Hematopoietic Cell–Expressed Endothelial Nitric Oxide Protects the Liver From Insulin Resistance


OBJECTIVE: Mice genetically deficient in endothelial nitric oxide synthase (Nos3−/−) have fasting hyperinsulinemia and hepatic insulin resistance, indicating the importance of Nos3 (nitric oxide synthase) in maintaining metabolic homeostasis. Although the current paradigm holds that these metabolic effects are derived specifically from the expression of Nos3 in the endothelium, it has been established that bone marrow–derived cells also express Nos3. The aim of this study was to investigate whether bone marrow–derived cell Nos3 is important in maintaining metabolic homeostasis.

APPROACH AND RESULTS: To test the hypothesis that bone marrow–derived cell Nos3 contributes to metabolic homeostasis, we generated chimeric male mice deficient or competent for Nos3 expression in circulating blood cells. These mice were placed on a low-fat diet for 5 weeks, a time period which is known to induce hepatic insulin resistance in global Nos3-deficient mice but not in wild-type C57Bl/6 mice. Surprisingly, we found that the absence of Nos3 in the bone marrow–derived component is associated with hepatic insulin resistance and that restoration of Nos3 in the bone marrow–derived component in global Nos3-deficient mice is sufficient to restore hepatic insulin sensitivity. Furthermore, we found that overexpression of Nos3 in bone marrow–derived component in wild-type mice attenuates the development of hepatic insulin resistance during high-fat feeding. Finally, compared with wild-type macrophages, the loss of macrophage Nos3 is associated with increased inflammatory responses to LPS and reduced anti-inflammatory responses to IL-4, a macrophage phenotype associated with the development of hepatic and systemic insulin resistance.

CONCLUSIONS: These results would suggest that the metabolic and hepatic consequences of high-fat feeding are mediated by loss of Nos3/nitric oxide actions in bone marrow–derived cells, not in endothelial cells.

N os3 (nitric oxide synthase) and eNOS (endothelial nitric oxide synthase) are well-established contributors to vascular homeostasis. Nevertheless, loss of Nos3 seems to result in metabolic derangements that contribute to insulin resistance. More specifically, a reduction in Nos3-derived nitric oxide (NO) during obesity precedes the development of insulin resistance1 and genetic deletion of Nos3 (Nos3−/− mouse) is associated with both systemic and hepatic insulin resistance.2–4 Moreover, pharmacological strategies that restore NO bioavailability during obesity (eg, PDE5 inhibition) and genetic strategies that increase NO production (eg, Nos3 overexpression) restore insulin sensitivity.4–7 Our laboratory and others have long attributed the salutary metabolic effects of Nos3 to endothelium-mediated improvement of blood flow that increases delivery of nutrients to insulin-sensitive tissues such as liver, muscle, and adipose tissue, thereby facilitating nutrient utilization or storage and maintaining metabolic homeostasis. We have previously proposed that Nos3/NO impairs NF-κB activation during high fat (HF) feeding, a critical step in the development of hepatic insulin resistance.4,7 Nos3 has been extensively studied in endothelial cells and conventional wisdom holds that the effects of Nos3 are mediated primarily by endothelial cell–expressed Nos3. However, Nos3 is also expressed in...
bone marrow–derived cells (leukocytes, red blood cells [RBCs], platelets).8,9 The biologic significance of Nos3 expression in bone marrow–derived cells is increasingly recognized; for example, Nos3/NO expression/function in bone marrow–derived cells has important effects on blood pressure8 and during ischemia reperfusion,10 functions which had been previously attributed to endothelial cell–dependent Nos3 but now seem to be independent of endothelial cell–expressed Nos3.

The aim of this work was to test the hypothesis that circulating bone marrow–derived cell Nos3 attenuates the development of hepatic insulin resistance during HF feeding. To test this hypothesis, we generated mice with Nos3 deletion or Nos3 overexpression exclusively in hematopoietic cells using bone marrow transplantation. The resulting chimeric mice were fed a low fat (LF) or HF diet, and these mice were then assessed for evidence of hepatic insulin resistance.

