

Inducible Nitric Oxide Synthase Induction Underlies Lipid-Induced Hepatic Insulin Resistance in Mice

Potential Role of Tyrosine Nitration of Insulin Signaling Proteins

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OBJECTIVE—The present study was undertaken to assess the contribution of inducible nitric oxide (NO) synthase (iNOS) to lipid-induced insulin resistance in vivo.

RESEARCH DESIGN AND METHODS—Wild-type and iNOS^{-/-} mice were infused for 6 h with a 20% intralipid emulsion, during which a hyperinsulinemic-euglycemic clamp was performed.

RESULTS—In wild-type mice, lipid infusion led to elevated basal hepatic glucose production and marked insulin resistance as revealed by impaired suppression of liver glucose production and reduced peripheral glucose disposal (R_d) during insulin infusion. Liver insulin resistance was associated with a robust induction of hepatic iNOS, reduced tyrosine phosphorylation of insulin receptor (IR) β , insulin receptor substrate (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 wild-type mice. In contrast to their wild-type 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 with their saline-infused controls. Importantly, lipid infusion induced tyrosine nitration of IR β , IRS-1, IRS-2, and Akt in wild-type 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. *Diabetes* 59:861–871, 2010

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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 (NO) synthase (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 and β -cell failure (1,2). Our laboratory first demonstrated that genetic deletion of iNOS protects against high-fat diet (HFD)-induced insulin resistance (1). We found that protection against obesity-linked insulin resistance in skeletal muscle was sufficient to improve whole-body insulin sensitivity and glucose tolerance in high-fat-fed iNOS^{-/-} mice (knockout). This was linked to the normalization of the insulin-induced phosphoinositide 3-kinase (PI3K)/Akt pathway in muscle of obese knockout mice as compared with their wild-type counterparts. Further studies confirmed that iNOS is a potential target for alleviating the adverse effects of obesity on insulin's glucoregulatory actions and vascular insulin resistance (3,4). Our group has also shown that iNOS-derived 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 peroxisome proliferator-activated receptor γ activity (7).

Additional examples of immuno-metabolic crossovers are apparent in the mechanisms by which free fatty acids (FFAs) induce insulin resistance. Indeed, short-term lipid infusion can impede insulin action in liver and skeletal muscle along with an accompanying inflammatory response (8). Recent studies (9–11) suggest that elevated circulating FFAs may exert their proinflammatory and insulin-desensitizing effects by binding to toll-like receptors. 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 insulin resistance has evolved in recent times, the detrimental role of iNOS in this metabolic impairment has yet to be investigated.

FFA-induced hepatic insulin resistance is often characterized by multiple serine and tyrosine phosphorylation defects on insulin receptor β (IR β), IRS-1, IRS-2, and Akt.

These alterations translate into increased hepatic glucose production due to an overactivation of phosphoenolpyruvate carboxykinase (Pepck) and glucose-6-phosphatase (G6pc). These enzymes are transcriptionally regulated by insulin via peroxisome proliferator-activated receptor- γ coactivator (PGC)-1- α and Forkhead box-O1 transcription factor (FoxO1) (12). Interestingly, no studies have explored the possible link between iNOS and the transcriptional regulation of glucose production in liver insulin resistance.

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

RESEARCH DESIGN AND METHODS

Experiments were performed using 8- to 12-week-old male C57BL/6 mice (wild-type) and iNOS^{-/-} (knockout) mice (Nos2tm1Lau;C57BL/6 backcrossed) purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were maintained in pathogen-free conditions and housed individually in a temperature-controlled environment under a 12-h light cycle. Animals were placed on a rodent diet (Harlan Teklad, Madison, 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 procedure. Wild-type and knockout mice were randomly assigned to either saline or 20% intralipid (20% soybean oil, 1.2% egg phospholipids, and 2.2% glycerin) 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 5 h before the clamp procedure. The intravenous infusion catheters were connected to a swivel 1 h prior to the infusion, and the mice were unrestrained and not handled thereafter to minimize stress. Before the onset of the infusion protocol ($t = -10$ min), blood samples were obtained to determine preinfusion FFA levels. At $t = 0$ min, the 6-h saline ($5 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$) or intralipid ($5 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$) (Baxter, Ontario, Canada) infusion protocol with 20 IU/ml heparin (LEO Pharma, Ontario, Canada) was initiated ($t = 0-360$ min). Two and a half hours into the infusion ($t = 150$ min), the hyperinsulinemic-euglycemic clamp (HIEC) was initiated according to previously described methodology (13). At the end of the clamp, mice were anesthetized, cardiac punctures were performed, and liver and hindlimb muscles were excised, freeze-clamped, and stored at -80°C until further analyses.

Calculations. Hepatic glucose production and peripheral R_d were determined using Mari's non-steady-state equations (14). Clamp glucose production was determined by subtracting the glucose infusion rate (GIR) from total glucose production.

Akt kinase assay. Procedures were performed according to Tremblay et al. (15) as detailed in the supplementary methodology, which can be found in the online appendix (available at <http://diabetes.diabetesjournals.org/cgi/content/full/db09-1238/DC1>).

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 (online appendix).

RNA extraction and Q-PCR. Total RNA was isolated from liver samples as previously described (7) as detailed in the supplementary methodology. Briefly, *G6pc*, *Pepck*, and β -*actin* were measured using 50 ng of each of the following primer pairs: *G6pc*: 5'-GCTTGGATTCTACCTGCTAC-3' and 5'-AAAGACTTCTTGTGTGTCTGTC-3'; *Pepck*: 5'-CCACAGCTGCTGCAGAACA-3' and 5'-AAAGACTTCTTGTGTGTCTGTC-3'; and β -*actin*: 5'-CTAGAAGCACTTGGCGTGCAC-3' and 5'-GAAATCGTGCCTGACATCAAA-3'.

