Protective Vascular and Cardiac Effects of Inducible Nitric Oxide Synthase in Mice with Hyperhomocysteinemia

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Abstract

Diet-induced hyperhomocysteinemia produces endothelial and cardiac dysfunction and promotes thrombosis through a mechanism proposed to involve oxidative stress. Inducible nitric oxide synthase (iNOS) is upregulated in hyperhomocysteinemia and can generate superoxide. We therefore tested the hypothesis that iNOS mediates the adverse oxidative, vascular, thrombotic, and cardiac effects of hyperhomocysteinemia. Mice deficient in iNOS (Nos2−/−) and their wild-type (Nos2+/+) littermates were fed a high methionine/low folate (HM/LF) diet to induce mild hyperhomocysteinemia, with a 2-fold increase in plasma total homocysteine (P<0.001 vs. control diet). Hyperhomocysteinemic Nos2−/− mice exhibited endothelial dysfunction in cerebral arterioles, with impaired dilatation to acetylcholine but not nitroprusside, and enhanced susceptibility to carotid artery thrombosis, with shortened times to occlusion following photochemical injury (P<0.05 vs. control diet). Nos2−/− mice had decreased rather than increased dilatation responses to acetylcholine (P<0.05 vs. Nos2+/+ mice). Nos2+/− mice fed control diet also exhibited shortened times to thrombotic occlusion (P<0.05 vs. Nos2+/+ mice), and iNOS deficiency failed to protect from endothelial dysfunction or accelerated thrombosis in mice with hyperhomocysteinemia. Deficiency of iNOS did not alter myocardial infarct size in mice fed the control diet but significantly increased infarct size and cardiac superoxide production in mice fed the HM/LF diet (P<0.05 vs. Nos2+/+ mice). These findings suggest that endogenous iNOS protects from, rather than exacerbates, endothelial dysfunction, thrombosis, and hyperhomocysteinemia-associated myocardial ischemia-reperfusion injury. In the setting of mild hyperhomocysteinemia, iNOS functions to blunt cardiac oxidative stress rather than functioning as a source of superoxide.

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Introduction

Hyperhomocysteinemia is an established risk factor for cardiovascular disease and stroke [1,2]. Even relatively mild elevations of plasma total homocysteine (e.g., 10 to 20 μmol/L) are associated with increased cardiovascular risk [1,2]. Several distinct cardiovascular abnormalities, including endothelial and cardiomycocyte dysfunction, and thrombosis, have been demonstrated in animal models of diet-induced hyperhomocysteinemia [3,4]. Hyperhomocysteinemia has been proposed to exert its adverse effects in the vasculature in part by generating vascular oxidative stress that diminishes the bioavailability of endothelial-derived nitric oxide (NO) [3]. NO is normally produced in a highly regulated manner by endothelial NO synthase (eNOS; Nos3), which is expressed constitutively in vascular endothelium. An inducible isoform of NOS (iNOS; Nos2) is expressed in vascular smooth muscle, endothelium, and myocardium in certain pathological conditions such as atherosclerosis, diabetes, and inflammatory disorders [5–9]. Experimental overexpression of iNOS in isolated murine blood vessels causes endothelial dysfunction [10–12], which may be due to iNOS uncoupling [13,14]. iNOS uncoupling results in the generation of superoxide rather than NO as the predominant reaction product and iNOS-derived superoxide can limit the bioavailability of endothelial NO [15]. Studies in diabetic mice revealed that deficiency of iNOS or reversal of iNOS uncoupling restores endothelium-dependent relaxation [16,17] and tolerance to myocardial ischemia/reperfusion (I/R) injury [18,19]. Collectively, these studies suggest a role for iNOS in the pathophysiology of multiple types of cardiovascular disease.

