iNOS induction underlies lipid-induced hepatic insulin resistance in mice: Potential role of tyrosine nitration of insulin signaling proteins

Running Title: iNOS and lipid-induced insulin resistance.

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**Objective:** The present study was undertaken to assess the contribution of inducible nitric oxide synthase (iNOS) to lipid-induced insulin resistance *in vivo.*

**Research Design and Methods:** Wild-type (WT) and iNOS−/− mice were infused for 6h with a 20% intralipid emulsion during which a hyperinsulinemic-euglycemic clamp was performed.

**Results:** In WT mice, lipid infusion led to elevated basal hepatic glucose production (GP) and marked insulin resistance as revealed by impaired suppression of liver GP and reduced peripheral glucose disposal (Rd) during insulin infusion. Liver insulin resistance was associated with a robust induction of hepatic iNOS, reduced tyrosine phosphorylation of IRβ, IRS-1 and IRS-2 but elevated serine phosphorylation of IRS proteins as well as decreased Akt activation. The expression of gluconeogenic enzymes Pepck and G6Pc was also increased in the liver of WT mice. In contrast to their WT counterparts, iNOS−/− mice were protected from lipid-induced hepatic and peripheral insulin resistance. Moreover, neither the phosphorylation of insulin signaling intermediates nor expression of gluconeogenic enzymes were altered in the lipid-infused iNOS−/− mice compared to their saline-infused controls. Importantly, lipid infusion induced tyrosine nitration of IRβ, IRS-1, IRS-2 and Akt in WT mice but not in iNOS−/− animals. Furthermore, tyrosine nitration of hepatic Akt by the NO derivative peroxynitrite blunted insulin-induced Akt tyrosine phosphorylation and kinase activity.

**Conclusions:** These findings demonstrate that iNOS induction is a novel mechanism by which circulating lipids inhibit hepatic insulin action. Our results further suggest that iNOS may cause hepatic insulin resistance through tyrosine nitration of key insulin signaling proteins.
In recent years the complex interplay occurring between immunity and energy metabolism has become increasingly evident as a growing number of factors once thought to be of sole importance for immune function have been shown to play essential roles in the regulation of glucose and lipid metabolism (1,2). One such molecule is the inducible nitric oxide synthase otherwise known as iNOS. First identified for its vital role in immunity, iNOS is now known to be expressed in metabolic tissues under various conditions of metabolic stress and has been implicated in the pathogenesis of obesity-linked insulin resistance (IR) and beta-cell failure (1,2). Our laboratory first demonstrated that genetic deletion of iNOS protects against high-fat diet (HFD)-induced IR (1). We found that protection against obesity-linked IR in skeletal muscle was sufficient to improve whole body insulin sensitivity and glucose tolerance in high-fat fed iNOS \textsuperscript{-/-} mice (KO). This was linked to the normalization of the insulin-induced PI3-kinase/Akt pathway in muscle of obese KO mice as compared to their WT counterparts. Further studies confirmed that iNOS is a potential target for alleviating the adverse effects of obesity on insulin's glucoregulatory actions and vascular IR (3,4). Our group has also shown that iNOS-derived nitric oxide (NO) not only influences the activity of proximal components of the insulin signaling pathway (1,5,6) but also modulates the transcription of metabolic genes through the intricate regulation of PPAR\textgamma activity (7).

Additional examples of immuno-metabolic crossovers are apparent in the mechanisms by which free fatty acids (FFA) induce IR. Indeed, short-term lipid infusion can impede insulin action in liver and skeletal muscle along with an accompanying inflammatory response (8). Recent studies suggest that elevated circulating FFA may exert their pro-inflammatory and insulin desensitizing effects by binding to toll-like receptors (9,10,11). This interaction activates intracellular inflammatory signaling pathways that impinge on key-components of the insulin signaling cascade. Although our understanding of the inflammatory mechanisms underlying FFA-induced IR has evolved in recent times, the detrimental role of iNOS in this metabolic impairment has yet to be investigated.

FFA-induced hepatic IR is often characterized by multiple serine and tyrosine phosphorylation defects on insulin receptor \textbeta (IR\textbeta), IRS-1, IRS-2 and Akt. These alterations translate into increase hepatic GP due to an overactivation of phosphoenulpyruvate carboxykinase (Pepck) and glucose-6-phosphatase (G6pc). These enzymes are transcriptionally regulated by insulin via peroxisome proliferator-activated receptor-\gamma coactivator-1-alpha (PGC-1\alpha) and Forkhead-box-O1 transcription factor (FoxO1) (12). Interestingly, no studies have explored the possible link between iNOS and the transcriptional regulation of GP in liver IR.

