eNOS Gene Therapy Exacerbates Hepatic Ischemia-Reperfusion Injury in Diabetes: A Role for eNOS Uncoupling

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Abstract—Previous studies indicate that endothelial nitric oxide synthase (eNOS) function is impaired in diabetes as a result of increased vascular generation of reactive oxygen species. We hypothesized that eNOS gene therapy would augment NO bioavailability and protect against hepatic ischemia–reperfusion (I-R) injury in type 2 diabetes mellitus. We developed a transgenic (Tg) diabetic mouse in which eNOS is systemically overexpressed. We also examined the effects of hepatic eNOS adenovirus therapy in diabetic mice. Diabetic (db/db) and nondiabetic mice were subjected to hepatic I-R injury. In nondiabetic mice, genetic overexpression of eNOS (both eNOS-Tg and eNOS adenovirus) resulted in hepatoprotection. In contrast, hepatic I-R injury was significantly increased in the db/db eNOS-Tg mouse, as serum alanine aminotransaminase (ALT) levels were increased by 3.3-fold compared with diabetic controls. Similarly, eNOS adenovirus treatment resulted in a 3.2-fold increase in serum ALT levels as compared with diabetic controls. We determined that hepatic eNOS was dysfunctional in the db/db mouse and increased genetic expression of eNOS resulted in greater production of peroxynitrite. Treatment with the eNOS cofactor tetrahydrobiopterin (BH₄) or the BH₄ precursor sepiapterin resulted in a significant decrease in serum ALT levels following I-R injury. We present clear examples of the protective and injurious nature of NO therapy in I-R. Our data indicate that eNOS exists in an “uncoupled” state in the setting of diabetes and that “recoupling” of the eNOS enzyme with cofactor therapy is beneficial. (Circ Res. 2006;99:78-85.)

Key Words: diabetes mellitus ■ tetrahydrobiopterin ■ eNOS phosphorylation ■ sepiapterin ■ peroxynitrite

Nitric oxide (NO) generated by endothelial nitric oxide synthase (eNOS) is critical for the maintenance of cardiovascular homeostasis. NO exerts potent vasodilator, antiplatelet, antiinflammatory, and cytoprotective effects in healthy animals. However, the role of NO in ischemic disease remains controversial, with a number of studies citing protective and deleterious effects of NO therapy. In the liver, eNOS appears to play a central role in protecting against ischemia–reperfusion (I-R) injury.

Experimental studies have revealed that eNOS-derived NO bioavailability is diminished in diabetic animals. The quenching of NO by advanced glycosylation products in diabetic patients is also thought to play a significant role in the pathogenesis of diabetes mellitus. Reports have implicated glycosylated and oxidized low-density lipoprotein as potent mediators in the downregulation of eNOS. Additionally, the generation of reactive oxygen species (ROS) has been shown to significantly inhibit eNOS production of NO and its downstream physiological actions. These pathogenic factors may impair eNOS function via a number of mechanisms including: decreased eNOS production, altered phosphorylation events at various residues, decreased substrate and/or cofactor availability, increased association with inhibitory proteins, and inhibition of dimerization allowing production of superoxide (uncoupling). Given that eNOS dysfunction and reduced NO bioavailability contribute to the pathogenesis of diabetes mellitus, we hypothesized that eNOS gene therapy, in the setting of diabetes, would increase nitric oxide bioavailability and thus ameliorate the severity of hepatic I-R injury.

Materials and Methods

Chemicals and Reagents

DETA NONOate (DETA/NO) was purchased from Alexis Biochemicals (San Diego, Calif) and administered (100 μg/kg) IP 15 minutes before reperfusion dissolved in normal saline. Sodium nitrite was obtained from Sigma Chemical Co (St Louis, Mo). Sodium nitrite was dissolved in phosphate buffered saline and the pH was adjusted to 7.4. In all experiments, 165 μg/kg sodium nitrite or vehicle was administered IP 15 minutes before reperfusion.
l-Sepiapterin was purchased from Alexis Biochemicals. Sepiapterin (5 mg/kg) was administered IP 30 minutes before ischemia and dissolved in normal saline. Tetrahydrobipterin (BH$_4$) was purchased from Alexis Biochemicals and administered at (5 mg/kg) IP 30 minutes before ischemia, dissolved in normal saline. N$_2$-nitro-L-arginine methyl ester (l-NAME) was purchased from Sigma-Aldrich (St. Louis, Mo) and administered at (50 mg/kg) 30 minutes before ischemia.