**MATERIALS AND METHODS**

The authors declare that all supporting data are available within the article and its online-only Data Supplement.

**Materials**

Anti-phospho-NF-κB p65 (ser536; #3033), anti-NF-κB p65 (#8242), anti-iNOS (#2982), Anti-AMPKα (#2532), Anti-phosphoAMPKα (Thr172; #2535), β-actin (#4967) antibodies were from Cell Signaling (Beverly, MA). Anti-eNOS mouse polyclonal antibody (#610296) was obtained from BD Bioscience (Lexington, KY). Anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase; FL-335) rabbit polyclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Mac2 (Cedarlane Laboratories). Total Akt, p-Akt (Ser 473), IRS-1, IRS-2 and p-IRS1, p-IRS-2 ELISA kits were purchased from Cell Signaling.

Quantitative Real-Time-PCR Analyses

RNA was extracted using an RNAase kit (Qiagen; Valencia, CA). For gene expression analysis, real-time-PCR reactions were conducted as described previously1 using TaqMan Gene Expression Analysis (Applied Biosystems; Foster City, CA).

Western Blotting

Cell lysis and tissue extraction were performed as described previously.11 All Western blots used equal amounts of total protein for each condition from individual experiments and were performed as described previously,12 some images were developed and quantified using LI-COR Odyssey Imaging System (Lincoln, NE).

Insulin and Hepatic Triglyceride Content

Insulin levels were determined using a mouse insulin ELISA kit (Crystal Chem, Downers Grove, IL). Hepatic TG content was enzymatically measured in liver lysates as previously described.14

Animal Experiments

eNOS transgenic (Nos3Tg) mice,15 kindly provided by Dr Hirata (Kobe University, Japan), express bovine eNOS under...
the control of the preproendothelin-1 promoter\textsuperscript{15} and were maintained on a C57BL/6 background. Nos3\textsuperscript{−/−} and wild-type (WT) mice were purchased from Jackson Laboratory. Control and experimental mice were fed either a LFD (10% fat; #D12450B; Research Diets, New Brunswick, NJ) or a high-fat diet (HFD; 60% of calories from fat; #D12492; Research Diets) and maintained for indicated periods. Only male mice were used for these studies since young female mice do not reliably become obese and insulin resistant during high fat feeding.

At the conclusion of the dietary intervention, one-half of the animals in each study group received an IP injection of either vehicle (normal saline) or regular insulin (0.06 U/g body weight in 200 µL of normal saline) after a 4-hour fast. Fifteen minutes later, mice were euthanized with an overdose of CO\textsubscript{2} followed by cervical dislocation. Liver, skeletal muscle, and adipose tissue were removed and snap-frozen on dry ice after dissecting away surrounding connective tissue. Protein was subsequently extracted from tissue samples and, after protein levels were quantified (Micro BCA Protein Assay Kit; Pierce, Rockford IL), equal amounts of protein were used for each condition in each assay. Total Akt, phospho-Akt (serine 473), and IRS-1, IRS-2 tyrosine phosphorylation levels were determined using ELISA assay kits (Cell Signaling). All study protocols were approved by the University of Washington Institutional Animal Care and Use Committee.

**Bone Marrow Transplant Protocol**

**Control BMT Experiment 1**

To control for potentially confounding effects of the transplant procedure, male WT mice (C57BL/6) age 6 to 8 weeks were lethally irradiated and transplanted with marrow from male WT mice (WT/WT) mice. Similarly, Nos3\textsuperscript{−/−} mice aged 6 to 8 weeks were lethally irradiated and transplanted with marrow from Nos3\textsuperscript{−/−} mice (Nos3\textsuperscript{−/−}/Nos3\textsuperscript{−/−}) mice.