Analytical methods. Plasma insulin was assessed by radioimmunoassay (Linco, St. Charles, MO). FFAs 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 [³H]glucose was determined as previously described (13).

Peroxynitrite treatment and hepatic Akt tyrosine nitration and phosphorylation. Wild-type mice were injected (intravenously) with saline or insulin (4 units/kg) for 5 min, and the liver was excised and homogenized as described above. Lysates were immunoprecipitated with antibodies specific for Akt and treated with 50 $\mu\text{mol/l}$ of peroxynitrite (ONOO⁻) or decomposed peroxynitrite (dONOO⁻) for 2 min (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 \pm SE. Statistical comparisons were performed using a two-way ANOVA for nonrepeated-measures design. Newman-Keuls post hoc test was used in the event of a significant ($P < 0.05$) F ratio.

RESULTS

Short-term intralipid infusion promotes iNOS expression and NO production in vivo. To establish the role of iNOS in the metabolic effects of elevated FFAs, we examined whether a 6-h intralipid infusion was sufficient to induce iNOS expression and accumulation of the NO derivative nitrite (NO₂⁻) in liver and skeletal muscle. Immunoblot analysis of hepatic iNOS protein levels in wild-type mice showed a robust (approximately threefold) induction following intralipid infusion ($P < 0.05$) compared with saline-infused controls (Fig. 1A). iNOS induction was associated with heightened NO₂⁻ accumulation (~ 2.2 -fold) in the liver of intralipid-infused wild-type 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, intralipid infusion caused nonsignificant iNOS induction in skeletal muscle. However, intralipid infusion increased muscle iNOS activity, as revealed by a significant accumulation of NO₂⁻ (~ 1.6 -fold, $P < 0.05$) in skeletal muscle of wild-type mice but not in their knockout 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 wild-type and knockout mice was determined during an HIEC. Physiological parameters measured at preinfusion, preclamp, 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, intralipid infusion led to a marked increase in plasma FFAs in both wild-type and knockout mice compared with their saline-infused counterparts ($P < 0.05$). Insulin infusion during the clamp led to a reduction in plasma FFAs in all groups. It is noteworthy that the magnitude of the intralipid-induced rise in plasma FFAs was comparable among the wild-type and knockout mice. Nevertheless, lack of iNOS prevented intralipid-induced elevations in both blood glucose and insulin concentrations. Indeed, intralipid-infused wild-type mice displayed significantly elevated glycemia (preclamp) compared with the corresponding knockout mice ($P < 0.05$). Furthermore, plasma insulin rose significantly after 150 min in lipid-infused wild-type mice compared with their saline-infused controls ($P < 0.05$). Since neither glycemia nor insulinemia varied among infusion groups in the knockout 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.