MnTMPyP a cell-permeable superoxide dismutase (SOD) mimic was purchased from Calbiochem (San Diego, Calif). MnTMPyP was administered at (1 mg/kg) 30 minutes before ischemia, dissolved in normal saline.

Adenovirus Vectors

Replication-deficient recombinant adenoviruses serotype 5—encoding nuclear-targeted β-galactosidase (AdCMVβgal) and eNOS (AdCMVeNOS), driven by a cytomegalovirus (CMV) promoter, were prepared as previously described.21 Adenovirus was delivered 48 hours before liver I-R experiments via IP injection of AdCMVβgal (1×10$^{10}$ parts), AdCMVeNOS (1×10$^{10}$ parts/mL), or AdCMV-Null (1×10$^{10}$ parts) diluted in normal saline.

Diabetic Mice

Cg-m$^{-/-}$Lepr$^{+/-}$/C57BL6/J (background db/db diabetic) mice were purchased from The Jackson Laboratory and maintained on a normal rodent chow diet. Age-matched nondiabetic littermate animals were used as controls.

Diabetic eNOS Transgenic Mice

We developed a diabetic eNOS transgenic mouse (db/db eNOS-Tg) exhibiting systemic overexpression of the bovine eNOS gene driven by the native eNOS promoter. This was accomplished by breeding the eNOS transgenic mouse (eNOS-Tg)$^{19}$ onto the db/db diabetic mouse (Jackson Laboratory). Genotype was confirmed by PCR analysis of the leptin receptor mutation and bovine eNOS transgene in all mice.

eNOS Knockout Mice were generated in our breeding colony as described previously.$^{16}$

eNOS Transgenic Mice

The eNOS transgenic mouse (eNOS-Tg) was developed on a C57BL6 background and has been characterized previously.$^{17}$ In all studies, male mice were used at 8 to 10 weeks of age. All experimental procedures complied with the NIH Guide for the Care and Use of Laboratory Animals (Publication No. 86-23, revised 1985).

Blood Glucose Determination

Blood obtained via tail snip was screened using a Sure Step glucose-monitoring system (Lifescan).

Hepatic I-R Protocol

The hepatic I-R protocol has been previously described.$^{18}$ Mice were anesthetized with the combination of ketamine (100 mg/kg) and xylazine (8 mg/kg). The left lateral and median lobes of the liver were rendered ischemic by completely clamping the hepatic artery and the portal vein. The duration of hepatic ischemia was 45 minutes, and reperfusion time was 5 hours in the studies of serum alanine aminotransaminase (ALT) levels.

Hepatic Enzyme Levels

Serum samples were analyzed for ALT using a spectrophotometric method (Thermo Electron Corp, Melbourne, Australia).

Measurement of Nitrotyrosine

Nitrotyrosine was determined by Western blotting and ELISA methods. Western blot was performed as described by Gao et al.$^{19}$ To determine protein nitration in a fully quantitative manner, liver tissue nitrotyrosine levels were determined by using an ELISA procedure reported by Tanaka et al.$^{20}$ with modification as previously described.$^{21}$ The amount of nitrotyrosine content in tissue samples was calculated using standard curves generated from nitrated BSA containing known amounts of nitrotyrosine and expressed as picomoles per milligram protein.

Western Blot Analysis of eNOS

Standard Western blotting procedure was used to analyze total eNOS and eNOS phospho residues as previously reported.$^{16}$ Low-temperature SDS-PAGE blots were performed as previously described$^{23}$ to assess eNOS monomer/dimer ratio.