In the present study, we tested the hypothesis that hepatic iNOS induction underlies lipid-induced IR in liver and skeletal muscle. Using a murine model of short-term lipid infusion to create conditions where FFA alone are sufficient to induce IR, we present data that clearly support a causal role for hepatic iNOS in lipid-induced alterations in glucose metabolism and insulin action \textit{in vivo}. Our results also uncover iNOS-dependent tyrosine nitration of IR\textbeta, IRS and Akt proteins as a potential novel mechanism whereby lipids regulate hepatic insulin action.

**RESEARCH DESIGN AND METHODS**

**Animals.** Experiments were performed using 8-12wk old male C57BL/6
mice (WT) and iNOS−/− (KO) (Nos2tm1Lau;C57BL/6-backcrossed) purchased from Jackson Laboratory (Ann Harbor, MI). Animals were maintained in pathogen-free conditions and housed individually in a temperature controlled environment under a 12h light cycle. Animals were placed on a rodent diet (Harlan Teklad, WI) and given free access to food and water. All protocols were in accordance with the Canadian Council on Animal Care.

**Paired infusion hyperinsulinemic-euglycemic clamp (HIEC) procedure.** WT and KO mice were randomly assigned to either saline (SAL) or 20% Intralipid® (20% soybean oil, 1.2% egg phospholipids, 2.2% glycerin) (LIP) infusion groups. Five days prior to the experiment, mice were anesthetized and catheters inserted into the left common carotid artery and the right jugular vein for blood sampling and infusions respectively. The free catheter ends were tunneled under the skin, externalized at the neck and sealed. Mice were fasted for 5h before the clamp procedure. The i.v. infusion catheters were connected to a swivel 1h prior to the infusion and the mice were unrestrained and not handled thereafter to minimize stress. Before the onset of the infusion protocol (t=−10min), blood sample was obtained to determine pre-infusion FFA levels. At t=0min, the 6h SAL (5ml.kg−1.h−1) or LIP-infusion protocol (5ml.kg−1.h−1) (Baxter Corporation, ON, Canada) with 20IU.ml−1 heparin (LEO Pharma®, ON, Canada) was initiated (t=0 to 360min). 2.5h into the infusion (t=150min), the HIEC was initiated according to previously described methodology (13). At the end of the clamp, mice were anesthetized, cardiac punctures was performed, and liver and hindlimb muscles were excised, freeze-clamped, and stored at −80°C until further analyses.

**Calculations.** Hepatic GP and peripheral Rd were determined using Mari’s non–steady-state equations (14). Clamp GP was determined by subtracting the glucose infusion rate (GIR) from total GP.

**Akt kinase assay.** Procedures were performed according to Tremblay et al. (15) as detailed in Supplementary Methodology which can be found in an online appendix at [http://diabetes.diabetesjournals.org](http://diabetes.diabetesjournals.org).

**Protein extraction, immunoprecipitation and immunoblotting analyses.** Livers and gastrocnemius muscles were processed as previously described (7) and detailed in Supplementary Methodology and Supplementary Table 1.

**RNA extraction and Q-PCR.** Total RNA was isolated from liver samples as previously described (7) as detailed in Supplementary Methodology.

**Analytical methods.** Plasma insulin was assessed by RIA (Linco, St-Charles, MI). FFA were measured by colorimetric assay (Wako Chemicals, Richmond, VA). NO2− concentrations were assessed with a DAN/NaOH-based kit (Cayman Chemical, Ann Arbor, MI). Liver glycogen was determined using the phenolsulphuric acid reaction (16). Plasma [3−3H]glucose was determined as previously described (13).

**Peroxynitrite treatment and hepatic Akt tyrosine nitration and phosphorylation.** WT mice were injected (i.v.) with saline or insulin (4U.kg−1) for 5min and the liver was excised and homogenized as described above. Lysates were immunoprecipitated with antibodies specific for Akt and treated with 50µM of peroxynitrite (ONOO−) or decomposed peroxynitrite (dONOO−) for 2min (Millipore, San Francisco, CA) according to previously described methodology (17,18). dONOO− was obtained by allowing peroxynitrite to decompose overnight in lysate buffer. Subsequently, lysates were subjected to immunoblot analysis as described above.

**Statistical analysis.** All data are reported as means ±SEM. Statistical
comparisons were performed using a two-way ANOVA for non-repeated measures design. Newman-Keuls post-hoc test was used in the event of a significant \((P<0.05)\) \(F\) ratio.