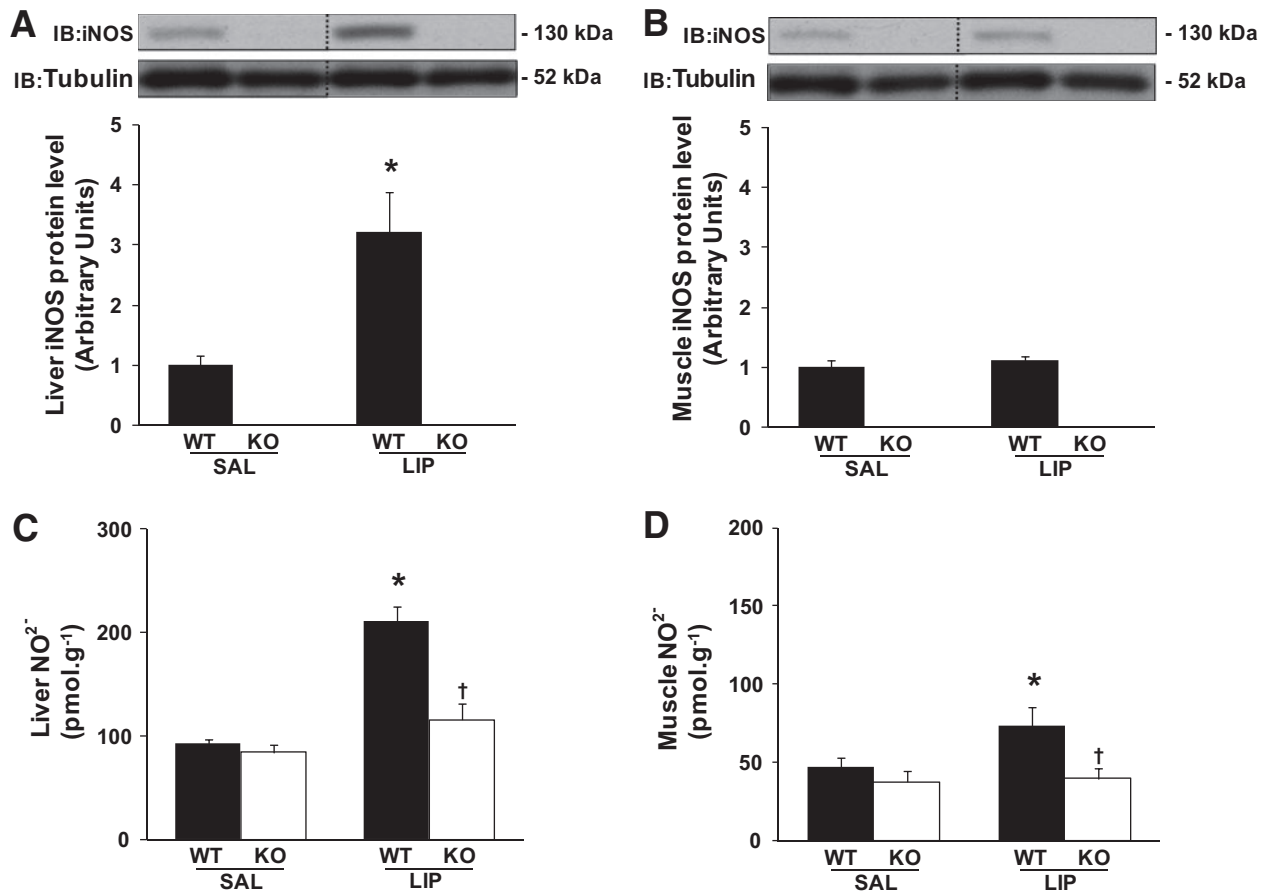


FIG. 1. Lipid infusion increases iNOS expression and NO production in liver and skeletal muscle. Immunoblots (IB) for iNOS (A and B) and nitrite concentrations (C and D) in liver and gastrocnemius extracts of wild-type (WT) and iNOS^{-/-} (KO) mice following an HIEC performed during the last 2 h of a 6-h saline or lipid infusion in 5-h fasted, chronically catheterized, conscious mice at least 5 days following surgical procedure. 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. Lanes were run on the same gel but were noncontiguous. Data are means \pm SE. Interactions between groups; * $P < 0.05$ vs. saline counterpart.

Genetic deletion of iNOS protects against lipid-induced insulin resistance. The HIEC was performed in unison with the intralipid 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, glucose production, and R_d were assessed

with arterial glycemia and insulinemia clamped at 6–7 mmol/l and 150–170 μ U/ml, respectively (Table 1). As suggested by the observed perturbations in preclamp glycemia and insulinemia, the HIEC data showed that intralipid infusion causes insulin resistance in wild-type mice, while knockout animals are protected from this

TABLE 1

Basal and clamp characteristics in wild-type and iNOS^{-/-} mice following a 6-h treatment of either saline or intravenous infusion of intralipid

	Wild type		Knockout	
	Saline	Intralipid	Saline	Intralipid
<i>n</i>	6	6	6	6
Weight (g)	24.0 \pm 0.9	25.2 \pm 1.1	24.5 \pm 1.3	23.9 \pm 1.4
Blood glucose (mmol/l)				
Basal	6.3 \pm 0.4	6.9 \pm 0.2	5.8 \pm 0.2	6.1 \pm 0.1 [†]
Clamp	6.9 \pm 0.4	6.6 \pm 0.3	6.7 \pm 0.3	6.9 \pm 0.4
Insulin (μ U/ml)				
Basal	15.9 \pm 4.2	20.4 \pm 2.5*	16.5 \pm 3	15.7 \pm 3.5
Clamp	155 \pm 27	164 \pm 29	167 \pm 29	173 \pm 33
FFA (mEq/l)				
Preinfusion	0.69 \pm 0.08	0.71 \pm 0.09	0.60 \pm 0.07	0.61 \pm 0.07
Basal	0.61 \pm 0.07	1.11 \pm 0.1*	0.56 \pm 0.08	1.05 \pm 0.3*
Clamp	0.26 \pm 0.03	0.72 \pm 0.06*	0.28 \pm 0.05	0.69 \pm 0.22*
Glycogen (mg/g liver)	79.2 \pm 6.4	40.0 \pm 7.6*	74.2 \pm 7.1	45.2 \pm 12.4*

Data are means \pm SE. * $P < 0.05$ vs. saline counterpart; [†] $P < 0.05$ vs. wild type.