High-Performance Liquid Chromatography

Analysis of BH$_4$

Hepatic BH$_4$ content was determined by modification of the method described by Howells et al.$^{23}$ At the time of analysis, the samples were diluted 1:10 in 0.1 mol/L perchloric acid containing 1 mg/mL dithioerythritol and 1 mg/mL diethylenetriaminepentaacetic acid. BH$_4$ levels were determined using a Waters 2695 (Milford, Mass), interfaced to a Coularray (ESA Inc, Chelmsford, Mass) electrochemical detector.

Statistical Analysis

Data were analyzed where appropriate by Student’s t test or 1-way ANOVA, with post hoc Tukey analysis using JMP IN statistical software (SAS Institute). Data are reported as mean±SEM. Probability values of <0.05 were considered significant.

Results

Phenotypic Profile of Diabetic and Transgenic Mice

Analysis of body weight, blood glucose, and glycosylated hemoglobin (HbA1C) are presented in Table I in the online data supplement, available at http://circres.ahajournals.org. The nondiabetic mice (wild type, eNOS$^{-/-}$, eNOS-Tg) all displayed similar weights, blood glucose, and HbA1C. In contrast, the db/db diabetic mouse is obese with an average weight ≈90% greater than the wild-type control. Both diabetic mice strains showed significantly elevated body weights, blood glucose, and HbA1C.

The Diabetic Role of Genetic eNOS Overexpression in Hepatic I-R Injury

The genetic overexpression of bovine eNOS in nondiabetic mice dramatically attenuated hepatic tissue injury, as assessed by serum ALT levels, following hepatic I-R injury (Figure 1A). This overexpression of eNOS in the nondiabetic mouse resulted in a 3.8-fold decrease in mean serum ALT level. To the contrary, overexpression of the same bovine eNOS transgene in the db/db diabetic mouse profoundly increased hepatic injury (Figure 1B). The db/db eNOS-Tg mouse displayed a 3.25-fold increase in serum ALT following hepatic I-R, suggesting that overexpression of eNOS in the setting of diabetes exacerbates hepatic I-R injury.

To further examine the role of eNOS gene therapy in I-R and rule out the possibility that some compensatory modification resulting from chronic overexpression was responsible for the increase in injury seen in the db/db eNOS-Tg mouse, we next used an adenoviral gene therapy approach to increase eNOS expression. Intraperitoneal delivery of AdCMVeNOS or AdCMV-Null was performed 48 hours before hepatic I-R. At 48 hours following eNOS adenovirus delivery eNOS expression increased ≈7-fold as compared with mice receiving the null adenovirus (supplemental Figure I). eNOS
adenoviral therapy in the nondiabetic (Figure 1C) significantly attenuated serum ALT levels by a 3.9-fold decrease similar to that observed in the nondiabetic eNOS-Tg animals. The protective action of the eNOS adenovirus was abrogated in the diabetic db/db mouse (Figure 1D), as assessed by serum ALT, increasing from 2312±260 in the adeno-null–treated animals to 9519±3428 U/L in adeno-eNOS mice. This represented a 4.1-fold increase in serum ALT following eNOS gene therapy mimicking the results found in the db/db eNOS-transgenic animal. These results clearly suggest that although genetically increasing eNOS in nondiabetic mice during I-R injury is advantageous, in the setting of diabetes mellitus eNOS gene therapy exacerbates hepatic injury.

Nitric Oxide Donors Are Protective in the Nondiabetic and Injurious in the Diabetic

DETA NONOate (100 μg/kg) reduced serum ALT from 482±65 U/L in nondiabetic mice subjected to hepatic I-R to 61±10 U/L (Figure 2A), however diabetic mice demonstrated a 1.7-fold increase in serum ALT post I-R injury (Figure 2B).

To validate these results a second NO’ source, sodium nitrite (165 μg/kg), was used. Figure 2C shows the protective nature of nitrite in the nondiabetic mouse subjected to I-R injury (2.3-fold decrease in serum ALT). Yet in the db/db diabetic mouse, sodium nitrite increased the severity of hepatic I-R injury (Figure 2D).