**BMT Experiment 2**

To investigate whether Nos3 signaling in bone marrow–derived cells is necessary for its anti-inflammatory effects in hepatic tissue, male WT mice age 6 to 8 weeks received marrow from Nos3\textsuperscript{−/−} mice (Nos3\textsuperscript{−/−}/WT) following lethal irradiation. To determine if normal bone marrow–derived cells are sufficient to reverse the vascular inflammation observed in mice lacking Nos3, male Nos3\textsuperscript{−/−} mice age 6 to 8 weeks were transplanted with marrow from WT mice (WT/Nos3\textsuperscript{−/−}) following lethal irradiation. Treated mice were allowed to recover for 6 weeks on standard chow diet, after which they were switched to a LFD at the beginning of the study.

**BMT Experiment 3**

To investigate whether Nos3 signaling is sufficient for the improved insulin resistance in hepatic tissue, male WT mice age 6 to 8 weeks received marrow-derived cells from Nos3\textsuperscript{−/−} mice following lethal irradiation.

Bone marrow–derived macrophages (BMDMs) were generated from male mice as described previously.\textsuperscript{16} For classical or alternative activation of macrophages, cells were stimulated with murine IFN-γ (12 ng/mL; STEMCELL Technologies, Vancouver, Canada) plus LPS (5 ng/mL; Sigma-Aldrich; St. Louis, MO) for 18 hours or murine IL-4 (10 ng/mL; R&D systems; Minneapolis, MN) for 18 hours, respectively.

**RESULTS**

**Bone Marrow–Derived Cell Nos3 Deficiency Promotes Development of Insulin Resistance and Hepatic Inflammation**

Since macrophages are implicated in the pathogenesis of both hepatic inflammation and hepatic insulin resistance,\textsuperscript{17} we hypothesized that the protective role of Nos3 with respect to hepatic inflammation involves an action in macrophages, monocytes, or other cells of bone marrow origin. To test this hypothesis, we sought to determine whether (1) deficiency of Nos3 specifically in marrow-derived cells is sufficient to induce hepatic inflammation in normal mice or (2) the presence of Nos3 in bone marrow–derived cells is sufficient to protect against hepatic inflammation in mice that otherwise lack the Nos3 gene. These goals were accomplished by generating chimeric mice in which the Nos3 gene was expressed either in bone marrow–derived cells only or in all cells excluding bone marrow–derived cells. Four different chimeric groups were studied: Nos3\textsuperscript{−/−}/WT (Nos3-deficient bone marrow–derived cells transplanted into a WT host), WT/Nos3\textsuperscript{−/−} (WT bone marrow–derived cells transplanted into Nos3-deficient mice) and 2 control groups, Nos3\textsuperscript{−/−}/Nos3\textsuperscript{−/−} (Nos3-deficient marrow transplanted into Nos3-deficient mice) and WT/WT (WT marrow transplanted into a WT mouse; Figure 1A).

To verify the efficacy of this approach, bone marrow–derived macrophage lysates from Nos3\textsuperscript{−/−}/WT and WT/WT mice were analyzed by Western blot following immunoprecipitation with an anti-Nos3 antibody (Figure 1B). As expected, Nos3 expression was absent in BMDM harvested from Nos3\textsuperscript{−/−}/WT mice, whereas vascular and liver content of Nos3 were similar (Figure 1F and 1G). RBC NO content as measured by DAF2DA in Nos3\textsuperscript{−/−}/WT was reduced compared with WT/WT controls (Figure 1C and 1D) and expression of Nos3 was reduced in RBC lysates from Nos3\textsuperscript{−/−}/WT mice as detected by Western blot (Figure 1E). These studies demonstrated the successful generation of chimeric mice with deficient Nos3 expression in bone marrow–derived cells.