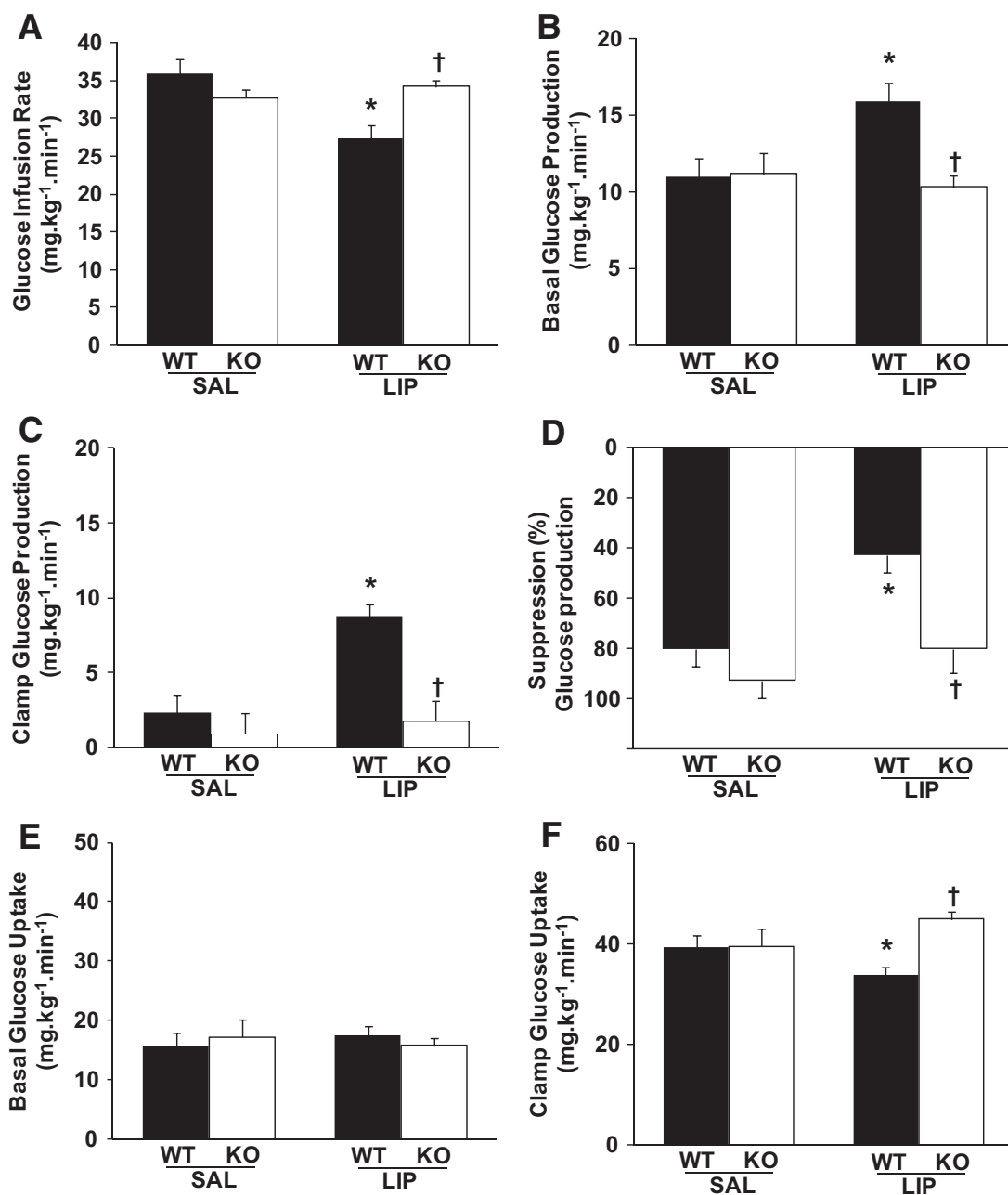


FIG. 2. HIEC characteristics in saline and lipid-infused wild-type (WT) and *iNOS*^{-/-} (KO) mice. Glucose infusion rate (A), basal glucose production (B), clamp glucose production (C), percentage of glucose production suppression (D), basal (E), and clamp (F) whole-body glucose uptake during an HIEC performed as described in the legend to Fig. 1. Data are means \pm SE. Interactions between groups; * $P < 0.05$ vs. saline counterpart; † $P < 0.05$ vs. wild type.

effect. Indeed, the GIR required to maintain euglycemia was reduced by $\sim 24\%$ ($P < 0.05$) in lipid-infused wild-type mice compared with saline-infused animals (Fig. 2A), whereas the GIR of lipid-infused knockout mice did not vary from that of their saline-infused counterparts. In line with *iNOS* induction, the liver appears to be the primary site of perturbed glucose metabolism in this short-term intralipid infusion model. Indeed, basal glucose production was elevated by $\sim 45\%$ ($P < 0.05$) (Fig. 2B) and insulin-mediated suppression of glucose production was impaired by $\sim 46\%$ ($P < 0.05$) (Fig. 2C and D) in lipid-infused wild-type mice. Although basal R_d was not affected by intralipid infusion, we did observe an $\sim 15\%$ reduction ($P < 0.05$) in insulin-mediated R_d during the clamp in wild-type mice (Fig. 2E and F). Importantly, knockout

mice were protected against lipid-induced liver and muscle insulin resistance, providing genetic evidence that *iNOS* is a mediator of lipid-induced insulin resistance in both tissues.

***iNOS* is key to lipid-induced perturbations in hepatic insulin signaling proteins.** Since the effects of intralipid infusion on *iNOS* and glucose metabolism were most pronounced in liver, we chose to explore the mechanisms underlying lipid-induced insulin resistance in this tissue. We first assessed the impact of lipid-induced *iNOS* expression on the phosphorylation state of *IR β* , *IRS-1*, and *IRS-2*, key transducers of insulin's metabolic actions in liver (19). Immunoblot analysis showed that tyrosine phosphorylation of both *IR β* and *IRS-2* is significantly diminished in

