Insulin Resistance and Altered Systemic Glucose Metabolism in Mice Lacking Nur77

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OBJECTIVE—Nur77 is an orphan nuclear receptor with pleotropic functions. Previous studies have identified Nur77 as a transcriptional regulator of glucose utilization genes in skeletal muscle and gluconeogenesis in liver. However, the net functional impact of these pathways is unknown. To examine the consequence of Nur77 signaling for glucose metabolism in vivo, we challenged Nur77 null mice with high-fat feeding.

RESEARCH DESIGN AND METHODS—Wild-type and Nur77 null mice were fed a high-fat diet (60% calories from fat) for 3 months. We determined glucose tolerance, tissue-specific insulin sensitivity, oxygen consumption, muscle and liver lipid content, muscle insulin signaling, and expression of glucose and lipid metabolism genes.

RESULTS—Mice with genetic deletion of Nur77 exhibited increased susceptibility to diet-induced obesity and insulin resistance. Hyperinsulinemic-euglycemic clamp studies revealed greater high-fat diet–induced insulin resistance in both skeletal muscle and liver of Nur77 null mice compared with controls. Loss of Nur77 expression in skeletal muscle impaired insulin signaling and markedly reduced GLUT4 protein expression. Muscles lacking Nur77 also exhibited increased triglyceride content and accumulation of multiple even-chained acylcarnitine species. In the liver, Nur77 deletion led to hepatic steatosis and enhanced expression of lipogenic genes, likely reflecting the lipogenic effect of hyperinsulinemia.

CONCLUSIONS—Collectively, these data demonstrate that loss of Nur77 influences systemic glucose metabolism and highlight the physiological contribution of muscle Nur77 to this regulatory pathway. ***Diabetes*** **58:2788–2796, 2009**

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The NR4A family includes three highly homologous isotypes, NR4A1, NR4A2, and NR4A3, also known as Nur77, Nurr1, and Nor1, respectively (1). Although these receptors possess a putative ligand-binding domain, X-ray crystallography studies suggest that the ligand-binding pocket is occluded by bulky hydrophobic residues and is unable to accommodate ligands (2,3). Instead, NR4A activity is regulated primarily at the transcriptional level by stimuli that signal through the cAMP pathway as well as posttranslational modification. NR4A receptors have been implicated in a range of biological processes, including apoptosis, dopaminergic neuron development, and tumorigenesis (4–6).

Several members of the nuclear receptor superfamily function as downstream regulators of metabolic pathways in response to nutritional perturbations. In particular, the ligand-responsive nuclear receptors of the peroxisomal proliferator–activated receptors (PPARs) family, liver X receptors, and glucocorticoid receptor have been characterized as transcriptional coordinators of specific metabolic programs (7–10). In addition, ligand-independent orphan nuclear receptors, such as estrogen-related receptor-α, chicken ovalbumin upstream transcriptional factors, and retinoic acid receptor–related orphan receptors have also been implicated in metabolic regulation (11–14). In recent years, the NR4A family of orphan nuclear receptors has also joined the cadre of nuclear receptors involved in metabolic regulation.

Work by our group and others have pointed to NR4A1 (Nur77) as a transcriptional regulator of glucose metabolism in liver and skeletal muscle (1,15–17). Ectopic expression of Nur77 in vivo enhances hepatic glucose production and elevates plasma blood glucose (16). In skeletal muscle, Nur77 regulates the expression of a battery of glucose utilization genes, including GLUT4 and multiple genes involved in glycolysis (17). However, whether Nur77-mediated changes in the expression of muscle-glucose utilization genes are sufficient to modulate systemic glucose metabolism is unknown. Furthermore, the relative contribution of liver versus muscle Nur77 to maintaining systemic glucose homeostasis has not been explored previously.

Here, we show that mice lacking Nur77 develop hepatic steatosis and exacerbated insulin resistance in both skeletal muscle and liver when challenged with a high-fat diet (HFD). In addition to diminished expression of GLUT4 and glycolytic genes, Nur77 null mice demonstrated impaired muscle insulin signaling and increased intramuscular lipid content. Our findings highlight the importance of skeletal muscle Nur77 in the regulation of systemic glucose metabolism.