**Statistical Analysis**

For all comparisons of data obtained using Western blot analysis, densitometry measurements were normalized to control values obtained from samples incubated with vehicle alone, and percent change relative to the control condition was calculated. Analysis of the results was performed using the STATA8 statistical package. Data are expressed as mean±SD, and values of \(P<0.05\) were considered statistically significant. For comparison of continuous data (eg, band intensities on Western blot) between 2 groups, the \(t\) test was applied after verifying normal distribution and equal variances. A 2-tailed \(t\) test was used to compare mean values from studies involving 2 experimental groups. To compare responses following treatment with vehicle or insulin across the 2 diets, data were analyzed by 2-way ANOVA followed by the Bonferroni post hoc comparison test.
Following a 6-week recovery from the bone marrow transplant procedure, (during which all mice were fed a standard chow diet), the 4 groups were placed on the LFD for 5 weeks, a time period which does not induce hepatic insulin resistance or hepatic inflammation in WT mice but is sufficient to induce fasting insulinemia, hepatic inflammation, and impairment of hepatic insulin signaling in mice with global deletion of Nos3.4

No significant differences in body weight or food intake were noted between the 4 groups in response to the LFD (Figure 2A and 2B). Fasting insulin levels at time of sacrifice, however, were significantly different. The absence of Nos3 in bone marrow–derived cells was associated with increased plasma insulin levels (Figure 2C), whereas the presence of WT marrow in Nos3−/− mice did not elevate fasting insulin levels. As expected,
the Nos3−/−/Nos3−/− chimeric mice exhibited elevated fasting insulin, whereas the WT/WT chimeric mice did not; an observation found in WT and global Nos3−/− mice.4,7 These changes in fasting insulin levels was not associated with significant changes in fasting glucose levels across the 4 experimental groups (Figure 2D).

We next examined markers of global hepatic inflammation as measured by phospho-p65, iNOS, IL-6, since elevation of these markers are associated with hepatic insulin resistance. As expected, the Nos3−/−/Nos3−/− mice demonstrated evidence for increased NF-κB activation compared with WT/WT mice (Figure 2E through 2G), an observation previously seen in global Nos3−/− and WT mice in response to a LFD.4

Deletion of Nos3 in the bone marrow–derived cell compartment, however, was sufficient to increase hepatic LF-dependent NF-κB responses, whereas the replacement of Nos3 in the bone marrow–derived cell compartment in Nos3-deficient mice did not (Figure 2E through 2G). These findings suggest that the protective role played by Nos3 with respect to hepatic inflammation is confined to cells derived from the bone marrow. Kupffer cells play an important role in the development of hepatic insulin resistance and we have previously
demonstrated that proinflammatory activation of Kupffer cells is evident in global Nos3 knock out mice. We next examined inflammatory activation in Kupffer cells isolated from Nos3−/−/WT and WT/WT mice (Figure I in the online-only Data Supplement) and found that the bone marrow transplant procedure did not alter Kupffer cell responses.

We next assessed hepatic insulin signaling by assessing insulin-mediated IRS-1, IRS-2, and Akt phosphorylation. The (Nos3−/−/Nos3−/−) mice exhibited impaired insulin-mediated IRS-1, IRS-2, and Akt phosphorylation (markers for hepatic insulin signaling) compared with the WT/WT mice fed a LFD (Figure 3A through 3C). This result was expected and similar to our previous work in WT and Nos3 global knock out mice. The absence of Nos3 in the bone marrow–derived cell compartment was also associated with impaired insulin-mediated IRS-1, IRS2, and Akt phosphorylation whereas the presence of Nos3 in the bone marrow compartment in Nos3-deficient mice restored hepatic insulin-mediated signaling. This observation suggests that absence of Nos3 in the bone marrow–derived cells impairs hepatic insulin signaling.

Under physiological conditions, insulin signaling in the liver acts to suppress gluconeogenesis, whereas during insulin resistant states, insulin fails to suppress 2 key gluconeogenic enzymes: Pck1 (phosphoenolpyruvate kinase) and G6p (glucose-6-phosphatase). This defect in gluconeogenesis regulation is also seen in global Nos3 knock out mice, and we found that the absence of bone marrow–derived Nos3 is associated with increased expression of Pck1 (Figure 3D), whereas the presence of Nos3 in the bone marrow compartment is not. This finding is consistent with the observed defect in hepatic insulin signaling in the Nos3−/−/WT mice as gluconeogenesis depends on intact Akt signaling.