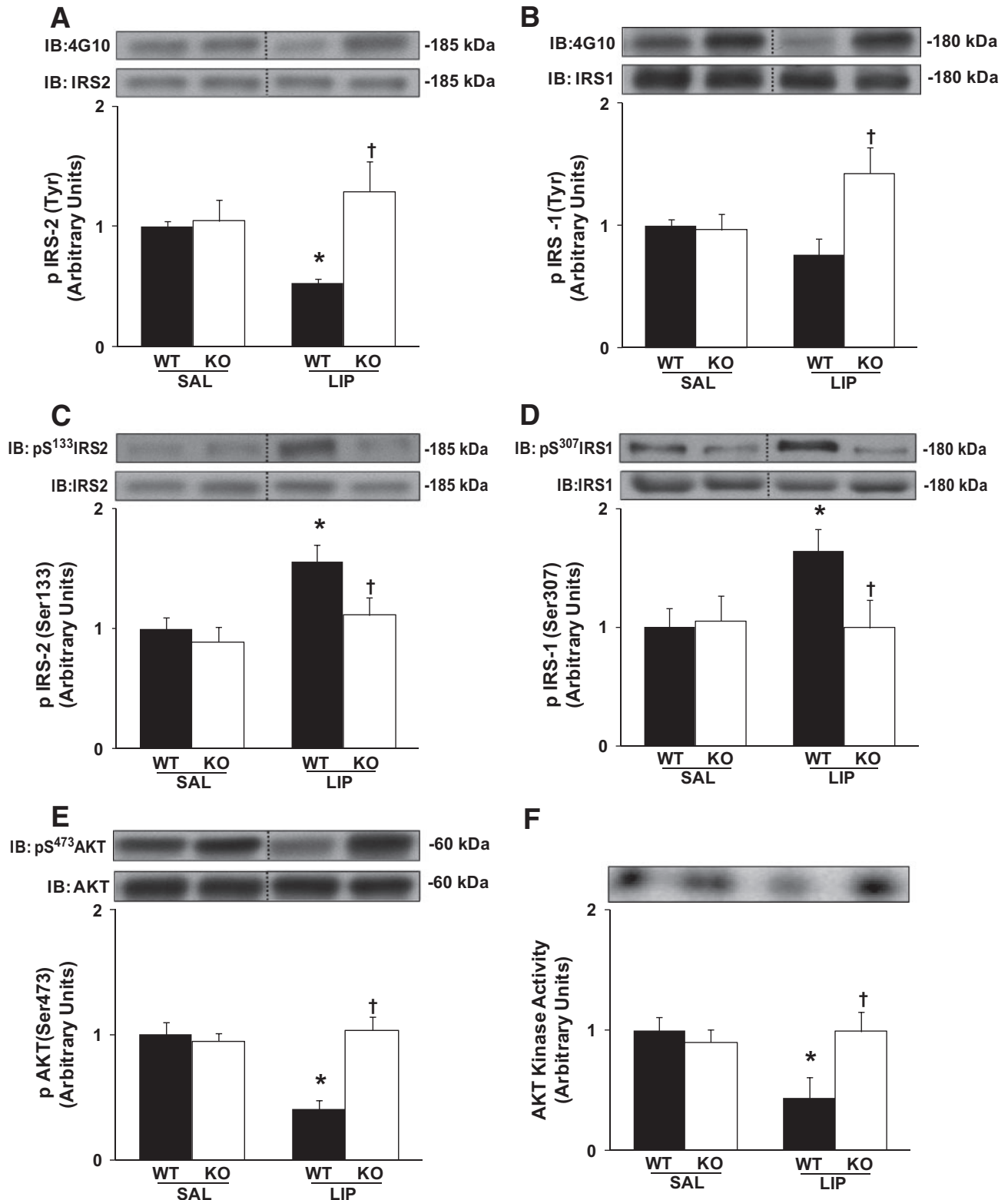


FIG. 3. Effects of lipid infusion on hepatic IRS-2, IRS-1, Akt phosphorylations, and Akt kinase activity. Immunoblots (IB) for pIRS-2 (Tyr) (A), pIRS-1 (Tyr) (B), pIRS-2 (Ser133) (C), pIRS-1 (Ser307) (D), pAkt (Ser473) (E), and Akt kinase activity (F) in liver extracts of wild-type (WT) and *iNOS*^{-/-} (KO) mice following an HIEC performed as described in the legend to Fig. 1. A and B: IRS-2 and IRS-1 were immunoprecipitated and subsequent immunoblots were performed with 4G10 antibody. Densitometric analyses were normalized for the expression of IRS-2 and IRS-1 in the same sample. 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. F: Akt was immunoprecipitated and kinase activity was determined using [γ -³²P]ATP as described in RESEARCH DESIGN AND METHODS. A–E: Lanes were run on the same gel but were noncontiguous. Data are means \pm SE. Interactions between groups; * P < 0.05 vs. saline counterpart; † P < 0.05 vs. wild type.