The potential role of iNOS in the etiology of hyperhomocysteinemia-induced cardiovascular abnormalities has not been directly explored, but several lines of evidence suggest that iNOS expression is upregulated in the setting of elevated homocysteine levels. In vitro studies with cultured endothelial and smooth
muscle cells treated with homocysteine showed increased expression of iNOS [20,21]. Similarly, upregulation of iNOS has been detected in the coronary and carotid artery, and kidneys of hyperhomocysteinemic rats [22–24]. Additionally, we and others have shown that iNOS inhibitors abrogate the increased production of reactive oxygen species (ROS) in carotid and coronary arteries of hyperhomocysteinemic rats or mice [22,25]. These observations suggest that the upregulation and uncoupling of iNOS, with increased production of superoxide, may be a mechanism that contributes to endothelial dysfunction in hyperhomocysteinemia. Consistent with this notion, impaired endothelium-dependent vasomotor responsiveness in the aorta of hyperhomocysteinemic rats could be reversed by tetrahydrobiopterin, an essential cofactor that promotes NOS coupling [26]. It remains unclear, however, whether iNOS directly and mechanistically contributes to the adverse vascular and cardiac effects of hyperhomocysteinemia.

We utilized a genetic approach to examine the mechanistic role of endogenous iNOS in the vascular phenotype of diet-induced hyperhomocysteinemia in mice. Using iNOS-deficient (Nos2−−) mice, we tested the hypothesis that iNOS contributes to endothelial dysfunction, thrombosis, and myocardial ischemia-reperfusion injury in hyperhomocysteinemia. Contrary to our hypothesis, our data suggest that endogenous iNOS protects against, rather than exacerbates, vascular oxidative stress, endothelial dysfunction, and thrombosis. Our findings also indicate that, during hyperhomocysteinemia, endogenous iNOS prevents myocardial injury by blunting cardiac oxidative stress.

Materials and Methods

Mice

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health. All animal protocols were approved by the University of Iowa Animal Care and Use Committee [approval # 1211243]. All surgery was performed under anesthesia (sodium pentobarbital) and all efforts were made to minimize suffering. Male homozygous iNOS deficient (Nos2−−) mice on the C57BL6/J background were obtained from the Jackson Laboratory and were backcrossed to C57BL6/J female mice (Jackson Laboratory) to generate male and female heterozygous iNOS deficient (Nos2+/−) breeders. These Nos2+/− breeder mice were then intercrossed to generate wild-type (Nos2+/*+ *) and Nos2−−/− littermate mice for study. Genotyping for the wild-type and targeted Nos2 alleles was performed by polymerase chain reaction (PCR) using the following primer sequences: for the wild-type Nos2 allele, 5′- TGA ACA TCT CCT GGT GGA AC -3′ and 5′- AGC ACA CAT GCA GAA GTA GTA -3′; for the targeted Nos2 allele, 5′- AAT ATG CGA AGT GGA CCT CG -3′ and 5′- AGC ACA CAT GCA GAA GTA GTA -3′. Starting from 4 weeks of age, mice were fed either a control diet (LM485, Harlan Teklad) that contains 4.0 g/Kg L-methionine and 6.7 mg/Kg of folic acid, or a high methionine/low folate (HM/LF) diet (TD00205, Harlan Teklad) that contains 8.2 g/Kg L-methionine and 6.7 mg/Kg of folic acid [27]. Mice were fed the diets for 5–11 months before study.

Dilator responses in cerebral arterioles

Dilatation of cerebral arterioles was measured as described previously [25]. Briefly, mice were anesthetized with sodium pentobarbital and ventilated mechanically. A cranial window was made over the left parietal cortex, and a segment of a randomly selected pial arteriole (∼30 μm in diameter) was studied. The diameter of the cerebral arteriole was measured, using a video microscope coupled to an image-shearing device, under control conditions and during superfusion with acetylcholine (1.0 μmol/L and 10.0 μmol/L), nitroprusside (0.1 μmol/L and 1.0 μmol/L), and papaverine (1.0 μmol/L and 10.0 μmol/L).

Carotid artery thrombosis

Carotid artery thrombosis was induced by photochemical injury as described previously [28]. Mice were anesthetized with sodium pentobarbital (70–90 mg/Kg intraperitoneally) and ventilated mechanically with room air and supplemental oxygen. The right femoral vein was cannulated for the administration of rose bengal. The right common carotid artery was dissected free and carotid artery blood flow was measured with a 0.5 PSB Doppler flow probe (Transonic Systems, Inc) and digital recording system (Gould Ponemah Physiology Platform version 3.33). To induce endothelial injury, the right common carotid artery was transilluminated continuously with a 1.5-mW, 540-nm green laser (Melles Griot) from a distance of 6 cm, and rose bengal (35 mg/
Kg) was injected via a femoral vein catheter. Blood flow was monitored continuously for 90 minutes or until stable occlusion occurred, at which time the experiment was terminated. Stable occlusion was defined as the time at which blood flow remained absent for ≥10 minutes.