We previously have shown that the global absence of Nos3 is associated with increased hepatic triglyceride content compared with WT controls after 4 weeks of a LFD. Similarly, we found increased hepatic triglyceride content following a LFD in WT mice lacking Nos3 in the bone marrow–derived cell compartment (Figure 3E).

The mechanism by which hepatic lipid levels are altered during insulin resistance is an area of intense investigation. Previous work in global Nos3−/− mice demonstrated increased circulating FFA levels, increased hepatic lipid synthesis, and decrease fatty acid oxidation, suggesting a significant role for Nos3 in regulating hepatic lipid content. We next examined the effect of bone marrow–derived cell deletion of Nos3 on hepatic lipid synthesis; we found no differences in expression of Srebp1C (sterol regulatory element-binding protein 1) and Scd1 (stearoyl-CoA desaturase-1) between the 4 groups (Figure 3F and 3G), suggesting that the absence of Nos3 does not impact hepatic lipid synthesis. Increased adipose lipolysis and increased FFA delivery to the liver has also been proposed as a mechanism by which HFD can increase hepatic TG content. We found no significant differences in plasma levels of total cholesterol and triglycerides (Figure 3H and 3I) or in adipose tissue lipolysis (hormone sensitive lipase, adipose triglyceride lipase; Figure II in the online-only Data Supplement) between the 4 groups.

Effect of Increased Bone Marrow–Derived Cell Expression of Nos3 on Hepatic Insulin Resistance

Previous work from our laboratory demonstrated that the Nos3Tg mouse is protected from the development of hepatic inflammation and insulin resistance during HF feeding. To further test our hypothesis, we next asked whether the overexpression of Nos3 in bone marrow–derived cells is sufficient to attenuate the development of HF-dependent hepatic insulin resistance. For these experiments, WT mice received bone marrow from Nos3Tg mice or WT mice. Following recovery, both sets of mice were fed a LFD or HFD for 8 weeks, a time period where HF-fed WT mice develop impaired insulin-mediate Akt phosphorylation and increased hepatic NF-kB signaling.

In response to the HFD, both the WT/WT and Nos3Tg/WT mice gained more weight than the LF-fed mice (Figure 4A); however, food intake was similar between the 4 groups (Figure 4B). As expected, the HF-fed WT/WT mice exhibited increased fasting insulin and increased HF-mediated NF-kB activation (Figure 4B and 4E), whereas the mice with increased Nos3 expression in the bone marrow–derived cells were protected from these HF-mediated changes. HF-mediated impairment of insulin-mediated Akt phosphorylation was reversed in mice with overexpression of Nos3 in bone marrow–derived cell lines (Figure 4F). Overexpression of Nos3 in the bone marrow cells also reduced HF-mediated increases in liver TG content (Figure 4G), in the absence of significant changes in plasma cholesterol or triglycerides. These data suggest that overexpression of Nos3 in the bone marrow compartment is sufficient to attenuate the deleterious effects of HF feeding on hepatic insulin signaling.

Role of Nos3 in Regulating M1 and M2 Responses

HF feeding is associated with accumulation of tissue macrophages in adipose tissue and in the liver with evidence for increased proinflammatory (M1) and decreased anti-inflammatory (M2) responses in these macrophages. We next asked whether the absence of Nos3 would alter macrophage responses to M1 or M2 stimulation. Bone marrow–derived macrophages were obtained from WT and Nos3−/− deficient mice and following differentiation, were treated with LPS (100 ng/mL) for 18 hours (M1) or IL-4 (10 ng/mL) for 18 hours (M2).
LPS stimulation results in known inflammatory responses including activation of NF-κB (inflammatory cytokine production), increased iNOS (inducible nitric oxide synthase) or Nos2 expression and increased iNOS-dependent NO production (involved in nitrosylation of proteins mediating electron transport) and increased

