)		Ca (Calcium)		Glucose	
	0 Gy	9.5 Gy	0 Gy	9.5 Gy	0 Gy	9.5 Gy	0 Gy	9.5 Gy	0 Gy	9.5 Gy
NOS1 ^{-/-}	148.3 ± 2.3	154.3 ± 1.7	10.6 ± 0.9	6.6 ± 0.2 *	112.5 ± 0.4	111.7 ± 0.9	9.8 ± 0.2	9.7 ± 0.2	241.7 ± 31.0	181.3 ± 14.5
C57BL/6NHsd	154.3 ± 1.2	152.0 ± 0.6	7.6 ± 0.6	7.6 ± 0.3	109.0 ± 0.6	111.0 ± 1.0	10.0 ± 0.1	9.7 ± 0.1	217.0 ± 17.2	203.0 ± 63.5
	Total bilirubin		ALT (alanine aminotransferase)		Albumin		Amylase		Lipase	
	0 Gy	9.5 Gy	0 Gy	9.5 Gy	0 Gy	9.5 Gy	0 Gy	9.5 Gy	0 Gy	9.5 Gy
NOS1 ^{-/-}	0.3 ± 0.1	0.3 ± 0.1	42.5 ± 4.5	112.3 ± 63.4	2.9 ± 0.0	2.7 ± 0.2	721.5 ± 13.5	830.3 ± 182.0	76.5 ± 3.7	127.3 ± 68.2
C57BL/6NHsd	0.2 ± 0.0	0.2 ± 0.0	85.0 ± 12.4	289.0 ± 134.8	3.3 ± 0.1	3.0 ± 0.1	763.3 ± 54.4	1134.7 ± 433.8	68.3 ± 6.2	225.0 ± 187.7
	BUN (Blood urea nitrogen)		Creatinine							
	0 Gy	9.5 Gy	0 Gy	9.5 Gy						
NOS1 ^{-/-}	31.0 ± 0.8	26.0 ± 1.2 *	0.2 ± 0.0	0.2 ± 0.0						
C57BL/6NHsd	27.7 ± 2.6	21.3 ± 3.3	0.2 ± 0.0	0.2 ± 0.0						

Notes. Whole blood was collected 6 days after irradiation and serum was isolated for analysis.

* $P < 0.05$.

TABLE 3
RT-PCR Analysis of Markers of Inflammatory Response in Irradiated Tissues of NOS1^{-/-} Mice

	TNF α	NF- κ B	IFN- γ	NOS2	NOS3
Esophagus					
C57BL/6	1.00	1.00	1.00	1.00	1.00
NOS1 ^{-/-}	0.63	0.69	0.31	0.46	0.75
C57BL/6 + TBI (Day 1)	0.56	1.35*	0.28	0.13	0.46
NOS1 ^{-/-} + TBI (Day 1)	0.67	0.62	0.17	0.40	1.48*
C57BL/6 + TBI (Day 6)	0.24	1.43*	0.15	0.57	0.75
NOS1 ^{-/-} + TBI (Day 6)	0.60	0.59	0.05	0.47	1.27*
Stomach					
C57BL/6	1.00	1.00	N.T.	N.T.	1.00
NOS1 ^{-/-}	0.47	0.57	N.T.	N.T.	1.18
C57BL/6 + TBI (Day 1)	0.92	0.61	N.T.	N.T.	0.95
NOS1 ^{-/-} + TBI (Day 1)	1.31	0.65	N.T.	N.T.	1.22
C57BL/6 + TBI (Day 6)	0.69	0.88	N.T.	N.T.	1.57
NOS1 ^{-/-} + TBI (Day 6)	1.46	0.54	N.T.	N.T.	0.56
Intestine					
C57BL/6	1.00*	1.00	1.00	1.00*	1.00
NOS1 ^{-/-}	0.25	0.68	N.T.	0.04	0.28
C57BL/6 + TBI (Day 1)	0.62*	0.39	0.49*	0.24	2.48
NOS1 ^{-/-} + TBI (Day 1)	0.15	2.06	0.06	0.02	0.33
C57BL/6 + TBI (Day 6)	0.21	1.28*	0.10	1.15	0.23
NOS1 ^{-/-} + TBI (Day 6)	0.35	0.34	0.08	2.69	0.14

Notes. NOS1^{-/-} and C57BL/6NHsd mice were either nonirradiated or received 9.5 Gy TBI and were killed after 1 or 6 days. Tissue from the esophagus, stomach and intestine was isolated and RT-PCR performed. All numbers are expressed relative to the established standard calibrated sample of each organ from control or irradiated C57BL/6NHsd mice. N.T. = not tested.

* $P < 0.05$.