**RESULTS**

**Short-term LIP-infusion promotes iNOS expression and NO production in vivo.** To establish the role of iNOS in the metabolic effects of elevated FFA, we examined whether a 6h LIP-infusion was sufficient to induce iNOS expression and accumulation of the NO derivative nitrite \((\text{NO}_2^-)\) in liver and skeletal muscle. Immunoblot analysis of hepatic iNOS protein levels in WT mice showed a robust (~3 fold) induction following LIP-infusion \((P<0.05)\) compared to SAL-infused controls (Fig.1A). iNOS induction was associated with heightened \(\text{NO}_2^-\) accumulation (~2.2 fold) in the liver of LIP-infused WT mice \((P<0.05)\) (Fig.1C). This is the first report that short-term lipid exposure is sufficient to induce iNOS and NO production in liver. In contrast to liver, LIP-infusion caused non-significant iNOS induction in skeletal muscle. However, LIP-infusion increased muscle iNOS activity, as revealed by a significant accumulation of \(\text{NO}_2^-\) (~1.6 fold, \(P<0.05\)) in skeletal muscle of WT mice but not in their KO counterparts (Fig.1B-D).

**Role of iNOS in lipid-induced modulation of plasma glucose and insulin:** The glucoregulatory influence of iNOS induction in lipid-challenged WT and KO mice was determined during a HIEC. Physiological parameters measured at pre-infusion, pre-clamp and during the HIEC are shown in Table 1. No effect of iNOS genetic deletion was observed on any of the parameters measured prior to the infusion protocol. As expected, prior to the clamp, LIP-infusion led to a marked increase in plasma FFA in both WT and KO mice compared to their SAL-infused counterparts \((P<0.05)\). Insulin infusion during the clamp led to a reduction in plasma FFA in all groups. It is noteworthy that the magnitude of the LIP-induced rise in plasma FFA was comparable among the WT and KO mice. Nevertheless, lack of iNOS prevented LIP-induced elevations in both blood glucose and insulin concentrations. Indeed, LIP-infused WT mice displayed significantly elevated glycemia (pre-clamp) compared to the corresponding KO mice \((P<0.05)\). Furthermore, plasma insulin rose significantly after 150min in lipid-infused WT mice compared to their SAL-infused controls \((P<0.05)\). Since neither glycemia nor insulinemia varied among infusion groups in the KO mice, these data provide initial evidence that iNOS plays a role in the lipid-induced perturbations of glucose metabolism *in vivo*. As expected, hepatic glycogen levels were decreased in lipid-infused animals (Table 1) and this parameter was independent of the genotype.

**Genetic deletion of iNOS protects against lipid-induced insulin resistance.** The HIEC was performed in unison with the LIP-infusion to assess differences in whole-body insulin sensitivity and to define the contribution of the liver and skeletal muscle to insulin's metabolic responses. GIR, GP, Rd were assessed with arterial glycemia and insulinemia clamped at 6-7mM and 150-170uU.ml\(^{-1}\) respectively (Table 1). As suggested by the observed perturbations in pre-clamp glycemia and insulinemia, the HIEC data showed that LIP-infusion causes IR in WT mice while KO animals are protected from this effect. Indeed, the GIR required to maintain euglycemia was reduced by ~24% \((P<0.05)\) in lipid-infused WT mice compared to SAL-infused animals (Fig.2A), whereas the GIR of lipid-infused KO mice did not vary from that of their SAL-infused counterparts. In line with iNOS induction, the liver appears to be the primary site of perturbed glucose metabolism in this short-term LIP-infusion model. Indeed, basal GP
was elevated by ~45% (P<0.05) (Fig.2B) and insulin-mediated suppression of GP was impaired by ~46% (P<0.05) (Fig.2C-D) in lipid-infused WT mice. Although basal Rd was not affected by LIP-infusion, we did observe a ~15% reduction (P<0.05) in insulin-mediated Rd during the clamp in WT mice (Fig.2E-F). Importantly, KO mice were protected against lipid-induced liver and muscle IR, providing genetic evidence that iNOS is a mediator of lipid-induced IR in both tissues.

**iNOS is key to lipid-induced perturbations in hepatic insulin signaling proteins:** Since the effects of LIP-infusion on iNOS and glucose metabolism were most pronounced in liver, we chose to explore the mechanisms underlying lipid-induced IR in this tissue. We first assessed the impact of lipid-induced iNOS expression on the phosphorylation state of IRβ, IRS-1 and IRS-2, key transducers of insulin's metabolic actions in liver (19). Immunoblot analysis showed that tyrosine phosphorylation of both IRβ and IRS-2 is significantly diminished in liver of lipid-infused WT mice compared to SAL-infused controls (P<0.05) (Supplementary Fig.1A and Fig.3A). Tyrosine phosphorylation of IRS-1 also tended to be reduced in these mice (Fig.3B). Importantly, tyrosine phosphorylation of all three proteins was found to be greater in lipid-infused KO mice compared to their WT counterparts (P<0.05) (Supplementary Fig.1A and Fig.3A-B).