![Figure 3](image-url). Effect of Nos3 (nitric oxide synthase 3) deletion in bone-derived cells on hepatic insulin signaling. Insulin-dependent signaling was assessed following intraperitoneal injection of insulin (0.6 U/g body weight). A–C, Liver protein lysates were analyzed for IRS-1 tyrosine phosphorylation [p-IRS-1], IRS-1, IRS-2 tyrosine phosphorylation [p-IRS-2], IRS-2, Akt serine phosphorylation [p-Akt], and Akt levels by ELISA). D, PCK-1 expression as measured by qPCR. E, Hepatic TG levels. F and G, Srebp1c and Scd1 expression as measured by qPCR. H, Plasma cholesterol. I, Plasma triglyceride. * P<0.05 (n=7–10 per group). Ins indicates insulin; and veh, vehicle.
expression of HIF1α (hypoxic inducible factor-1). Comparing these responses in the WT versus Nos3-deficient BMDMs revealed that Nos3 deficiency enhances the induction of these proinflammatory responses (Figure 5A through 5D).

As the biological effect of IL-4 receptor stimulation involves activation of the IL-4/STAT6 pathway, we evaluated whether signaling via this pathway is disrupted in BMDM from Nos3−/− mice. Consistent with this hypothesis, treatment of WT BMDM with IL-4 increased STAT6 phosphorylation (Figure 5E), but this effect was attenuated in Nos3-deficient macrophages. Other markers of an anti-inflammatory macrophage phenotype, increased Arg1 (Arginase 1) expression, increased AMPK (adenosine 5'-monophosphate-activated protein kinase) phosphorylation, and PFKFB1 (6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 1) expression were also attenuated in the Nos3-deficient macrophages compared with the WT controls (Figure 5F through 5H). Collectively, these data suggest that the

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**Figure 4.** Effect of Nos3 (nitric oxide synthase 3) overexpression in bone marrow–derived cells on hepatic inflammation and insulin resistance.

C57BL/6 wild-type (WT) mice were irradiated and bone marrow reconstituted with either WT or Nos3Tg bone marrow creating 2 groups of chimeric mice: WT-WT (WT bone marrow transplanted into WT mice) and Nos3Tg/WT (Nos3Tg bone marrow transplanted into WT mice). After recovery from the bone marrow transplant, the chimeras were placed on a low-fat or high-fat diet for 8 wks. A, Body weight (g). B, Food intake. C, Fasting glucose. D, Fasting insulin levels. E, Liver lysates were analyzed for p65 phosphorylation and total p65 levels by Western blot. F, IL-6 levels by qPCR. G, Vehicle or insulin (0.6 U/g body weight) was injected intraperitoneally and fold increase of pAKT/AKT were measured by ELISA. H, Liver triglyceride levels. I, Plasma cholesterol. J, Plasma triglyceride. *P<0.05, n=7 to 10 for each group.
absence of Nos3 in bone marrow–derived cells reduces M2 responses and increases M1 responses.

DISCUSSION

In the present study, cross transplanted chimeric mice demonstrate an unexpected role for bone marrow–derived cell Nos3 in the development of hepatic insulin resistance. The absence of bone marrow–derived cell Nos3 is associated with fasting insulinemia, hepatic NF-κB activation, and impaired insulin-mediated IRS-2/Akt signaling following a 5-week LFD, whereas overexpression of bone marrow–derived cell Nos3 is associated with attenuation of HF-mediated hepatic NF-κB activation and impaired insulin-mediated IRS-2/Akt signaling following a 5-week HFD. Furthermore, these data suggest that endothelial Nos3 is unable to compensate for the depletion of bone marrow–derived cell Nos3 in terms of reduction in hepatic NF-κB activation or impaired insulin-mediated signaling. These studies demonstrate a critical role of bone marrow–derived Nos3 during the development of hepatic insulin resistance.