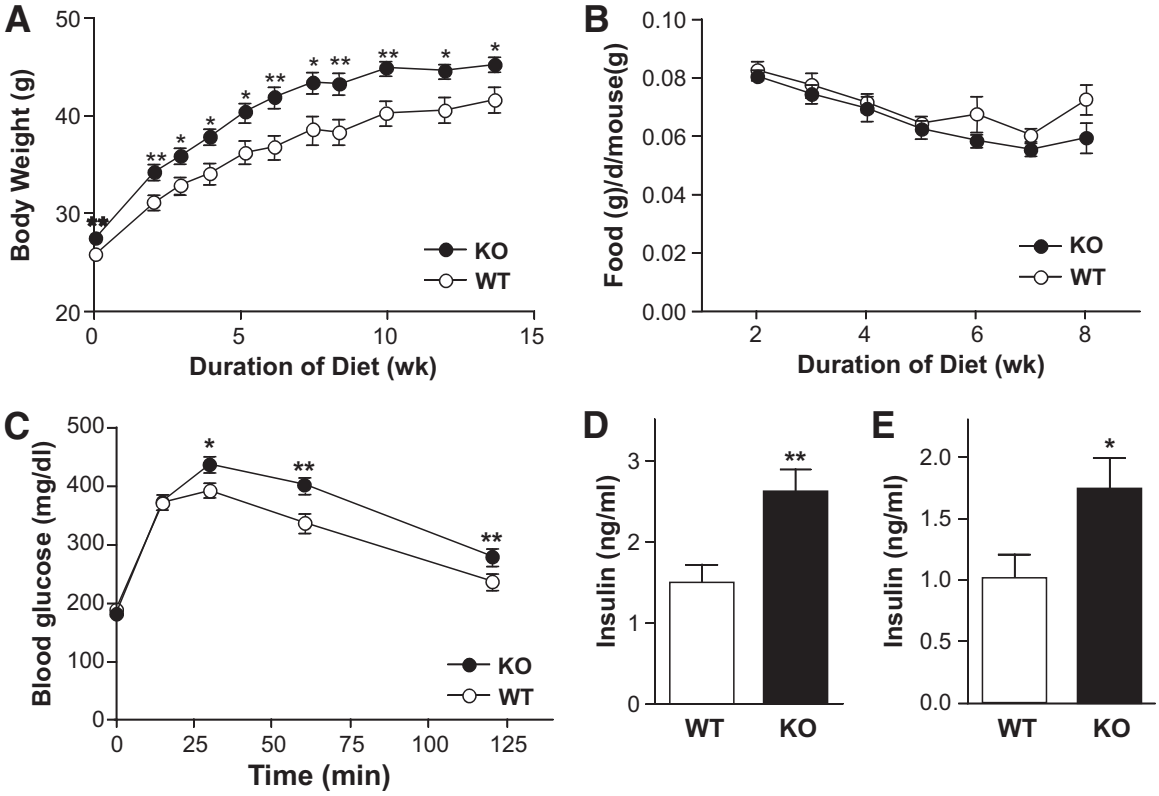

FIG. 1. Nur77 null mice develop diet-induced obesity and insulin resistance on a HFD. Body weight (*A*) and food intake (*B*) of wild-type (WT, ○) and Nur77 knockout (KO, ●) mice. *C*: Intraperitoneal glucose tolerance test (1 g/kg) performed after 1 month of HFD. *D* and *E*: Fasting plasma insulin concentrations 6 weeks after high-fat feeding. *A–D*: Male, $n = 8–9$, 11- to 14-week-old mice. *E*: male, $n = 7–10$, 17–18 weeks old at the start of high-fat feeding. *$P < 0.05$, **$P < 0.01$.

RESULTS

Nur77 null mice develop worsened glucose tolerance after high-fat feeding. Prior work has shown that Nur77 regulates metabolic pathways that have opposing effects on peripheral glucose clearance. In the liver, Nur77 is a transcriptional modulator of gluconeogenesis (16). In skeletal muscle, Nur77 regulates the expression of a panel of genes that promote glucose utilization (17). To investigate the net functional outcome of Nur77 activity on systemic glucose metabolism, we performed metabolic analysis on Nur77 null mice. Global Nur77 knockout mice (4), devoid of Nur77 in both skeletal muscle and liver, exhibited no obvious difference in glucose tolerance when fed a standard rat diet (data not shown). To determine whether Nur77 affected the development of diet-induced obesity and diabetes, we challenged male control and Nur77 null mice with a HFD (60% calories from fat; Research Diets, New Brunswick, NJ) for a period of 3 months. As shown in Fig. 1*A*, Nur77 null mice exhibited slightly greater body weight at baseline and continued to gain more weight than wild-type controls over the course of the high-fat feeding, despite similar food intake (Fig. 1*B*). After 1 month of high-fat feeding, Nur77 null mice had worsened glucose tolerance compared with wild-type controls (Fig. 1*C*). In addition, Nur77 null mice displayed markedly higher fasting insulin levels (Fig. 1*D*), indicating that the glucose intolerance observed was attributable to insulin resistance rather than insulinopenia. This difference in fasting insulin level was reproduced in a separate cohort of high-fat–fed Nur77 null mice despite comparable body weight, adiposity, and adiponectin level (Fig. 1*E*, supplementary Table 1 and supplementary Fig. 1, available in an online appendix at http://diabetes.diabetesjournals.org/cgi/content/full/db09-0763/DC1), suggesting that the insulin resistance we observed was not entirely attributable to differences in adiposity.