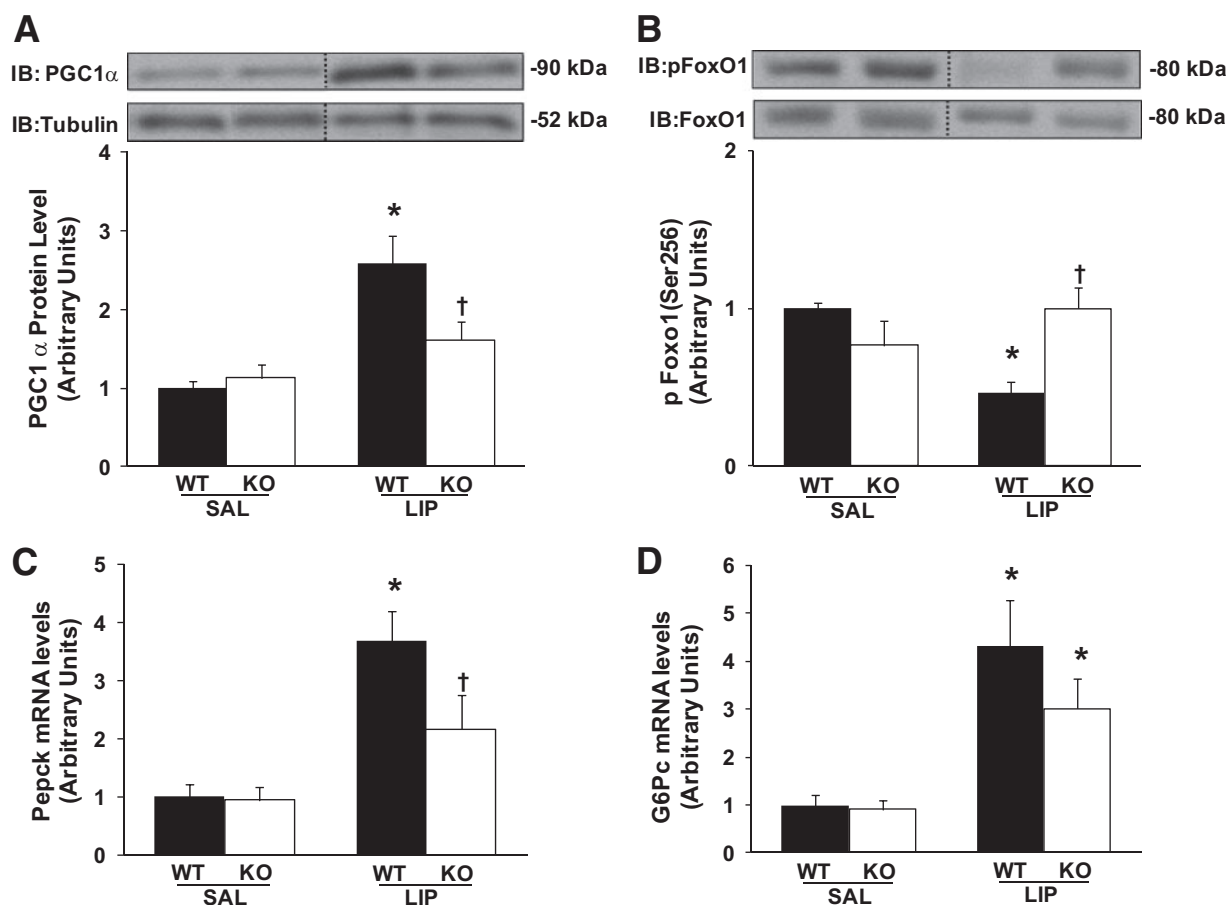


FIG. 4. Effects of lipid infusion on hepatic gluconeogenesis enzymes and gene transcripts. Immunoblots (IB) for PGC1 α (A), pFoxO1 (Ser256) (B), and expression of mRNA for phosphoenolpyruvate carboxykinase (*Pepck*) (C) and glucose-6-phosphatase (*G6Pc*) (D) in liver extracts of wild-type (WT) and iNOS^{-/-} (KO) mice following an HIEC performed as described in the legend to Fig. 1. A and B: Immunoblottings of tissue lysates were performed with PGC1 α 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. C and D: *Pepck* and *G6Pc* mRNA expressions were evaluated by quantitative RT-PCR, and values were corrected for housekeeping β -actin transcript levels. Data are means \pm SE. Interactions between groups; * $P < 0.05$ vs. saline counterpart; † $P < 0.05$ vs. wild type.

liver of lipid-infused wild-type mice compared with saline-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 knockout mice compared with their wild-type counterparts ($P < 0.05$) (supplementary Fig. 1A and Fig. 3A and B).

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

To determine whether the perturbations in phosphorylation of IR β , 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 wild-type mice compared with saline-infused controls ($P < 0.05$) (supplementary Fig. 2A and Fig. 3E and 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 glucose production, we studied transcriptional regulators of hepatic gluconeogenesis, PGC1 α and FoxO1. In line with the elevated glucose production witnessed during the clamp, PGC1 α protein content was increased in lipid-infused wild-type mice compared with saline-infused controls ($P < 0.05$) (Fig. 4A). Importantly, iNOS genetic disruption prevented the rise in PGC1 α protein expression in the lipid-infused knockout mice ($P < 0.05$) (Fig. 4A). Phosphorylation of FoxO1 by Akt in response to insulin is known to abolish FoxO1 binding to PGC1 α , 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 wild-type mice as compared with saline-infused controls ($P < 0.05$) (Fig. 4B). However, this was not observed in knockout animals where FoxO1 Ser256 phosphorylation was significantly increased in liver of lipid-