In line with the lipid-induced reduction in IRS-1/2 tyrosine phosphorylation, lipid-infused WT mice also displayed elevated inhibitory phosphorylation of Ser133 in IRS-2 and Ser307 in IRS-1 compared to their SAL-infused controls (P<0.05) (Fig.3C-D). In contrast to tyrosine phosphorylation, serine phosphorylation of IRS-1 and IRS-2 is known to inhibit downstream insulin signaling (20). Interestingly, KO mice were completely protected from these detrimental effects of LIP-infusion.

To determine whether the perturbations in phosphorylation of IRβ, IRS-1 and IRS-2 translated in reduced downstream signaling, we examined Akt Ser473/Thr308 phosphorylation and kinase activity. We found that Akt phosphorylation on Ser473 and Thr308 as well as Akt kinase activity were reduced in lipid-infused WT mice compared to SAL-infused controls (P<0.05) (Supplementary Fig.2A and Fig.3E-F). Genetic deletion of iNOS partially, but not significantly, restored Akt Thr308 phosphorylation in lipid-infused animals while fully reversing impairments in both Akt Ser473 phosphorylation and kinase activity, further suggesting a key role for iNOS in the lipid-mediated inhibition of hepatic insulin signaling.

**iNOS is involved in the regulation of lipid-induced elevations of hepatic gluconeogenesis:** To gain a more precise understanding of the role of iNOS in lipid-induced elevations in GP, we studied transcriptional regulators of hepatic gluconeogenesis, PGC1α and FoxO1. In line with the elevated GP witnessed during the clamp, PGC1α protein content was increased in lipid-infused WT mice compared to SAL-infused controls (P<0.05) (Fig.4A). Importantly, iNOS genetic disruption prevented the rise in PGC1α protein expression in the lipid-infused KO mice (P<0.05) (Fig.4A). Phosphorylation of FoxO1 by Akt in response to insulin is known to abolish FoxO1 binding to PGC1α leading to reduced expression of the gluconeogenic genes Pepck and G6Pc (12). We found that FoxO1 phosphorylation on Ser256, a marker of its nuclear exclusion and inactivation by Akt, was decreased in lipid-infused WT mice as compared to SAL-infused controls (P<0.05) (Fig.4B). However, this was not observed in KO animals where FoxO1 Ser256 phosphorylation was significantly increased.
in liver of lipid-infused KO mice compared to their WT counterparts.

In line with the regulation of PGC1α and FoxO1, LIP-infusion increased the expression of Pepck and G6Pc transcripts in WT mice compared to the SAL-infused controls (P<0.05) (Fig.4C-D). LIP-infusion also increased Pepck and G6Pc mRNA expression in KO mice but to a lesser extent as compared to their lipid-infused WT counterparts. Taken together, these data are the first to demonstrate a role for iNOS in the transcriptional regulation of hepatic gluconeogenesis in conditions of lipid excess.

iNOS modulates GP through tyrosine nitration of IRβ, IRS and Akt proteins: To further explore the mechanisms underlying hepatic IR in lipid-infused mice, we assessed whether insulin signaling proteins were modified through iNOS-mediated protein tyrosine nitration. Tyrosine nitration is a covalent post-translational protein modification that is derived from the reaction of proteins with nitrating agents such as ONOO−, a potent oxidant derivative of NO (21). Tyrosine nitration has been used as a biomarker of nitrosative stress in several pathological conditions including IR (21,22). Growing evidence suggest that this modification can alter protein function by preventing functional phosphorylation (23,24,25). Since iNOS induction promotes the generation of NO-derived ONOO−, we hypothesized that IRS-1/2 tyrosine nitration could underlie iNOS-dependent inhibition of IRS-1/2 tyrosine phosphorylation and hepatic IR in lipid-challenged mice.

Since Akt activity is also regulated by the phosphorylation of key tyrosine residues (26) we also assessed whether Akt is tyrosine nitrated in lipid-infused WT mice. Immunoblot analysis of IRβ, IRS-1, IRS-2 and Akt immunoprecipitates with a 3-nitrotyrosine antibody revealed that tyrosine nitration of all four proteins were significantly increased in liver of lipid-infused WT mice as compared to SAL-infused controls (P<0.05) (Supplementary Fig.1B and Fig.5A-C). Akt tyrosine nitration was associated with impaired kinase tyrosine phosphorylation (Fig.5D). Notably, iNOS gene disruption significantly reduced IRβ, IRS-1 and Akt tyrosine nitration in liver of lipid-infused mice suggesting that the inability of insulin to suppress GP in lipid-infused WT mice is linked to iNOS-dependent tyrosine nitration of key insulin signaling proteins.