Nur77 null mice have reduced oxygen consumption. To determine the effect of Nur77 deletion on energy expenditure, we subjected high-fat–fed Nur77 knockout mice to indirect calorimetry. Mice were housed individually in the Comprehensive Lab Animal Monitoring System (CLAMS, Columbus Instruments, OH) and were fed ad libitum. As shown in Fig. 2*A*, Nur77 null mice showed reduced oxygen consumption (a measure of energy expenditure) during both light and dark cycles. This reduction in energy expenditure may contribute to the increased weight accretion in Nur77 null mice on a HFD, despite comparable ambulatory activities (Fig. 2*B*) and food intake. Although Nur77 regulates the expression of glycolytic genes in skeletal muscle, there was no significant effect of Nur77 deletion on substrate preference based on comparable respiratory exchange ratios between the genotypes, either during fasting or fed states (data not shown).

High-fat feeding elicits insulin resistance in both skeletal muscle and liver of Nur77 null mice. To determine tissue-specific contributions to the impaired insulin sensitivity of Nur77 null mice, we performed hyperinsulinemic-euglycemic clamps. In diet-fed animals, there was no difference in basal glucose turnover between wild-type and Nur77 null mice in the fasted state (Table 1 and Fig. 3*B*). Furthermore, Nur77 null and wild-type control mice had comparable insulin-stimulated glucose disposal rates (IS-GDR), reflecting similar skeletal muscle insulin sensitivity (Fig. 3*A*). In contrast, Nur77 null mice

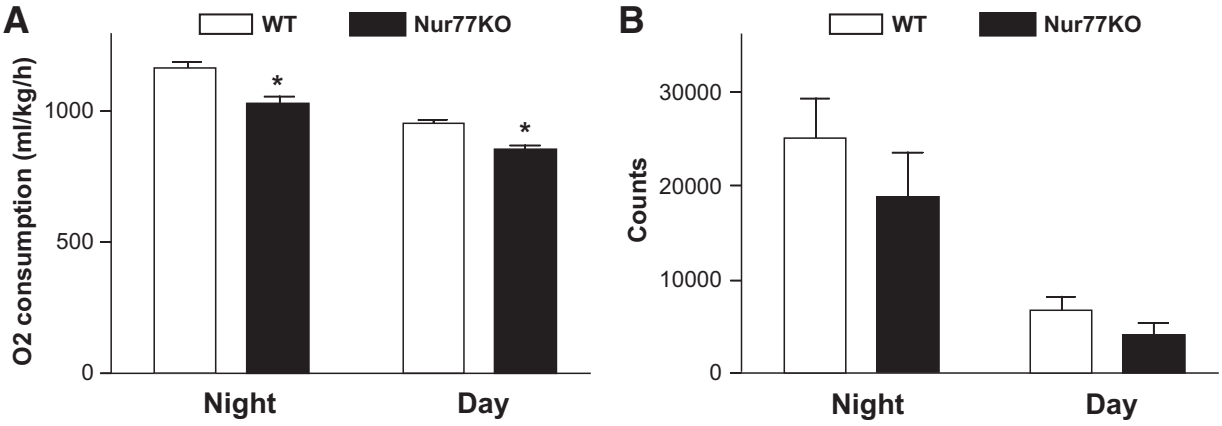

FIG. 2. Indirect calorimetry of Nur77 null mice on HFD. Oxygen consumption (*A*) and ambulatory activity (*B*) of wild-type (WT) and Nur77 knockout (KO) mice after 3 months of high-fat feeding. Mice were fed ad libitum, male, $n = 7–8$. *$P < 0.05$.

subjected to high-fat feeding exhibited marked reduction in IS-GDR, indicative of impaired skeletal muscle insulin sensitivity (Fig. 3*C*). Thus, these results highlight Nur77 as an important regulator of insulin sensitivity in skeletal muscle.

Although muscle insulin sensitivity was unchanged in diet-fed Nur77 null mice, Nur77 deletion enhanced liver insulin sensitivity. As shown in Fig. 3*B*, insulin suppressed hepatic glucose production more effectively in diet-fed Nur77 knockout mice, reflecting enhanced hepatic insulin sensitivity. The increased hepatic insulin sensitivity is also supported by a higher glucose infusion rate required to maintain euglycemia during the clamp (Table 1). This result confirmed our previous finding that Nur77 regulates hepatic gluconeogenesis (16). Surprisingly, however, the protective effect of Nur77 on hepatic insulin sensitivity was not preserved during high-fat feeding, as Nur77 null mice showed a blunted suppression of hepatic glucose production (HGP) by insulin (Fig. 3*D*). When integrating both diet and genotype in two-way ANOVA, high-fat–fed Nur77 null livers actually exhibited worsened insulin sensitivity.