A body of work suggests that insufficient levels of Nos3-derived NO relates directly to defects in metabolism in key peripheral tissues such as the liver. Our laboratory has previously shown that the global absence of Nos3 is associated with impaired insulin-mediated signaling in hepatic tissue, increased hepatic NF-κB activation, and increased proinflammatory Kupffer cell activation. These responses occurred in the absence of a HF-stimulus, which is required in control, WT mice, and suggests that the global absence of Nos3 is sufficient to cause hepatic insulin resistance. Conversely, the 4- to 5-fold global overexpression of Nos3 in mice (Nos3 Tg mice) attenuates HF-mediated impairment of hepatic insulin signaling, activation of NF-κB and inflammatory effects on Kupffer cells. Others have shown that the global deletion of Nos3 has profound metabolic effects on the liver. The Nos3 knockout mice demonstrates reduced hepatic fat oxidation, increased hepatic lipid synthesis, and increased hepatic lipid content compared with WT mice. Hepatic glucose production as measured by clamp studies was reduced in the Nos3 knockout mice without evidence for hyperglycemia. Systemically, the absence of global Nos3 is associated with elevated plasma levels of cholesterol, triglyceride, and free fatty acids compared with WT mice.

The original goal of the current work was to determine the cellular source of Nos3 with the expectation that endothelial Nos3, not bone marrow–derived cell Nos3, would drive the metabolic phenotype. We unexpectedly found that bone marrow–derived cell Nos3 recapitulates many of the metabolic findings described in the global Nos3 mice. We found increased fasting insulin levels,
increased hepatic TG content, and impairment of hepatic insulin signaling at the level of IRS-1, IRS-2, Akt phosphorylation and failure to suppress gluconeogenesis in mice deficient of Nos3 in the bone marrow compartment. We, however, did not see differences in hepatic lipid synthesis, plasma levels of cholesterol and triglycerides or in adipose lipolysis in the control or in the Nos3-deficient mice. There are few potential explanations.

The HFD or diet-induced obesity model of insulin resistance relies on both the time on the diet and the fat content to induce obesity and the subsequent development of insulin resistance. We and others have shown that insulin-sensitive tissues such as muscle, liver, and adipose tissue have different susceptibility to the HFD. Adipose tissue requires up to 14 weeks of HFD whereas liver tissue only require 8 weeks. The current work demonstrates that the absence of Nos3 decreases the necessary time on the diet (now only 4–5 weeks) and also that lowered fat content of the diet (now LFD) are sufficient to impair insulin signaling in hepatic tissue. The regulation of lipid metabolism and development of fatty liver require insulin signaling via Akt resulting in the activation of mTOR and lipogenic transcription factors. It is also known that impairment of hepatic insulin signaling occurs earlier than the development of systemic insulin resistance and the development of hepatosteatosis, thus a longer time period on the LFD is necessary for changes in hepatic lipid synthesis to become evident in our model.

In other insulin sensitive tissues, such as adipose and skeletal muscle from mice fed a LFD for 5 weeks (Figure III through V in the online-only Data Supplement), the absence of Nos3 and only the LFD stimulus was not sufficient to impair insulin signaling. However, in the presence of HFD for 5 weeks, the absence of Nos3 in the bone marrow compartment was sufficient to increase adipose NF-κB activation and impair insulin-mediated Akt signaling (Figure VB and VC in the online-only Data Supplement). These findings are consistent with the idea that the absence of Nos3 lowers the threshold for the development of impaired insulin signaling.

Since previous studies utilized global deletion or overexpression of Nos3, the cellular source of Nos3/NOS3 remained unanswered. Nos3 has traditionally been thought of as an endothelial cell-specific enzyme and the dogma has been that most of the metabolic effects were dependent on endothelial-specific Nos3. Most bone marrow-derived cells including leukocytes, RBCs, and platelets have been shown to contain Nos3, and the biologic significance of Nos3 expression in bone marrow-derived cells is increasingly recognized. For example, Nos3/NO expression and activity in bone marrow-derived cells has important effects on blood pressure and during ischemia reperfusion, functions which have been previously attributed to endothelial cell-expressed Nos3. The current study identifies another potentially key role for bone marrow–derived Nos3 in maintaining hepatic insulin sensitivity and supports the idea that bone marrow–derived Nos3 is involved in responses previously attributed to endothelial specific Nos3.