ONOO− inhibits insulin-stimulated Akt activity through tyrosine nitration: We next determined whether treatment of liver Akt with ONOO− ex vivo could trigger Akt tyrosine nitration and impede Akt activity as seen in liver extracts from lipid-infused WT mice. Akt was immunoprecipitated from liver lysates of saline or insulin-treated mice and incubated with ONOO− in vitro for 2min. ONOO− treatment was found to increase Akt tyrosine nitration while decreasing Akt Tyr/Ser473 phosphorylations and Akt kinase activity (Fig.6A-D). Importantly, these effects were not observed in control experiments with dONOO−. It is noteworthy that although ONOO− treatment significantly decreased phosphorylation of Akt Ser473, it did not affect phosphorylation of Akt Thr308 (Supplementary Fig. 3C) suggesting that the reactive NO derivative does not interact non-specifically with the hydroxyl moiety of any residues. These data demonstrate that Akt kinase activity is modulated in a tyrosine nitration-dependent manner and strongly suggest that iNOS-mediated tyrosine nitration of Akt is a novel mechanism of hepatic IR upon LIP-infusion.

DISCUSSION

A pathogenic immune response to nutrient excess is believed to be involved in the development of obesity-linked IR (27). Indeed, lipid-induced IR in muscle and liver is linked to overactivation of inflammatory signaling pathways known to impede insulin
signal transduction (27,28,29). We and others have shown that iNOS is overexpressed in metabolic tissues of both dietary and genetic models of obesity and plays a pivotal role in the pathogenesis of IR and glucose intolerance (1,3,4,28). However, it remained unclear whether increased lipid availability, in the absence of other chronic changes that accompany the obese state, can induce iNOS expression and NO production in metabolic tissues of non-obese animals. This is key to determine whether iNOS should be considered as a primary candidate for triggering IR in obesity or as a late onset exacerbating factor in the complex inflammatory sequelae that characterizes the obese state. Using the paired-infusion HIEC procedure, we report that iNOS can be induced within a few hours in liver and to a lesser extent in muscle of lipid-infused WT mice. Using iNOS−/− mice, we further show that iNOS underlies lipid-induced IR in both liver and skeletal muscle.

The present work also unravels a previously unrecognized role for iNOS in the transcriptional regulation of hepatic gluconeogenesis. iNOS disruption restored insulin’s ability to suppress GP and prevented lipid-induced elevations in basal GP that account for the initial hyperglycemia seen in WT mice. Since, glycogen levels were unaffected by iNOS lack, this provides clear evidence that iNOS induction is involved in the regulation of hepatic gluconeogenesis and not glycogenolysis in response to lipid availability in vivo. Importantly, we were able to tie the physiological alterations of hepatic glucose metabolism following iNOS induction to the intrinsic regulation of the expression of the gluconeogenic enzymes Pepck and G6Pc. Indeed, iNOS gene disruption prevented lipid-induced increases in gluconeogenic gene expression as well as defects in their transcriptional regulators. This is the first time that iNOS is reported to be involved in the lipid-mediated increase in liver PGC1α expression, a well established mechanism of augmented gluconeogenic gene expression in altered metabolic states (12). Our finding that iNOS expression was associated with PGC1α induction may also suggest that NO can trigger an adaptive metabolic response to chronic lipid excess in liver. Indeed, it has previously been shown that NO promotes mitochondrial biogenesis by stimulating PGC1α induction (30). It is therefore conceivable that the iNOS-NO-PGC1α axis coordinates hepatic metabolism in lipid-challenged conditions through stimulation of gluconeogenesis and a later increase in mitochondriogenesis and FFA oxidation capacity.

It has been previously reported that iNOS induction in metabolic tissues and insulin target cells interferes with the IRβ/IRS/PI3K/Akt insulin signalling pathway (1,5,6,28). Accordingly, we found that iNOS impairs insulin action on GP by altering insulin signaling to IRβ, IRS-1/2 and Akt. Indeed, iNOS−/− mice were protected from lipid-induced inhibitory phosphorylation of IRS-1 Ser307 and IRS-2 Ser133, two well established target sites of ser/thr kinases known to be activated by lipids through activation of inflammatory pathways (e.g. IKK-NFkB,JNK,PKC) (8,31,32). Conversely, IRβ and IRS-1/2 tyrosine phosphorylation were preserved in liver of lipid-challenged iNOS−/− mice. Similarly, lack of iNOS prevented the lipid-induced impairment in ser/tyr phosphorylation of Akt observed in WT mice, resulting in normalization of hepatic Akt kinase activity. Normalization of IRβ and IRS-1/2 signaling and Akt activation likely explains the improved action of insulin to inhibit gluconeogenic enzymes in liver of iNOS−/− mice since phosphorylation of the key transcription factor FoxO1, which is mediated by Akt (33) was restored in lipid-infused KO mice. Taken together with the increased expression of PGC1α, these results suggest that iNOS is a key inflammatory mediator.
linking excessive lipid levels to activation of the gluconeogenic transcription programme in the liver of models of elevated FFA. In future studies, it will be interesting to determine whether iNOS increases hepatic gluconeogenic enzymes by modulating the ability of PGC1α to target O-GlcNAc transferase for GlcNAcylation of FoxO1, another mechanism by which PGC1α serves as a transcriptional coactivator of gluconeogenic enzymes (34).