Figure 1. eNOS gene therapy in hepatic I-R. Non-diabetic eNOS transgenic mice (A), diabetic eNOS transgenic mice (B), eNOS adenoviral therapy in nondiabetic mice (C), and eNOS adenoviral therapy in diabetic mice (D). *P<0.05, **P<0.01.

Figure 2. Nitric oxide donor therapy in hepatic I-R. A and B, DETA/NONOate administered IV 15 minutes before reperfusion in nondiabetic mice (A) or diabetic mice (B). C and D, Sodium nitrite administered IV 15 minutes before reperfusion in nondiabetic mice (C) or in diabetic mice (D). **P<0.01 vs vehicle.
The Diabetic Liver Displays Altered Phosphorylation of Key eNOS Regulatory Residues

To more clearly understand the state of eNOS in the diabetic liver Western blots were performed. Figure 3A shows representative blots using antibodies against total eNOS, threonine 495 (Thr495) phospho residue, and the serine 1177 (Ser1177) phospho residue. Total eNOS expression was semiquantitatively assessed by densitometric analysis. Figure 3B demonstrates no significant difference in total eNOS protein expression in the diabetic liver as compared with nondiabetic liver. Densitometric analysis of the ratio of phospho:total eNOS revealed a 2-fold decrease in liver eNOS Thr495 phosphorylation in the diabetic versus the nondiabetic (Figure 3C). We also found that diabetic mice had a 2.9-fold reduction in liver eNOS Ser1177 phosphorylation versus nondiabetic mice (Figure 3D). Low-temperature SDS-PAGE Western blots were also performed to assess the monomer/dimer ratio of eNOS in diabetic mice (supplemental Figure II). Nondiabetic mice were found to have a 3.9-fold increase in the ratio of eNOS monomer/dimer ratio versus diabetic mice, suggesting eNOS may exist in an altered state in diabetic mice.

Nitrated Tyrosine Is Increased in the Diabetic and eNOS Overexpression Augments Nitrotyrosine Formation in the Liver

We first performed Western blots to qualitatively assess the amount of nitrotyrosine formation (Figure 4A). To quantitatively assess nitrated tyrosine residues, we performed nitrotyrosine ELISAs. Figure 4B shows low nitrotyrosine levels in wild-type and eNOS transgenic mice liver samples. However, the livers of diabetic (db/db) eNOS-Tg mice demonstrated a significant increase in nitrotyrosine formation over those of nondiabetic control and eNOS-Tg mice.

Inhibition of NOS Further Exacerbates Liver I-R Injury

First, we subjected eNOS−/− animals to the hepatic I-R protocol and found that deletion of the eNOS gene in nondiabetic animals resulted in a 3.1-fold increase in serum ALT levels (Figure 5A). Next, we pharmacologically inhibited eNOS in the db/db diabetic and nondiabetic control mice with the NOS inhibitor 1-NAME. Nondiabetic mice receiving 1-NAME exhibited increased serum ALT levels 7.6-fold higher than mice receiving the vehicle normal saline (Figure 5A). Following hepatic I-R, serum ALT levels increased more than 5-fold higher in diabetic mice receiving 1-NAME, rather than vehicle, before hepatic I-R (Figure 5B).

The eNOS Cofactor BH₄ Is Reduced in the Diabetic Liver

We examined the eNOS cofactor BH₄. We found that BH₄ levels in the nondiabetic liver averaged 332.91 ± 40.29 pmol/mg protein (Figure 6). Likewise, BH₄ levels in the eNOS-Tg mice averaged 362.41 ± 86.05 pmol/mg. However, BH₄ levels in db/db mouse liver samples were significantly reduced by 2.2-fold to 149.61 ± 17.19 pmol/mg protein. These results suggest that decreased levels of BH4 may be responsible for eNOS dysfunction and help to explain the deleterious effects of eNOS gene therapy in the diabetic liver.
Increasing Bioavailability of BH₄ or Sepiapterin Protects Against Hepatic I-R Injury in the Diabetic