The mechanism by which Nos3 attenuates the development of insulin resistance remains an important question. Some have argued that endothelial specific Nos3 could alter metabolism by altering blood flow to muscle or insulin sensitive tissue, thus providing a mechanistic explanation for Nos3’s role in metabolic regulation. Others have proposed that Nos3 and NO attenuate the activation of NF-κB signaling, a critical step in the development of insulin resistance. In this model, endothelial cell specific Nos3 and NO reduces tissue macrophage activation in liver and adipose tissue via a paracrine effect. Coculture studies involving endothelial cells and bone marrow–derived WT macrophages seem to support this model. Reduction of endothelial-dependent NO production (by reducing endothelial Nos3 expression) was associated with increased LPS-dependent NF-κB activation compared macrophages cocultured with endothelial cells with normal Nos3 expression. Furthermore, the addition of NO donors in BMDM culture was associated with reduced LPS-dependent NF-κB activation. These previous experiments, however, do not exclude the possibility that endogenous macrophage Nos3 may play a significant role in attenuating inflammatory activation of NF-κB.

Macrophages undergo phenotypic changes in response to various environmental signals that can dramatically alter their function. The clinical relevance of this phenomenon stems in part from accumulating evidence that proinflammatory (M1) polarization of macrophages contributes to metabolic impairment associated with obesity and diabetes mellitus. Thus, deletion of proinflammatory M1 tissue macrophages normalizes sensitivity to insulin in obese mice, while conversely, reduction of anti-inflammatory M2 macrophages predisposes lean mice to the development of insulin resistance, implying a critical role for M2 polarization of macrophages in metabolic homeostasis. For example, although the IL-4/STAT6 immune axis is essential in helminth immunity and allergy, disruption of STAT6 also decreases insulin action and enhances peroxisome proliferator-activated receptor driven program of oxidative metabolism. Our current study reveals a previously unrecognized role of endogenous macrophage Nos3 in the regulation of M2 signaling through its effect on STAT6 signaling. Together, these findings provide compelling evidence that under physiological conditions, maintenance of macrophage M2 polarization depends upon sufficient bone marrow–derived NO signaling.

In addition, activation of AMPK has been reported to prevent proinflammatory cytokine production by macrophages, whereas AMPK β1 subunit loss
enhances adipose tissue macrophage inflammation and hepatic insulin resistance during HF feeding. Indeed, we found that the loss of Nos3 in macrophages is associated with reduced AMPK activity in response to IL-4 (Figure 5), which is consistent with previous results.

The current work (Figure 5) demonstrates that the absence of Nos3 in macrophages increases proinflammatory and decreases anti-inflammatory responses, suggesting one potential mechanism by which bone marrow–derived Nos3 may modulate metabolic homeostasis. Other potential mechanisms may involve the known effect of NO to alter gene expression by epigenetic regulation through histone modification, and recent studies implicate this type of mechanism in the control of macrophage polarization.

Finally, erythrocytes are highly abundant cells and the major role of these cells is respiratory gas exchange; however, their potential contribution in immune responses, and it is possible that RBC Nos3 may contribute to these responses and is an area of active investigation.

In summary, the present study provides the first in vivo evidence that circulating blood Nos3 is a critical regulator of the development of hepatic insulin resistance and hyperinsulinaemia during HF feeding.

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Affiliations
From the Department of Medicine, University of Washington, Seattle (B.P.D., R.M., L.B., Y.L., J.C.S., Y.C.F., M.R.), and Ben May Department for Cancer Research, University of Chicago, IL (L.B.).

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Disclosures
None.

REFERENCES
21. Sag D, Carling D, Stout RD, Suttles J. Adenosine 5’-monophosphate-activated protein kinase promotes macrophage polarization to an


35. Li Q, Sarna SK. Nitric oxide modifies chromatin to suppress ICAM-1 expression during colonic inflammation. *Am J Physiol Gastrointest Liver Physiol* 2012;303:G103–G110. doi: 10.1152/ajpgi.00381.2011