The present data further suggest that iNOS activation in lipid-infused mice may impede insulin signaling through tyrosine nitration of key insulin signaling proteins. Tyrosine nitration is a major covalent post-translational protein modification and a marker of nitrosative stress in several pathological disorders associated with iNOS induction (21,35,36,37,38). However, no studies have explored whether protein tyrosine nitration regulates hepatic insulin signaling and glucose metabolism in vivo. Our results show that LIP-infusion leads to tyrosine nitration of IRβ, IRS-1 and IRS-2, as well as Akt in liver of WT mice. Importantly, iNOS−/− animals are protected from these nitrosative modifications and exhibited improved hepatic insulin sensitivity, strongly suggesting a key role for iNOS in the tyrosine nitration of IRS-1/2 and Akt proteins in lipid-induced hepatic IR.

iNOS likely promotes tyrosine nitration of IRβ, IRS-1/2 and Akt proteins through ONOO− formation (39). Indeed, although the role of ONOO− in causing protein tyrosine nitration is still debated, it is still recognized as the most efficient mechanism for nitrating tyrosine residues under biologically relevant conditions (21,25,39). In vitro studies have showed that ONOO− induces tyrosine nitration of IRS-1 interfering with its tyrosine phosphorylation and activation of downstream insulin signaling (40). To further confirm the functional impact of tyrosine nitration on the insulin signaling pathway, we exposed immunopurified Akt from liver of insulin-treated WT mice to ONOO−. We found that ONOO− robustly increased Akt tyrosine nitration, leading to reduced ser/tyr phosphorylation and inhibition of Akt kinase activity. Nitration of Tyr315 and Tyr326, which are of critical importance for Akt activation by growth factors (26), are likely targets of nitration in these studies. On the other hand, the reduction of Ser473 phosphorylation by ONOO−-mediated tyrosine nitration was somewhat unexpected. Phosphorylation of Ser473 in the hydrophobic motif (HM:site 469-474) of Akt is considered crucial for activation of the enzyme by insulin (41). Interestingly, Ser473 is immediately adjacent to a tyrosine residue positioned at site 474. It is therefore tempting to speculate that a nitrated adducts on Tyr474 could displace a phosphate on the adjacent Ser473 and contribute to defective hepatic Akt activity. Another possible mechanism that could affect Akt activation is the potential nitration of three tyrosine residues (sites 18,26,38) in the pleckstrin homology domain (PH:site 6-107) which would impede Akt translocation to the plasma membrane. Further mass spectrometry/proteomic studies are warranted to delineate the precise tyrosine residues within Akt that are targeted by iNOS-generated ONOO− and to demonstrate their functional significance in models of hepatic IR.

We considered the possibility that ONOO− might also directly nitrate serine residues but this is unlikely given that ONOO− is thought to only interact with protein tyrosyl groups in tyrosine or sulfhydryls groups within cysteine and tryptophan (42), and that neither serine nor threonine residues contain tyrosyls or thiols. Accordingly, ONOO−-exposure failed to affect Thr308 phosphorylation, a key residue for insulin-dependent Akt activation (43) that is not in close contact with any tyrosine residues. On
the other hand, we found that unlike in vitro ONOO• exposure, LIP-infusion in vivo impedes both Ser473 and Thr308 phosphorylation in WT mice while iNOS genetic deletion only partially and not significantly restored Akt Thr308 phosphorylation. This suggests that iNOS is not implicated in the mechanism leading to reduced Thr308 phosphorylation in liver of lipid-infused mice, consistent with the selective effect of ONOO• on Akt Ser473 phosphorylation in vitro. Nevertheless, Akt kinase activity and liver insulin action were fully normalized in lipid-infused iNOS KO mice indicating that only restoring Akt Ser473 phosphorylation was sufficient to prevent liver Akt dysfunction in this model of lipid-induced insulin resistance.

The present results do not rule out S-nitrosylation as an alternative mechanism negatively regulating insulin signal transduction in this model of lipid-induced insulin resistance. Indeed, iNOS-mediated S-nitrosylation of cysteine residues in key insulin signaling intermediates such as IR, IRS-1 and Akt has been previously observed in models of inflammation and obesity-related insulin resistance (28,44). Whether nitrosylation and nitration of insulin signaling proteins occur simultaneously or sequentially in different models of insulin resistance clearly warrants further investigations.