Impaired insulin receptor phosphorylation in Nur77 null mice. We performed immunoblot analysis on quadriceps muscle from HFD fed mice to examine insulin-signaling pathways. Caveolin 3, a muscle-specific plasma membrane protein (18), was used as a loading control. As shown in Fig. 4*A*, GLUT4 protein level was markedly reduced in Nur77 null muscle, consistent with our previous finding that GLUT4 is a direct transcriptional target of Nur77 (17). We found that insulin receptor phosphorylation was reduced in Nur77 null muscle (Fig. 4*A*). Insulin receptor phosphorylation was unaffected in Nur77 null livers (supplementary Fig. 2). Both the reduced GLUT4 protein level and insulin receptor phosphorylation would be predicted to contribute to the observed impairment in muscle insulin sensitivity. Interestingly, Akt phosphorylation was not appreciably altered in Nur77 null mice. Although this result was unexpected, dissociation between insulin resistance and diminished Akt phosphorylation has been reported in several models of insulin resistance (19–21). As well, the level of insulin-regulated aminopeptidase, a GLUT4 vesicle protein (22), was unchanged, thus confirming the specificity of Nur77 regarding genes of glucose utilization.

Nur77 stimulates glycolysis in C2C12 muscle cells. The observation that Nur77 null mice display impaired insulin-stimulated glucose disposal is concordant with our previous finding that Nur77 regulates the expression of glycolytic genes in skeletal muscle (17). Indeed, the expression of several glycolytic genes (enolase 3, 2,3-bisphosphoglycerate mutase, phosphoglycerate kinase 1) was reduced in tibialis anterior muscle of Nur77 null mice on a HFD (Fig. 4*B*). This defect would be expected to diminish insulin-stimulated glycolysis and glucose disposal. We found that the expression of lipin1 was reduced in Nur77 null muscle. Although the physiologic function of

TABLE 1
Metabolic parameters

	Diet-WT	Diet-KO	*P*	HFD-WT	HFD-KO	*P*
n	8	7		7	7	
Age (weeks)	16.7 ± 0.39	17.4 ± 0.18	0.12	27.1 ± 0.1	28.4 ± 0.3	0.001
Body wt (g)	29.1 ± 0.8	30.6 ± 0.70	0.08	39.4 ± 0.80	43.6 ± 0.90	0.004
Fasting blood glucose (mg/dl)	109 ± 3.5	113 ± 5.9	0.58	119 ± 3.4	126 ± 1.9	0.07
Clamp blood glucose (mg/dl)	106 ± 1.8	108 ± 2.4	0.59	114 ± 1.7	117 ± 2.1	0.35
Basal insulin (ng/ml)	0.39 ± 0.04	0.41 ± 0.04	0.72	1.5 ± 0.2	2.6 ± 0.3	0.007
Basal glucose turnover ($mg \cdot kg^{-1} \cdot min^{-1}$)	20 ± 1.6	20 ± 1.6	0.9	18 ± 0.9	18 ± 1.0	0.9
Hepatic glucose production ($mg \cdot kg^{-1} \cdot min^{-1}$)	5.8 ± 1.2	3.8 ± 0.8	0.007	6.1 ± 0.8	8.6 ± 1.5	0.156/0.013*
GIR ($mg \cdot kg^{-1} \cdot min^{-1}$)	52 ± 2.3	58 ± 1.7	0.04	40 ± 2.2	18 ± 4.4	0.001
Clamp insulin (ng/ml)	9 ± 2	8 ± 2	0.68	11 ± 2	16 ± 2	0.11
Basal FFA (mM)	0.87 ± 0.04	0.77 ± 0.06	0.21	0.82 ± 0.03	0.88 ± 0.06	0.41
Clamp FFA (mM)	0.20 ± 0.02	0.21 ± 0.03	0.73	ND	ND	

All comparisons done by Student *t* test except as noted. *Two-way ANOVA of interaction between diet and genotype. FFA, free fatty acid; GIR, glucose infusion rate; KO, knockout; ND, not done; WT, wild type.