**Myocardial I/R injury**

Regional myocardial I/R injury was performed using a procedure modified from Gandhi et al [29]. Male mice were anesthetized with sodium pentobarbital and supplemented every hour (sodium pentobarbital, 20 mg/Kg IP) as needed to maintain adequate depth of anesthesia. Body temperature was monitored with a rectal probe and maintained at 36.5–37.5°C with a heating pad. A tracheotomy was performed and a volume-controlled Harvard respirator (model 681) was used to mechanically ventilate mice with room air (stroke volume 0.24 mL, 105 strokes per minute). A thoracotomy was performed in the fourth intercostal space, and the left coronary artery was ligated 2 mm below the tip of the left atrium using 8.0 Ethicon mounted on a tapered needle. A short (1 mm) section of PE-10 tubing was placed into the ligature to facilitate later reperfusion. Successful occlusion of the left coronary artery was verified by visual inspection (development of paleness of the anterolateral wall of the left ventricle, color change of left coronary artery from bright-red to violet, and regional akinesis). After occlusion for 30 minutes, reperfusion was induced by cutting the ligature on the top of the PE-10 tubing and was visually confirmed by a return of pink-red color of the

![Figure 2. Dilator responses in cerebral arterioles.](image) Dilatation of cerebral arterioles to (A) 1.0 μmol/L acetylcholine, (B) 10.0 μmol/L acetylcholine, (C) 0.1 μmol/L nitroprusside, (D) 1.0 μmol/L nitroprusside, (E) 1.0 μmol/L papaverine and (F) 10.0 μmol/L papaverine were measured in Nos2+/+ (open bars) or Nos2−/− (filled bars) mice. 10–15 mice were studied in each group. *P<0.05 compared with mice of same genotype fed the control diet by two-way ANOVA. doi:10.1371/journal.pone.0107734.g002

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DHE for 30 minutes at room temperature in a dark chamber. Fluorescence was detected by laser-scanning confocal microscopy. Plasma homocysteine and methionine (10 μmol/L), and plasma was flash frozen. Plasma total homocysteine, defined as the total concentration of homocysteine after quantitative reductive cleavage of all disulfide bonds [30], was measured by high performance liquid chromatography (HPLC) using ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBDF) fluorescence detection [31]. Plasma methionine was measured by HPLC coupled to fluorescence detection after pre-column derivatization using o-phthaldialdehyde as described previously [32].

Detection of ROS

The oxidative fluorescent dye dihydroethidium (DHE) (Molecular Probes) was used to detect ROS in sections of cerebral arterioles as described previously [33]. Frozen transverse sections (10 μm) of cerebral arterioles were incubated with 10 μmol/L DHE for 30 minutes at room temperature in a dark chamber. Fluorescence was detected by laser-scanning confocal microscopy, imaged, and quantified using Image J software and reported as mean fluorescence relative to NoS2+/+ mice fed the control diet.

Real-time PCR

Levels of mRNA for NoS2, neuronal and endothelial nitric oxide synthase (NoS1 and NoS3, respectively), and 18S were measured by quantitative real-time PCR as described previously [34]. Total RNA was isolated from frozen hearts using Trizol reagent (Invitrogen, Carlsbad, CA) and treated with DNase I to remove contaminating genomic DNA. RNA (325 ng) was then reverse transcribed using Taqman reverse transcriptase and random hexamer primers. PCR primers and 6-carboxy fluorescein-labeled probes for 18S (Mm00465190_m1), NoS2 (Mm01309892_g1), and NoS3 (Mm00435204_m1) were purchased from Applied Biosystems. Reverse transcribed cDNA was incubated with TaqMan Universal PCR mix (Applied Biosystems) and PCR primers and probes at 50°C for 2 minutes and then at 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute using the Applied Biosystems 7700 sequence detection system. For quantitative analysis of mRNA, the comparative threshold cycle (ΔΔCt) method [35] was used, with values normalized to 18S and expressed relative to levels in NoS2+/+ mice fed the control diet. Validation experiments were performed to confirm equal amplification efficiency for all primers sets.