In nondiabetic mice, acute administration of sepiapterin, an intermediate in the pterin salvage pathway, was used to increase BH₄ biosynthesis. Sepiapterin administered before ischemia reduced serum ALT levels by 4.5-fold compared with mice receiving vehicle (Figure 7A). Injection of BH₄ also very significantly reduced serum ALT levels in nondiabetic mice from 678±61 U/L to 225±45 U/L. Figure 7B displays the 3.7-fold reduction in serum ALT in the diabetic receiving sepiapterin versus vehicle. BH₄ delivery mimicked these results decreasing serum ALT levels post I-R injury from 2986±274 U/L in the vehicle to 1419±275 U/L in mice given BH₄.

BH₄ Supplementation Plus the Addition of Nitrite Further Protects Against Hepatic I-R Injury

We next treated both nondiabetic and diabetic mice with BH₄ before ischemia, followed by nitrite administration 15 minutes before reperfusion. In the nondiabetic mouse administration of BH₄ before the delivery of nitrite lowered serum ALT by 3.2-fold following hepatic I-R (Figure 7C). In the db/db diabetic mouse, we observed the greatest protection of all therapeutic interventions with a reduction of serum ALT from 2933±490 U/L in the vehicle to 768±121 U/L in the BH₄+nitrite group. This represents a 3.8-fold decrease in ALT injury (Figure 7D).

A Cell-Permeable SOD Mimetic With the Addition of a Nitric Oxide Donor Protects Against Hepatic I-R Injury in the Diabetic

Experiments in the nondiabetic revealed a significant reduction in serum ALT with the administration of MnTMPyP (Figure 8A). In diabetic mice, SOD therapy lowered serum ALT levels, although not significantly, from 3173±694.3 U/L in vehicle-treated mice to 2088±490.6 U/L following hepatic I-R (Figure 8B). Next, db/db diabetic mice were given MnTMPyP before ischemia followed by the administration of the NO donor DETA NONOate. This combination of therapy significantly lowered serum ALT following hepatic I-R by 2.4-fold.

Discussion

There have been numerous reports citing protective effects of eNOS gene therapy. In the present study, we found that eNOS gene therapy protects the nondiabetic liver against I-R injury. In stark contrast, and disproving our initial hypothesis, we established that genetic overexpression of eNOS in diabetic (db/db) mice significantly exacerbates hepatic I-R injury. These results were validated by 2 different approaches. We first developed a diabetic (db/db) mouse with systemic overexpression of the bovine eNOS transgene. Secondly, to account for possible compensatory mechanisms in the transgenic mouse, we used an adenoviral approach.

To further investigate the deleterious nature of eNOS gene therapy in the diabetic mouse, we examined direct NO therapy using various nitric oxide donor compounds. In the nondiabetic animals DETA/NO significantly reduced I-R induced hepatic injury. This result is predictable given the numerous experimental studies citing the protective effects of NO donor therapy in hepatic I-R. In a second series of experiments, db/db diabetic mice received DETA/NO before reperfusion. The NO donor significantly increased serum ALT levels in diabetic mice. A second agent that has been shown to increase NO following ischemic insult, sodium nitrite, was examined to further evaluate the role of NO therapy on hepatic I-R injury in diabetes. Nitrite is a biological storage form of NO that releases NO following hypoxia or ischemic insult. Nitrite significantly increased serum ALT levels following I-R in the db/db diabetic mouse. This further corroborated the injurious nature of increasing NO levels in the diabetic mouse experiencing liver I-R injury. Although these results were quite surprising, we can only speculate that a direct bolus of NO just before reperfusion interacted with superoxide anion and formed the potent free radical peroxynitrite, thereby resulting in increased hepatic injury. This seems a plausible explanation given the

Figure 5. eNOS Inhibition in hepatic I-R. A, Nondiabetic, eNOS⁺/⁻, and nondiabetic mice receiving 50 mg/kg L-NAME were subjected to hepatic I-R injury. B, Diabetic mice received either vehicle or L-NAME 50 mg/kg and were subjected to hepatic I-R injury. ∗P<0.05 vs vehicle, **P<0.01 vs vehicle.