Overall these data provide genetic evidence that iNOS is a key factor in the regulation of insulin sensitivity and hepatic glucose metabolism by FFA in vivo. Our data further suggest that iNOS causes hepatic insulin resistance by impairing insulin signaling through the coordinated action of three independent mechanisms. By promoting 1) inhibitory serine phosphorylation of IRS proteins 2) tyrosine nitration of IRβ, and IRS-1/2 as well as by 3) directly impairing Akt activity through its tyrosine nitration. The latter two nitrosative modifications are potentially triggered by iNOS-linked ONOO• suggesting that limitation of ONOO• generation should be considered as a potential target for combating IR in obesity and metabolic conditions associated with excess lipid availability.

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Table 1. Basal and clamp characteristics in WT and iNOS\(^{-/-}\) mice following a 6h treatment of either saline (SAL) or i.v. infusion of intralipid (LIP)

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<td>40.0 ± 7.6(^{*})</td>
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Data are mean ± SEM. \(^{*}\) \(p < 0.05\) vs saline counter-part; \(^{†}\) \(p < 0.05\) vs WT
FIGURE LEGENDS

Figure 1: Lipid infusion increases iNOS expression and NO production in liver and skeletal muscle. Immunoblots for iNOS (A,B) and nitrite concentrations (C,D) in liver and gastrocnemius extracts of wild-type (WT) and iNOS\(^{-/-}\) (KO) mice following a HIEC performed during the last 2h of a 6h saline or lipid infusion in 5 h fasted, chronically catheterized, conscious mice at least 5 days following surgical procedure. In A and B, immunoblottings of tissue lysates were performed with iNOS antibody and densitometric analyses were normalized for the expression of tubulin content in the same sample. For (A) and (B), lanes were run on the same gel but were noncontiguous. Data are mean ±SEM. Interactions between groups; *p < 0.05 vs saline counter-part.

Figure 2: Hyperinsulinemic-euglycemic clamp characteristics in saline and lipid-infused wild-type (WT) and iNOS\(^{-/-}\) (KO) mice. Glucose infusion rate (A), basal glucose production (B), clamp glucose production (C), percentage of glucose production suppression (D), basal (E) and clamp (F) whole body glucose uptake during a HIEC performed as described in the legend to Fig. 1. Data are mean ±SEM. Interactions between groups; *p < 0.05 vs saline counter-part; †p < 0.05 vs WT.

Figure 3: Effects of lipid infusion on hepatic IRS-2, IRS-1, Akt phosphorylations and Akt kinase activity. Immunoblots for pIRS-2 (Tyr) (A), pIRS-1 (Tyr) (B), pIRS-2 (Ser133) (C), pIRS-1 (Ser307) (D), pAkt (Ser473) (E), Akt kinase activity (F) in liver extracts of wild-type (WT) and iNOS\(^{-/-}\) (KO) mice following a HIEC performed as described in the legend to Fig. 1. In A and B, IRS-2 and IRS1 were immunoprecipitated (IP) and subsequent immunoblots were performed with 4G10 antibody. Densitometric analyses were normalized for the expression of IRS-2 and IRS1 in the same sample. In C and D, immunoblottings of tissue lysates were performed with pIRS-2 (Ser133) and pIRS-1 (Ser307) antibody and densitometric analyses were normalized for the expression of tubulin content in the same sample. In F, Akt was immunoprecipitated and kinase activity was determined using [γ\(^{32}\)P]ATP as described in Methods. For A to E, lanes were run on the same gel but were noncontiguous. Data are mean ±SEM. Interactions between groups; *p < 0.05 vs saline counter-part; †p < 0.05 vs WT.

Figure 4: Effects of lipid infusion on hepatic gluconeogenesis enzymes and gene transcripts Immunoblots for PGC1α (A), pFoxO1 (Ser256) (B) and expression of mRNA for (C) phosphoenolpyruvate carboxykinase (Pepck) and (D) glucose-6-phosphatase (G6Pc) in liver extracts of wild-type (WT) and iNOS\(^{-/-}\) (KO) mice following a HIEC performed as described in the legend to Fig. 1. In A and B, immunoblottings of tissue lysates were performed with PGC1α and pFoxO1 (Ser256) antibody and densitometric analyses were normalized for the expression of tubulin and FoxO1 content in the same sample respectively. Lanes were run on the same gel but were noncontiguous. In C and D, Pepck and G6Pc mRNA expressions were evaluated by quantitative RT-PCR and values were corrected for house-keeping β-actin transcript levels. Data are mean ±SEM. Interactions between groups; *p < 0.05 vs saline counter-part; †p < 0.05 vs WT.