Statistical analysis

Two-way analysis of variance (ANOVA) with the Tukey test for multiple comparisons was used to analyze the effects of NoS2
Results

Deficiency of iNOS does not protect against endothelial dysfunction in hyperhomocysteinemic mice

To investigate the potential contribution of iNOS to endothelial dysfunction during hyperhomocysteinemia, Nos2−/− mice deficient in iNOS were placed on a HM/LF diet. Plasma levels of total homocysteine were elevated approximately 2-fold in both Nos2+/+ and Nos2−/− mice fed the HM/LF diet compared with mice fed the control diet (Figure 1, P<0.001). Plasma levels of methionine also were elevated significantly in mice fed the HM/LF diet compared with mice fed the control diet (Figure 1, P<0.05). Deficiency of iNOS had no impact on plasma levels of homocysteine or methionine (P = 0.5).

Hyperhomocysteinemia is a risk factor for small vessel disease in the brain [36]. Thus, we next measured responses of small cerebral arterioles to endothelium-dependent and -independent dilators (Figure 2). Dilator responses to 10.0 μmol/L acetylcholine, an endothelium-dependent dilator, were decreased in Nos2+/+ mice fed the HM/LF diet compared with Nos2−/− mice fed the control diet (P<0.05; Figure 2B). Deficiency of iNOS failed to protect cerebral arterioles from this effect of hyperhomocysteinemia, as demonstrated by the similar dilator responses to acetylcholine in Nos2+/+ and Nos2−/− mice fed the HM/LF diet. These data suggest that iNOS does not play a causative role in the endothelial vasomotor dysfunction associated with hyperhomocysteinemia. In mice without hyperhomocysteinemia, the data suggest a possible protective effect of endogenous Nos2, indicated by a strong trend towards decreased dilator responses to acetylcholine in Nos2−/− mice compared with Nos2+/+ mice fed the control diet (P = 0.053; Figure 2B). The endothelium-independent vasodilators nitroprusside (Figure 2C and 2D) and papaverine (Figure 2E and 2F) also produced dilatation of cerebral arterioles, and the responses were not influenced by either diet or Nos2 genotype.

Absence of iNOS does not reverse the prothrombotic phenotype produced by HM/LF diet

To further examine the vascular effects of iNOS deficiency in hyperhomocysteinemic mice, experimental thrombosis of the carotid artery was induced by photochemical injury. Nos2+/+ mice fed the HM/LF diet exhibited a significant shortening in the time to stable occlusion compared with Nos2−/− mice fed the control diet (P<0.05, Figure 3). A similar shortening of the time to stable occlusion was observed in Nos2−/− mice fed the HM/LF diet, indicating that deficiency of iNOS did not prevent this prothrombotic effect of hyperhomocysteinemia. In mice fed the control diet, the mean time to stable occlusion was significantly shortened in Nos2−/− mice compared with Nos2+/+ mice (P<0.01), again suggesting a protective effect of endogenous Nos2.

Figure 5. Regional myocardial ischemia-reperfusion injury. The left coronary artery was ligated for 30 minutes and then reperfused for 2 hours. Panel A illustrates representative 2,3,5-triphenyltetrazolium chloride (TTC) stained images for each of the four groups of mice. The percent of (B) area at risk over total area (AR/TA), (C) area of necrosis over total area (AN/TA), and (D) area of necrosis over area at risk (AN/AR) were calculated in Nos2+/+ (open bars) or Nos2−/− mice (filled bars) by staining with Evans blue and TTC. Values are mean ±SE. 8–12 mice were studied in each group.

*P<0.01 compared with Nos2+/+ mice fed the same diet, #P<0.05 compared with mice of the same genotype fed the control diet by two-way ANOVA.

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Increased vascular ROS was detected with the HM/LF diet and iNOS deficiency

Previous work has suggested a possible mechanistic role for vascular ROS in both the endothelial vasomotor dysfunction and prothrombotic state of diet-induced hyperhomocysteinemia [3]. Therefore, we utilized laser-scanning confocal microscopy to detect ROS in cerebral arteries by DHE fluorescence. We found that DHE fluorescence was significantly (P<0.05) higher in Nos2+/+ mice fed the HM/LF diet (Figure 4C) compared with Nos2+/+ mice fed the control diet (Figure 4A). Deficiency of iNOS tended to increase DHE fluorescence in Nos2−/− mice fed the control diet (P = 0.07) (Figure 4B) and was increased further in Nos2−/− mice fed the HM/LF diet (Figure 4D). The quantitative data are summarized in Figure 4E. These findings suggest that endogenous Nos2 protects from ROS generation rather than acting as a source of vascular ROS. Our findings also suggest that both iNOS deficiency and hyperhomocysteinemia independently contribute to increased vascular ROS.