Figure 6. Hepatic BH₄ levels. BH₄ levels (pmol/mg protein) in nondiabetic, diabetic, and eNOS-Tg livers. **P<0.01 vs all other groups.

Figure 8A. In diabetic mice, SOD therapy lowered serum ALT levels, although not significantly, from 3173±694.3 U/L in vehicle-treated mice to 2088±490.6 U/L following hepatic I-R (Figure 8B). Next, db/db diabetic mice were given MnTMPyP before ischemia followed by the administration of the NO donor DETA NONOate. This combination of therapy significantly lowered serum ALT following hepatic I-R by 2.4-fold.
high levels of reactive oxygen species, such as superoxide, found in the diabetic, especially during I-R injury. These data are supported by our findings that peroxynitrite (ONOO\(^-\)) levels are significantly elevated in the db/db mouse at baseline and that a SOD mimetic decreased hepatic I-R injury.

To evaluate the molecular status of eNOS in the diabetic liver, we examined various phospho residues. We found that although there was no difference in total hepatic eNOS protein between nondiabetic and diabetic animals, there was a significant alteration in the phosphorylation of key regulatory residues. We discovered decreased phosphorylation of both threonine 495 and serine 1177 residues in diabetic mice as compared with nondiabetic control animals. It has been suggested that phosphorylation status of Thr495 is critical to the coupling of the eNOS enzyme for efficient production of nitric oxide. Lin et al.\(^{29}\) have shown that Thr495 may be the “intrinsic switch mechanism” that determines whether eNOS produces either NO\(^-\) (phospho 495) or superoxide (dephospho 495). The phosphorylation state of Ser1177 is indicative of eNOS enzyme activity. Decreased phosphorylation of Ser1177 is associated with a decrease in enzyme activity, whereas increased phosphorylation of Ser1177 has been shown by many to increase catalytic activity.\(^{30,31}\) In a study of diabetic animals,\(^{32}\) it has also been shown that this regulatory site may be subject to posttranslational modification, thereby decreasing eNOS activity. We also performed low-temperature SDS-PAGE Western blots to assess the monomer/dimer ratio of the eNOS enzyme in diabetic mice. We found a striking difference in the monomer/dimer ratio in the diabetic mice as compared with nondiabetic controls, further suggesting that eNOS exists in an altered state in the diabetic mice.

Our present findings that eNOS is “uncoupled” in the diabetic mouse support our data demonstrating that eNOS gene therapy enhanced hepatic I-R injury in db/db diabetic mice. We therefore sought to further this notion by examining the possibility that increases in eNOS in the diabetic mouse resulted in increased ONOO\(^-\) via interactions between superoxide and NO\(^-\). To indirectly identify peroxynitrite formation, we examined hepatic nitrotyrosine formation using 2 distinct methods. Qualitative assessment of nitrotyrosine via Western blot analysis and quantitative assessment with an ELISA revealed an increase in nitrotyrosine in the diabetic as compared with nondiabetic animals. Remarkably, these levels were increased to an even greater extent in the db/db eNOS-Tg animals under baseline conditions. These results are very suggestive that eNOS therapy in the diabetic further enhances the pathologic consequences of increased nitrotyrosine formation.

**Figure 7.** eNOS cofactor therapy in hepatic I-R. A and B, Nondiabetic (A) or diabetic (B) mice received either sepiapterin or BH\(_4\) (5 mg/kg) 30 minutes before ischemia. C and D, nondiabetic (C) or diabetic (D) mice received either nitrite (165 \(\mu\)g/kg) 15 minutes before reperfusion or BH\(_4\) (5 mg/kg) 30 minutes before ischemia plus nitrite (165 \(\mu\)g/kg) 15 minutes before reperfusion. **\(P<0.01\) vs vehicle.