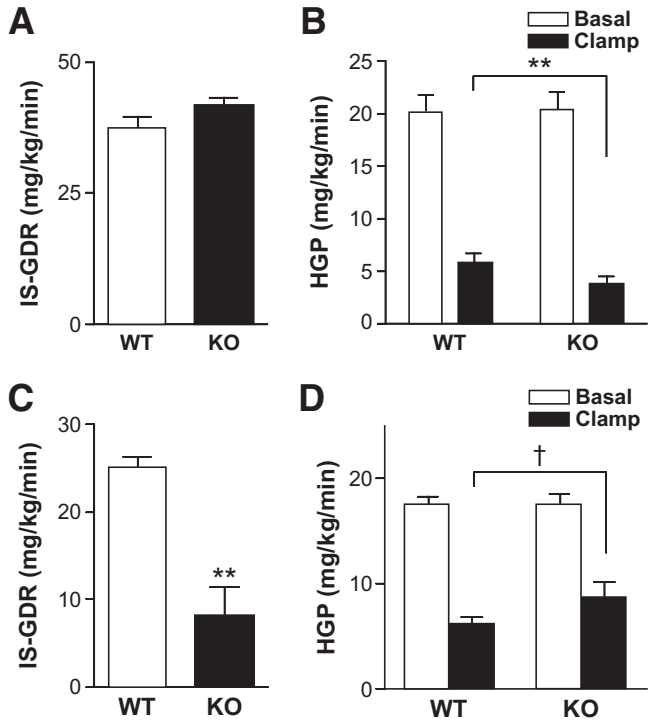

FIG. 3. Muscle and liver insulin sensitivity of Nur77 null mice. *A* and *B*: IS-GDR and HGP of diet-fed wild-type (WT) and Nur77 knockout (KO) mice; male, $n = 7–8$. *C* and *D*: Basal state, white bars; postclamp, black bars. IS-GDR and HGP of mice on HFD for 3.5 months; $n = 7$, male. $^{**}P < 0.01$ by one-way ANOVA; †$P < 0.05$ by two-way ANOVA.

lipin1 in skeletal muscle remains unclear, our finding is consistent with the observation that muscle lipin1 expression is reduced in other models of insulin resistance (K. Reue, unpublished results).

At least in the Nur77 null model, regulation of glycolytic genes by Nur77 appears to be specific to skeletal muscle, as there was no appreciable loss of glycolytic gene expression in other metabolically active tissues such as brain and heart (supplementary Fig. 3), although we cannot exclude the potential compensatory actions of the remaining two NR4A receptors. In the liver, the expression of these enzymes was upregulated in high-fat–fed Nur77 null mice (supplementary Fig. 3). However, as most glycolytic enzymes also catalyze the reverse gluconeogenic reactions, the induction of these enzymes may reflect excessive gluconeogenesis observed in the high-fat–fed Nur77 knockout mice (Fig. 3*D* and supplementary Fig. 4). Interestingly, we also observed an increase in muscle Nur77 expression in response to HFD feeding (supplementary Fig. 5).

To test the hypothesis that Nur77-dependent regulation of glycolytic gene expression is sufficient to alter glycolytic activity, we expressed Nur77 in mouse C2C12 muscle cells with an adenoviral vector and measured in vivo glycolytic activity 3 days after infection. Glycolysis was measured by the Extracellular Flux Analyzer (Seahorse Bioscience, Chicopee, MA). Lactate accumulation during glycolysis lowers extracellular pH; extracellular acidification rates reflect glycolytic activity (23). Glycolytic activity can be further augmented by the addition of an uncoupling agent such as 2,4-dinitrophenol (DNP). As shown in Fig. 4*C*, Nur77-expressing cells exhibited enhanced glycolysis both at baseline and after DNP treatment. The specificity of