Hyperhomocysteinemia exacerbates myocardial I/R injury in iNOS-deficient mice

We next examined the effect of Nos2 deficiency on regional myocardial I/R injury (Figure 5A). In Nos2−/− mice, the ischemic area at risk (AR) following I/R injury was significantly increased compared with Nos2+/+ littermate mice, regardless of diet (P<0.01, Figure 5B). In Nos2+/+ mice, the HM/LF diet did not alter infarct size, calculated either as area of necrosis per total area (AN/TA) (Figure 5C) or area of necrosis per area at risk (AN/AR) (Figure 5D). In Nos2−/− mice, however, the HM/LF diet caused a significant increase in infarct size (Figure 5C and 5D; P<0.05 vs. Nos2+/+ mice fed the HM/LF diet; P<0.05 vs. Nos2−/− mice fed the control diet). These data suggest that endogenous iNOS protects against reperfusion injury in the setting of hyperhomocysteinemia.

To further explore the relationship between NOS and ROS in the heart, we measured cardiac levels of Nos1, Nos2 and Nos3 mRNA by quantitative real-time PCR. The data demonstrate a 50% increase in Nos2 mRNA levels in hearts from Nos2+/+ mice fed the HM/LF diet compared with the control diet (Figure 6A; P<0.05). This finding indicates that hyperhomocysteinemia induces the upregulation of iNOS expression in the heart, as has been previously reported in vascular tissue [22] and kidney [23]. As expected, no Nos2 mRNA was detected in Nos2−/− mice (Figure 6A). There was no significant difference in Nos3 mRNA expression between Nos2+/+ mice fed the control diet vs. the HM/LF diet (Figure 6B). We did detect a small but significant increase in Nos3 mRNA levels in Nos2−/− mice fed the HM/LF diet (Figure 6B; P<0.05 vs. Nos2−/− mice fed the control diet). Nos1 mRNA was not detected in cardiac tissue from any of the groups of mice (not shown). We next determined whether Nos2 deficiency during hyperhomocysteinemia alters cardiac ROS production. A significant increase in cardiac ROS was observed in Nos2−/− mice fed the HM/LF diet (Figure 6C; P<0.05 compared with Nos2−/− mice fed the control diet; P<0.01 compared with Nos2+/+ mice fed the HM/LF diet), consistent with the larger infarct size in this group (Figure 5B and 5C). These findings suggest that during hyperhomocysteinemia, endogenous iNOS protects cardiac tissue from oxidative stress and I/R injury.

Discussion

In certain pathological conditions such as diabetes and atherosclerosis, iNOS has been proposed to be a mediator of increased oxidative stress and impaired vascular function. In diabetic mice, deficiency of iNOS protects against endothelial dysfunction [16,17] and myocardial I/R injury [18,19]. The absence of iNOS also decreases oxidative stress and protects against atherosclerosis in apolipoprotein E-deficient (ApoE−/−) mice [37]. Because hyperhomocysteinemia causes upregulation of vascular iNOS expression and increases vascular oxidative stress [22,34], we hypothesized that genetic deficiency of iNOS also might protect against hyperhomocysteinemia-induced vascular
dysfunction and thrombosis. To address this hypothesis, we examined the mechanistic role of iNOS in endothelial dysfunction, thrombosis and myocardial I/R injury in mice with or without diet-induced hyperhomocysteinemia. The major findings of our study are: 1) the absence of iNOS failed to protect mice against endothelial dysfunction or accelerated thrombosis produced by hyperhomocysteinemia; and 2) deficiency of iNOS exacerbated, rather than protected against myocardial ischemia-reperfusion injury and cardiac oxidative stress in hyperhomocysteinemic mice. These findings demonstrate that, unlike what has been described in models of diabetes or atherosclerosis, iNOS is not a mediator of the adverse vascular and endothelial effects of hyperhomocysteinemia.