**Figure 8.** SOD mimetic in hepatic I-R. Nondiabetic mice received MnTMPyP (A) and diabetic mice received MnTMPyP 30 minutes before ischemia or MnTMPyP 30 minutes before ischemia plus DETA/NO 15 minutes before reperfusion (B). *\(P<0.05\) vs vehicle, **\(P<0.01\) vs vehicle.
increased oxidative stress in these animals via the formation of ONOO\(^-\). Current studies\(^{22,33}\) have cited ONOO\(^-\) as a mediator of eNOS uncoupling. This presents a possible positive feedback in the db/db diabetic mouse whereby increased superoxide production further increases ONOO\(^-\) formation, which in turn acts to further uncouple eNOS perpetuating superoxide production. Similarly, the addition of exogenous NO\(^-\) (DETA/NO or nitrite), which we found to increase hepatic injury, could result in increased ONOO\(^-\) formation by interaction with high levels of superoxide anion following I-R.

To further implicate eNOS as a pathogenic mediator of I-R injury in the diabetic, we next pharmacologically inhibited eNOS with \(\text{l-NAME}\). First, nondiabetic controls and eNOS \(^{-/-}\) animals were subjected to hepatic I-R. We found that genetic deletion of the eNOS enzyme in the nondiabetic increased serum ALT by 3.1-fold following I-R. This result was anticipated given previous studies\(^{16,34}\) demonstrating increased I-R injury in eNOS \(^{-/-}\) animals. Pharmacological inhibition with \(\text{l-NAME}\) was also found to increase serum ALT levels following I-R in nondiabetic mice. However, the administration of \(\text{l-NAME}\) to the nondiabetic caused ALT levels to rise more than 2-fold higher than those seen in the eNOS \(^{-/-}\), suggesting \(\text{l-NAME}\) was not simply inhibiting eNOS but was also exerting additional effects that resulted in enhanced hepatic I-R injury. The administration of \(\text{l-NAME}\) in the diabetic was the most injurious intervention in the present study and given the results noted in the nondiabetic, clearly \(\text{l-NAME}\) is not solely specific for the inhibition of eNOS.

In an effort to further our understanding of eNOS function in the db/db diabetic mouse, we next measured the eNOS cofactor BH\(_4\) levels in these animals. BH\(_4\) availability has been shown to be important for both catalytic activity and the coupling of the enzyme for efficient production of nitric oxide.\(^{35,36}\) High-performance liquid chromatography (HPLC) analysis revealed a substantial 2-fold decrease in BH\(_4\) levels in the diabetic liver. This result supports other published studies\(^{37,38}\) and provides another possible mechanism by which eNOS may be uncoupled in the diabetic liver. Decreases in BH\(_4\) availability has been suggested\(^{37,39}\) to be directly linked to superoxide production in diabetes. We administered either BH\(_4\) or sepiapterin before induction of hepatic I-R injury. Sepiapterin is a precursor in the BH\(_4\) salvage pathway\(^{40}\) and has been shown to increase BH\(_4\) levels and augment eNOS function in vivo.\(^{38}\) The acute administration of either BH\(_4\) or sepiapterin decreased serum ALT levels in the both the nondiabetic and diabetic following I-R injury. These data suggest that the restoration of BH\(_4\) levels in the diabetic liver is beneficial via augmenting eNOS function.

Working on the hypothesis that increasing BH\(_4\) levels in the diabetic “recouples” eNOS and thereby reduces superoxide production, we again attempted NO\(^-\) donor therapy following the supplementation of BH\(_4\). Treatment of diabetic mice with BH\(_4\) before sodium nitrite therapy significantly reduced hepatic injury. ALT levels with this combination therapy decreased hepatic injury to the lowest seen in the current study in db/db diabetic mice. This was in stark contrast to the previous finding that nitrite administration alone tended toward an increase in hepatic injury in the db/db mouse. A possible mechanism for this protection is that increased levels of BH\(_4\) recoupled eNOS, thus decreasing the production of superoxide. The subsequent administration of NO\(^-\) thereby did not yield the potent oxidizing species ONOO\(^-\) but rather increased NO\(^-\) bioavailability, leading to protection against I-R injury.