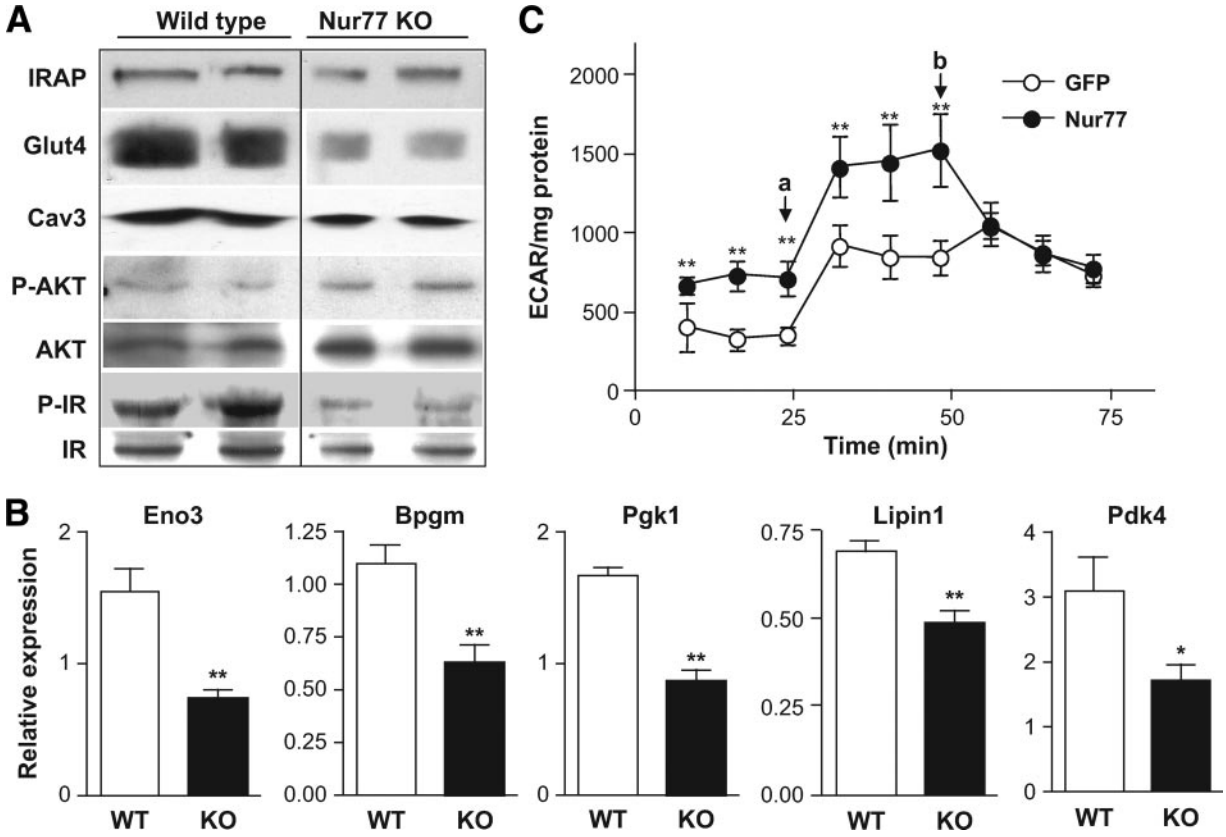

FIG. 4. Effect of Nur77 on glycolysis and insulin signaling. *A*: Immunoblot of cell lysates prepared from high-fat–fed Nur77 null quadriceps muscle. *B*: Gene expression of wild-type (WT) and Nur77 knockout (KO) tibialis anterior muscle from high-fat–fed mice; male, $n = 7$. *C*: Glycolytic activity of Ad-GFP (○)- vs. Ad-Nur77 (●)-infected C2C12 myoblasts. a, 2,4-DNP; b, 2-DG. $^{*}P < 0.05$, $^{**}P < 0.01$.

DNP-stimulated glycolysis was confirmed by inhibiting glycolysis, using excess 2-deoxyglucose, which is phosphorylated by hexokinase but cannot be further metabolized in the glycolytic pathway. These results demonstrate that Nur77-driven changes in glycolytic gene expression increase cellular glycolytic function. Conversely, lactate and citrate content, two byproducts of glycolytic flux, was reduced in Nur77 null muscles (supplementary Fig. 6). These data support our hypothesis that impaired muscle insulin sensitivity in high-fat–fed Nur77 null mice is in part attributable to attenuated glycolysis.

Nur77 deletion increases intramuscular lipid content. Increased intramuscular lipid content has been associated with development of skeletal muscle insulin resistance in human and rodent models (24,25). Recent evidence suggests that although intramuscular triglyceride content correlates with skeletal muscle insulin resistance, it is the proinflammatory lipid intermediates such as diacylglycerol (DAG) and ceramides that may contribute to lipid-induced insulin resistance (26–29). We sought to determine whether impairment in muscle insulin sensitivity of high-fat–fed Nur77 null mice was associated with increased intramuscular lipid accumulation. Intramuscular triacylglycerol (TAG) and DAG content in tibialis anterior muscle of Nur77 null mice was increased, whereas ceramide level was unchanged (Fig. 5*A*). In addition, as shown in Fig. 5*B*, Nur77 null mice had increased accumulation of multiple even-chain acylcarnitine species, reflecting accumulation of the cognate acyl CoA species in muscle mitochondria. Possibly related to this finding, the expression of lipoprotein lipase was upregulated in Nur77 null mice (Fig. 5*C*), suggesting that fatty acid uptake was increased in Nur77 null muscle. The expression of PPARα and other mitochondrial fatty acid metabolism genes (including carnitine palmitoyltransferase 1b, long-chain acyl-CoA dehydrogenase, and medium-chain acyl-CoA dehydrogenase) was unchanged. Interestingly, the expression of peroxisomal bifunctional β-oxidation enzyme, 3-hydroxyacyl CoA dehydrogenase (Ehhadh), was reduced by 54% ($P = 0.01$) in Nur77 null muscle (Fig. 5*C*). The expression of peroxisomal acyl-CoA oxidase also tended to be lower in Nur77 null mice. The reduction in peroxisomal β-oxidation enzyme suggests that long-chain fatty acid oxidation (>C16) was impaired in Nur77 null mice. The expression of lipogenic enzymes was largely unchanged (supplementary Fig. 7). In addition, the expression of pyruvate dehydrogenase kinase, isozyme 4 (Pdk4), was reduced in Nur77 null muscle (Fig. 4*A*). Pdk4 inactivates the pyruvate dehydrogenase complex, limiting the flux of pyruvate through the Kreb cycle, effectively reducing carbohydrate oxidation and favoring lipid catabolism (30). The decrease in Pdk4 and the increase in lipoprotein lipase may represent attempts to compensate for reduced glucose uptake and glycolytic activity in Nur77 null muscle, as changes in expression of these genes might enhance glucose entry into the trichloroacetic acid cycle and fatty acid uptake into the cell to provide alternative sources of energy.