Figure 5: Effects of lipid infusion on hepatic IRS-2, IRS-1, Akt tyrosine nitration and Akt tyrosine phosphorylation. Immunoblots for IRS-2 nitration (Tyr) (A), IRS-1 nitration (Tyr) (B), Akt nitration (Tyr) (C) and Akt tyrosine phosphorylation in liver extracts of wild-type (WT) and iNOS\(^{-/-}\) (KO) mice following a HIEC performed as described in the legend to Fig. 1. In A-D,
IRS-2, IRS1 and Akt were immunoprecipitated (IP) and subsequent immunoblots were performed with 3-NT antibody for IRS-2 (A), IRS-1 (B), and either 3-NT (C) or 4G10 (D) for Akt. Densitometric analyses were normalized for the expression of IRS-2, IRS1, Akt and tubulin in the same sample. For C, lanes were run on the same gel but were noncontiguous. Data are mean ±SEM. Interactions between groups; *p < 0.05 vs saline counter-part; †p < 0.05 vs WT.

**Figure 6: Effects of peroxynitrite on insulin-stimulated Akt activity**

5h fasted wild-type (WT) mice were treated for 5 min with either saline or insulin (tail i.v.-4 U.kg⁻¹). In A-C, following saline or insulin treatment, Akt from liver lysates was immunoprecipitated and incubated for 2 min with peroxynitrite (ONOO⁻) (50µM) or an equimolar amounts of decomposed ONOO⁻ (dONOO⁻). Subsequent immunoblots were performed with 3-NT, 4G10 and pAkt (Ser473) antibody for Akt. In D, following incubation, Akt kinase activity was determined using [γ⁻³²P]ATP as described in Methods. For (A) and (B), lanes were run on the same gel but were noncontiguous. Data are mean ±SEM. Interactions between groups; *p < 0.05 vs saline counter-part; †p < 0.05 vs WT.
Figure 1

A
IB:iNOS
IB:Tubulin
Liver iNOS protein level (Arbitrary Units)

B
IB:iNOS
IB:Tubulin
Muscle iNOS protein level (Arbitrary Units)

C
Liver NO$_2^-$ (pmol.g$^{-1}$)

D
Muscle NO$_2^-$ (pmol.g$^{-1}$)

WT KO WT KO
SAL LIP SAL LIP

- 130 kDa
- 52 kDa
- 130 kDa
- 52 kDa

* p < 0.05
† p < 0.01
Figure 2

A

B

C

D

E

F

iNOS and lipid-induced insulin resistance
**Figure 3**

- **A** and **B**: Comparison of AKT kinase activity between WT and KO mice under saline (SAL) and lipopolysaccharide (LIP) conditions.

- **C** and **D**: Comparison of IRS-2 and IRS-1 phosphorylation levels at Tyr and Ser307 between WT and KO mice under saline and LIP conditions.

- **E** and **F**: Comparison of AKT phosphorylation at Ser473 between WT and KO mice under saline and LIP conditions.
Figure 4

A
IB: PGC1α
IB: Tubulin

B
IB: pFoxO1
IB: FoxO1

C

D

PGC1α Protein Level
(Arbitrary Units)

p Foxo1 (Ser256)
(Arbitrary Units)

Pepck mRNA levels
(Arbitrary Units)

G6Pc mRNA levels
(Arbitrary Units)

WT
KO
SAL
LIP
WT
KO
SAL
LIP
WT
KO
SAL
LIP
WT
KO
SAL
LIP
WT
KO
SAL
LIP

*  
†
Figure 5

A

IB:3-NT

IB:IRS2

IRS2 Nitration (Tyr) (Arbitrary Units)

WT KO

WT KO

B

IB:3-NT

IB:IRS1

IRS1 Nitration (Tyr) (Arbitrary Units)

WT KO

WT KO

C

IB:3-NT

IB:AKT

AKT Nitration (Tyr) (Arbitrary Units)

WT KO

WT KO

D

IB:4G10

IB:AKT

P-AKT (Tyr) (Arbitrary Units)

WT KO

WT KO
iNOS and lipid-induced insulin resistance

Figure 6

A

IB:3-NT
IB:AKT

AKT Nitration (Tyr)
(Arbitrary Units)

0 1 2 3

\( \text{dONOO}\text{-ONOO}^- \) \( \text{SAL} \)
\( \text{dONOO}\text{-ONOO}^- \) \( \text{INS} \)

†

B

IB:4G10
IB:AKT

P AKT (Tyr)
(Arbitrary Units)

0 1 2 3

\( \text{dONOO}\text{-ONOO}^- \) \( \text{SAL} \)
\( \text{dONOO}\text{-ONOO}^- \) \( \text{INS} \)

* †

C

IB:pAKT
IB:AKT

P AKT (Ser473)
(Arbitrary Units)

0 1 2 3

\( \text{dONOO}\text{-ONOO}^- \) \( \text{SAL} \)
\( \text{dONOO}\text{-ONOO}^- \) \( \text{INS} \)

* †

D

Akt Kinase Activity
(Arbitrary Units)

0 1 2 3

\( \text{dONOO}\text{-ONOO}^- \) \( \text{SAL} \)
\( \text{dONOO}\text{-ONOO}^- \) \( \text{INS} \)

* †