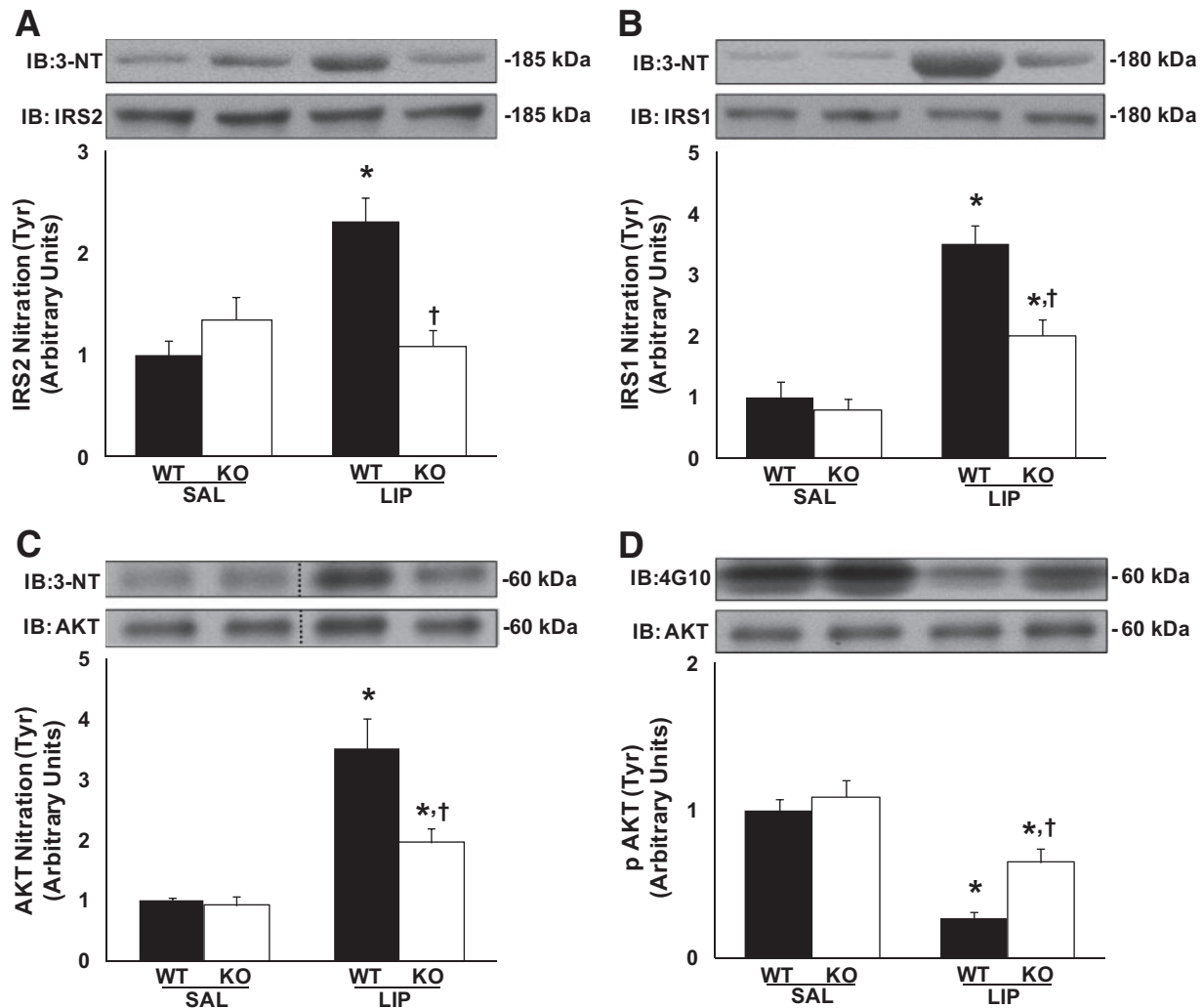


FIG. 5. Effects of lipid infusion on hepatic IRS-2, IRS-1, Akt tyrosine nitration, and Akt tyrosine phosphorylation. Immunoblots (IB) for IRS-2 nitration (Tyr) (A), IRS-1 nitration (Tyr) (B), Akt nitration (Tyr) (C), and Akt tyrosine phosphorylation in liver extracts of wild-type (WT) and *iNOS*^{-/-} (KO) mice following an HIEC performed as described in the legend to Fig. 1. A–D: IRS-2, IRS-1, and Akt were immunoprecipitated 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, IRS-1, Akt, and tubulin in the same sample. C: Lanes were run on the same gel but were noncontiguous. Data are means \pm SE. Interactions between groups; * $P < 0.05$ vs. saline counterpart; † $P < 0.05$ vs. wild type.

infused knockout mice compared with their wild-type counterparts.

In line with the regulation of PGC1 α and FoxO1, intralipid infusion increased the expression of *Pepck* and *G6Pc* transcripts in wild-type mice compared with the saline-infused controls ($P < 0.05$) (Fig. 4C and D). Intralipid infusion also increased *Pepck* and *G6Pc* mRNA expression in knockout mice but to a lesser extent compared with their lipid-infused wild-type 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 glucose production through tyrosine nitration of IR β , IRS, and Akt proteins. To further explore the mechanisms underlying hepatic insulin resistance in lipid-infused mice, we assessed whether insulin signaling proteins were modified through iNOS-mediated protein tyrosine nitration. Tyrosine nitration is a covalent posttranslational 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 insulin resistance (21,22). Growing evidence suggests that this modification can alter protein function by preventing functional phosphorylation (23–25). 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 insulin resistance 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 wild-type mice. Immunoblot analysis of IR β , 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 wild-type mice as compared with saline-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 IR β , IRS-1, and Akt tyrosine nitration in liver of lipid-infused mice, suggesting that the inability