Hepatic steatosis in high-fat–fed Nur77 null mice. In addition to increased intramuscular lipid content, high-fat–fed Nur77 null mice also developed hepatic steatosis (Fig. 6*A*). Quantitation of liver lipid content confirmed that liver from Nur77 null mice accumulated more triglyceride and cholesterol than wild-type mice (Fig. 6*B*). Not surprisingly, quantitative real-time PCR confirmed that the expression of lipogenic genes including sterol regulatory element binding protein (SREBP-1c), fatty acid synthase, and stearoyl-CoA desaturase 1 was increased ~twofold in Nur77 null livers. To investigate whether the hepatic steatosis observed in high-fat–fed Nur77 null mice could be linked to a direct effect of Nur77 on SREBP pathway, we overexpressed a dominant-negative form of Nur77 (Nur77-898) (16) using adenoviral vectors in HepG2 cells and primary murine hepatocytes. As shown in supplementary Fig. 8, Nur77 overexpression did not suppress SREBP-1c or stearoyl-CoA desaturase 1 expression. Likewise, Nur77 had no effect on lipogenic gene expression when the liver X receptor was activated by ligand GW3965 (1 μmol/l). To test the effect of Nur77 on SREBP-1c activity, we also performed transient transfection assays in HEK293T cells, using the rat fatty acid synthase promoter that contains the SREBP-1c binding site as the reporter. As shown in supplementary Fig. 8*C*, cotransfecting Nur77 had little effect on SREBP-1c activity with or without liver X receptors-α and retinoid X receptor-α. Collectively, our data suggest that the hepatic steatosis observed in high-fat–fed Nur77 null mice is not attributable to loss of Nur77 suppression of lipogenesis but rather the lipogenic effect of hyperinsulinemia.

DISCUSSION

To determine the net effect of tissue-specific Nur77 regulation of glucose metabolism, we studied the effect of high-fat feeding on Nur77 null mice. We found that Nur77 null mice developed exacerbated skeletal muscle insulin resistance after high-fat feeding. Skeletal muscle insulin resistance in Nur77 null mice may be explained by defects in multiple steps of the glucose utilization pathway (Fig. 7). As Nur77 is a transcriptional regulator of GLUT4 and multiple glycolytic enzymes, reduction in the abundance of these glucose uptake and glycolytic genes would be predicted to diminish insulin-stimulated glucose disposal. The reduced glucose utilization may contribute to a compensatory increase in lipid uptake in skeletal muscle. In addition, we found that insulin receptor phosphorylation was reduced in Nur77 null skeletal muscle, which may in part be attributed to increased intramuscular lipid accumulation. In the liver, Nur77 deletion was unable to protect high-fat–fed mice from hepatic insulin resistance. Rather, Nur77 null livers became more steatotic than wild-type livers because of increased lipogenesis, likely secondary to hyperinsulinemia. These findings highlight skeletal muscle Nur77 as a physiologic regulator of systemic glucose metabolism.

The metabolic benefit of oxidative metabolism in skeletal muscle is well established. Studies in patients with diabetes suggest that insulin resistance correlates with decreased oxidative enzyme activity in skeletal muscle (31,32). Muscle-specific overexpression of PPARδ results in not only increased abundance of slow-twitch oxidative fibers but also protects mice from diet-induced obesity and diabetes (33). On the other hand, the metabolic impact of glycolytic activity in skeletal muscle is less clear. Recent evidence suggests that selective fast-twitch/glycolytic muscle fiber growth also protects mice from diet-induced obesity and diabetes (34). We have previously shown that Nur77-mediated regulation of glycolytic genes occurred selectively in fast-twitch/glycolytic, not slow-twitch/oxidative, fibers (17). Our current finding that Nur77 deletion exacerbates diet-induced insulin resistance further sup-