To examine the possibility that superoxide anion modulates the deleterious effects of NO\(^-\) therapy we investigated a cell-permeable SOD mimic. Treatment of nondiabetic mice with the SOD mimetic before ischemia resulted in a decrease in serum ALT. This finding supports work by Hines et al.,\(^{41}\) who found significant protection against hepatic I-R injury following treatment with an SOD mimetic. Treatment of diabetic mice with SOD therapy before ischemia trended to lower serum ALT levels, although not significantly. However, cotreatment with SOD therapy followed by the administration of the NO\(^-\) donor DETA/NO significantly lowered ALT levels following I-R injury. This result further supports the likelihood that decreasing superoxide levels in the setting of diabetic I-R injury allows for nitric oxide therapy to be beneficial rather than injurious. Taken together, these data suggest that recoupling eNOS provides protection in an additive fashion by both decreasing superoxide production and increasing NO\(^-\) production.

The controversy of nitric oxide therapy has been at the forefront of the I-R injury field for more than 2 decades. We present a clear example of both the protective and injurious nature of NO\(^-\) therapy in an in vivo model of hepatic I-R injury. We show that eNOS gene therapy and direct nitric oxide therapy are very protective in nondiabetic animals subjected to I-R injury. In contrast, in the setting of type 2 diabetes mellitus, these therapies are pathogenic. The injurious nature of NO\(^-\) therapy could however be reversed with prior administration of BH\(_4\) or a SOD mimic. These results suggest that superoxide may play a pivotal role by combining with NO\(^-\) to form the potent free radical ONOO\(^-\) and that eNOS is a major source of superoxide anion generation in the diabetic liver. Our study suggests that eNOS cofactor therapy may restore eNOS function in diabetes mellitus and may be beneficial in the setting of ischemic disorders.

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Disclosure

D.J.L. is a participant on a pending U.S. patent, filed on October 14, 2003 through NIH (patent no. 60/511, 244), regarding the use of sodium nitrite in cardiovascular disease.

References


SUPPLEMENTAL FIGURES

Supp. Figure 1. eNOS Protein expression 48 hr. post adenoviral delivery. eNOS protein expression increased ~7 fold in mice receiving adeno-eNOS as compared to mice receiving adeno-null as assessed by the presence of the anticipated 140 kD band.

Supp. Figure 2. eNOS monomer/dimmer ratio in non-diabetic and diabetic mice. Low-temperature SDS page western blots were utilized to assess the status of the eNOS enzyme in non-diabetic and diabetic mice. Diabetic mice were found to have a significant decrease in eNOS monomer/dimmer ratio as assessed by the relative band density of the expected 280 kD (dimmer) and 140 kD (monomer) immunoreactive bands.
**Online Table 1. Body weights, blood glucose and HbA1C levels in non-diabetic and diabetic mice.**

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Mice were examined for phenotypical markers of type 2 diabetes mellitus.

Body weight in grams (g); serum blood glucose levels (mg/dL); and percent of hemoglobin A1C in non-fasted: non-diabetic, eNOS knock-out (−/−), eNOS transgenic (Tg), db/db diabetic, and db/db diabetic eNOS transgenic (Tg) mice.

** p < 0.01 vs. non-diabetic controls.
Supplemental Figure 1.

Hepatic eNOS Protein Expression

![Graph showing protein expression levels for Adeno-Null and Adeno-eNOS]

- **eNOS**
  - Adeno-Null
  - Adeno-eNOS

- **Relative Optical Density**
  - Adeno-Null: 3
  - Adeno-eNOS: 70

- **140 kd**

**Statistical Significance:**
- Adeno-eNOS vs. Adeno-Null: ** **
Supplemental Figure 2.

eNOS Western Analysis

Comparison of eNOS Western Analysis between Non-Diabetic (c57BL6) and Diabetic (db/db) groups. The bar graph shows the monomer/dimer ratio with a significant difference indicated by **.