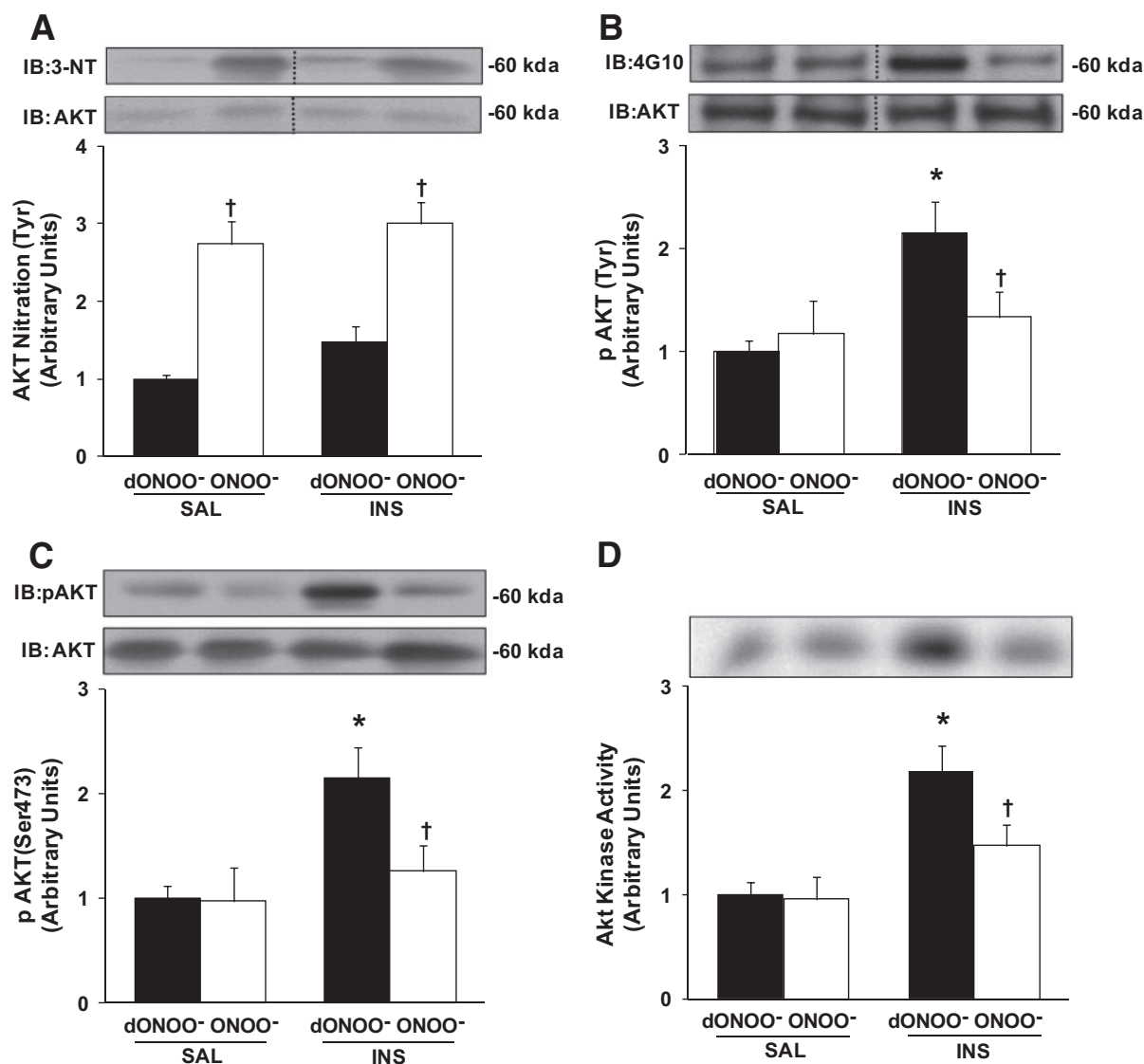


FIG. 6. Effects of peroxynitrite on insulin-stimulated Akt activity. Five-hour fasted wild-type (WT) mice were treated for 5 min with either saline or insulin (tail intravenous -4 units/kg). *A–C*: Following saline or insulin treatment, Akt from liver lysates was immunoprecipitated and incubated for 2 min with peroxynitrite (ONOO⁻) (50 μ mol/l) or an equimolar amounts of decomposed ONOO⁻ (dONOO⁻). Subsequent immunoblots (IB) were performed with 3-NT, 4G10, and pAkt (Ser473) antibody for Akt. *D*: Following incubation, Akt kinase activity was determined using [γ -³²P]ATP as described in RESEARCH DESIGN AND METHODS. *A* and *B*: Lanes were run on the same gel but were noncontiguous. Data are means \pm SE. Interactions between groups; * $P < 0.05$ vs. saline counterpart; † $P < 0.05$ vs. wild type.

of insulin to suppress glucose production in lipid-infused wild-type 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 wild-type mice. Akt was immunoprecipitated from liver lysates of saline or insulin-treated mice and incubated with ONOO⁻ in vitro for 2 min. ONOO⁻ treatment was found to increase Akt tyrosine nitration while decreasing Akt Tyr/Ser473 phosphorylations and Akt kinase activity (Fig. 6*A–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. 3*C*), suggesting that the reactive NO derivative does not interact nonspecifically with the hy-

droxyl 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 insulin resistance upon intralipid infusion.

DISCUSSION

A pathogenic immune response to nutrient excess is believed to be involved in the development of obesity-linked insulin resistance (27). Indeed, lipid-induced insulin resistance in muscle and liver is linked to overactivation of inflammatory signaling pathways known to impede insulin signal transduction (27–29). 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 insulin resistance 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 nonobese animals. This is key to determining whether iNOS should be considered as a primary candidate for triggering insulin resistance 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 wild-type mice. Using iNOS^{-/-} mice, we further show that iNOS underlies lipid-induced insulin resistance 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 glucose production and prevented lipid-induced elevations in basal glucose production that account for the initial hyperglycemia seen in wild-type mice. Since glycogen levels were unaffected by the absence of iNOS, 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 PGC1 α expression, a well-established mechanism of augmented gluconeogenic gene expression in altered metabolic states (12). Our finding that iNOS expression was associated with PGC1 α 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 PGC1 α induction (30). It is therefore conceivable that the iNOS-NO-PGC1 α 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 IR β /IRS/PI3K/Akt insulin signaling pathway (1,5,6,28). Accordingly, we found that iNOS impairs insulin action on glucose production by altering insulin signaling to IR β , 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., I κ B kinase-nuclear factor κ B, c-Jun NH₂-terminal kinase, protein kinase C) (8,31,32). Conversely, IR β 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 wild-type mice, resulting in normalization of hepatic Akt kinase activity. Normalization of IR β 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 knockout mice.

Taken together with the increased expression of PGC1 α , these results suggest that iNOS is a key inflammatory mediator linking excessive lipid levels to activation of the gluconeogenic transcription program in the liver of models of elevated FFAs. In future studies, it will be interesting to determine whether iNOS increases hepatic gluconeogenic enzymes by modulating the ability of PGC1 α to target O-GlcNAc transferase for GlcNAcylation of FoxO1, another mechanism by which PGC1 α 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 posttranslational protein modification and a marker of nitrosative stress in several pathological disorders associated with iNOS induction (21,35–38). However, no studies have explored whether protein tyrosine nitration regulates hepatic insulin signaling and glucose metabolism *in vivo*. Our results show that intralipid infusion leads to tyrosine nitration of IR β , IRS-1, and IRS-2, as well as Akt in liver of wild-type 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 insulin resistance.

iNOS likely promotes tyrosine nitration of IR β , 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 wild-type 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 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, and 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 insulin resistance.

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 sulfhydryl 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, intralipid infusion in vivo impedes both Ser473 and Thr308 phosphorylation in wild-type 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 knockout 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 insulin resistance, 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 FFAs 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 insulin resistance in obesity and metabolic conditions associated with excess lipid availability.

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