Our data also suggest a baseline antithrombotic effect of endogenous iNOS in mice without hyperhomocysteinemia, demonstrated by a marked shortening of the time to thrombotic occlusion of the carotid artery in Nos2-/- mice fed the control diet (Figure 3). This observation is consistent with a prior study in which iNOS was found to provide protection against injury-induced thrombosis in female mice [38]. In our study, loss of endogenous iNOS also appeared to decrease dilatation responses to acetylcholine (Figure 2) and increase levels of ROS in cerebral arterioles (Figure 4) in the absence of hyperhomocysteinemia. Interestingly, a previous study found that Nos2-/- mice exhibited enhanced oxidative stress after traumatic brain injury [39]. These observations suggest that, rather than contributing to oxidative vascular injury, endogenous iNOS may protect against endothelial dysfunction and thrombosis by suppressing vascular oxidative stress.

Clinical evidence for increased troponin levels after coronary artery bypass in hyperhomocysteinemic patients suggests a relationship between elevated homocysteine and cardiac injury [40]. Ex vivo studies in isolated Langendorff-perfused hearts from mice with heterozygous deficiency of cystathionine β-synthase suggested that elevated homocysteine results in impaired relaxation and contractile function and increased apoptosis following I/R injury [41]. Unexpectedly, we did not observe a difference in infarct size in wild-type mice fed control vs. HM/LF diet. Potential reasons for the different findings between the ex vivo Langendorff results and our in vivo data include the different endpoints for myocardial injury examined, and that the ex vivo studies were conducted with a genetic model of hyperhomocysteinemia [4], whereas we used a diet-induced model of hyperhomocysteinemia.

Our findings in the myocardial I/R model are particularly interesting, since prior reports have suggested contrastingly that either deficiency or overexpression of iNOS can protect against I/R injury in mouse models [19,41,42]. We observed that the myocardial area at risk was increased with iNOS deficiency regardless of diet, suggesting that endogenous iNOS may limit the area of myocardial ischemia by promoting vasodilatation of collateral blood vessels. We also found that cardiac iNOS mRNA was significantly upregulated by hyperhomocysteinemia in Nos2+/+ mice (Figure 6A). Our data further suggest that the elevated levels of cardiac iNOS in hyperhomocysteinemic mice are protective against oxidative stress and I/R injury, because Nos2−/− mice had increased cardiac ROS production (Figure 6C) and increased infarct size (Figure 5) compared with Nos2+/+ mice in the setting of hyperhomocysteinemia. This protective effect of iNOS is in accordance with previous studies in which gene transfer of iNOS protected against myocardial injury [42].

Our study has some limitations, including the inherent weaknesses of mouse models of human disease. The mouse model of diet-induced hyperhomocysteinemia, and the experimental models of vascular injury utilized herein may only approximate conditions that occur in human patients. Moreover, it is possible that some or all of the vascular effects observed in this study may be driven by metabolic consequences of the HM/LF diet that are independent of hyperhomocysteinemia. We note that the HM/LF diet produced only modest hyperhomocysteinemia in mice, with levels of plasma tHcy (10.3 ± 0.9 μmol/L) somewhat lower than those seen in human patients with moderate hyperhomocysteinemia (10–20 μmol/L) and vascular diseases [1,2]. It may be interesting in future studies to explore the vascular effects of additional dietary and genetic models of altered homocysteine, methionine and folate metabolism.

In summary, our data suggest a model in which, under normal or minimally pathological conditions (e.g. mild hyperhomocysteinemia, which is common in the general population), iNOS expressed at low levels in vascular cells is primarily protective against vascular oxidative stress and its complications. This vasoprotective effect of iNOS contrasts with its harmful effects in more severe pathological conditions (e.g. diabetes, atherosclerosis, or sepsis), in which upregulation of iNOS can mediate increased oxidative stress and related vascular pathology [16–19,37,43]. Our study design was comprehensive in that we examined the modulating effects of iNOS deficiency in both vascular and cardiac tissues in mice with diet-induced hyperhomocysteinemia. Our findings argue strongly against a role for iNOS as a mediator of the vascular, thrombotic, or cardiac complications of hyperhomocysteinemia in this mouse model.

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Author Contributions

Conceived and designed the experiments: SD SRL. Performed the experiments: SD RAE MJ IOB EA JWS. Analyzed the data: SD TB FMS L. Contributed to the writing of the manuscript: SD IOB JWS.

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