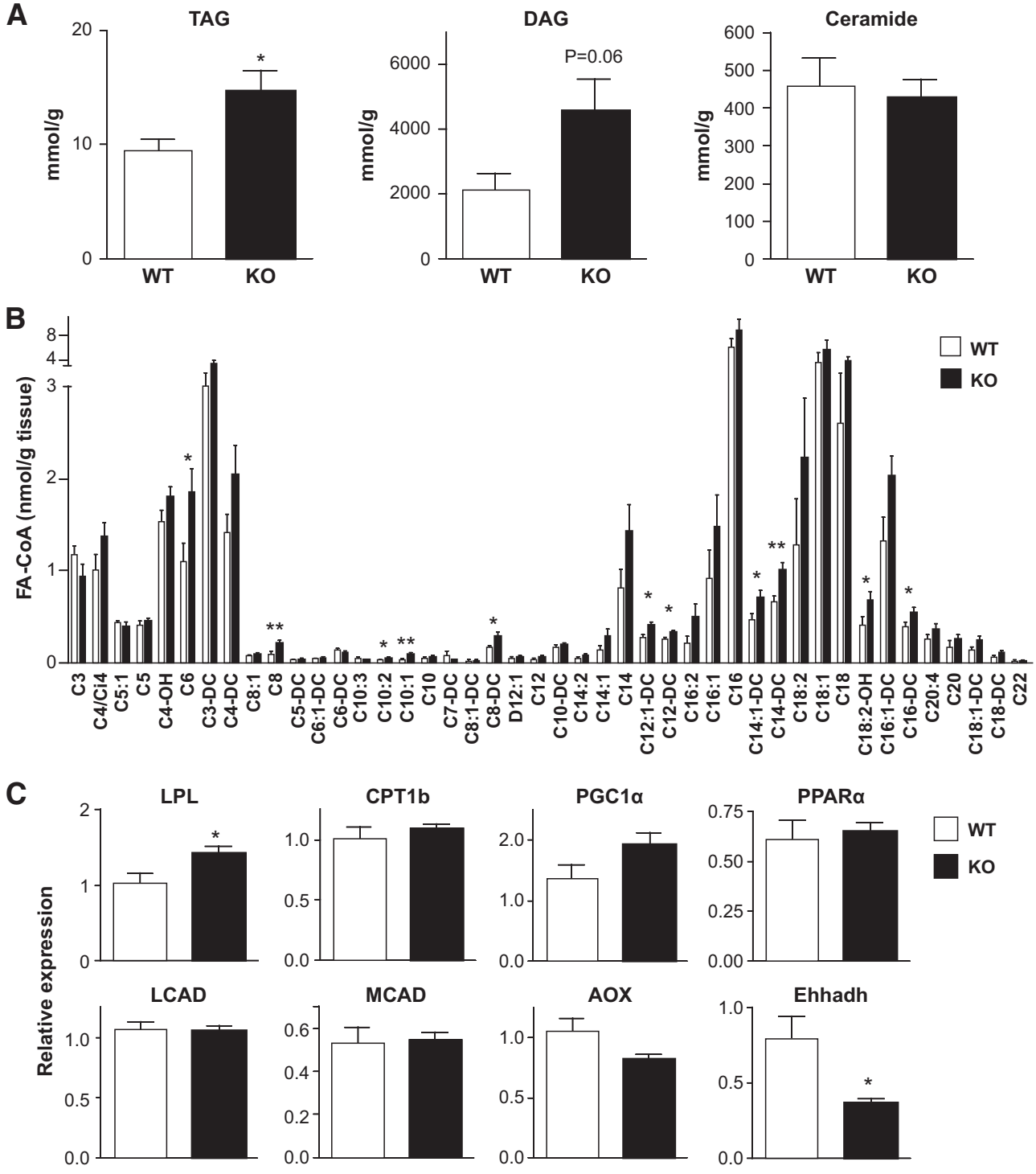

FIG. 5. Intramuscular lipid content and expression of lipid metabolism genes in high-fat–fed Nur77 null mice. *A*: TAG, DAG, and ceramide concentrations from tibialis anterior muscle. *B*: Quantitation of fatty-acylcarnitine species in gastrocnemius muscle. *A* and *B*: Male, $n = 7$. *C*: Expression of lipid metabolism genes from gastrocnemius muscle; female, $n = 9$. Wild-type (WT), white bars; Nur77 null, black bars. *$P < 0.05$, **$P < 0.01$. KO, knockout.

ports the notion that enhancing glycolytic activity in skeletal muscle is metabolically advantageous.

Lipotoxicity has been implicated as a contributor to insulin resistance in both skeletal muscle and liver, although the relationship between increased lipid accumulation and tissue insulin responsiveness is not always direct. Increased muscle lipid content, particularly long-chain acyl-CoAs, DAGs, and ceramides, has been implicated in activation of various kinases that phosphorylate insulin receptor substrate on serine residues and thereby impairs insulin signaling (35,36). An implication of these studies is that decreased fatty acid oxidation contributes to insulin resistance and impaired glucose metabolism. In support of this idea, mice with global acetyl-CoA carboxylase 2 deletion have diminished malonyl-CoA levels, increased CPT-1 activity, and are protected from diet-

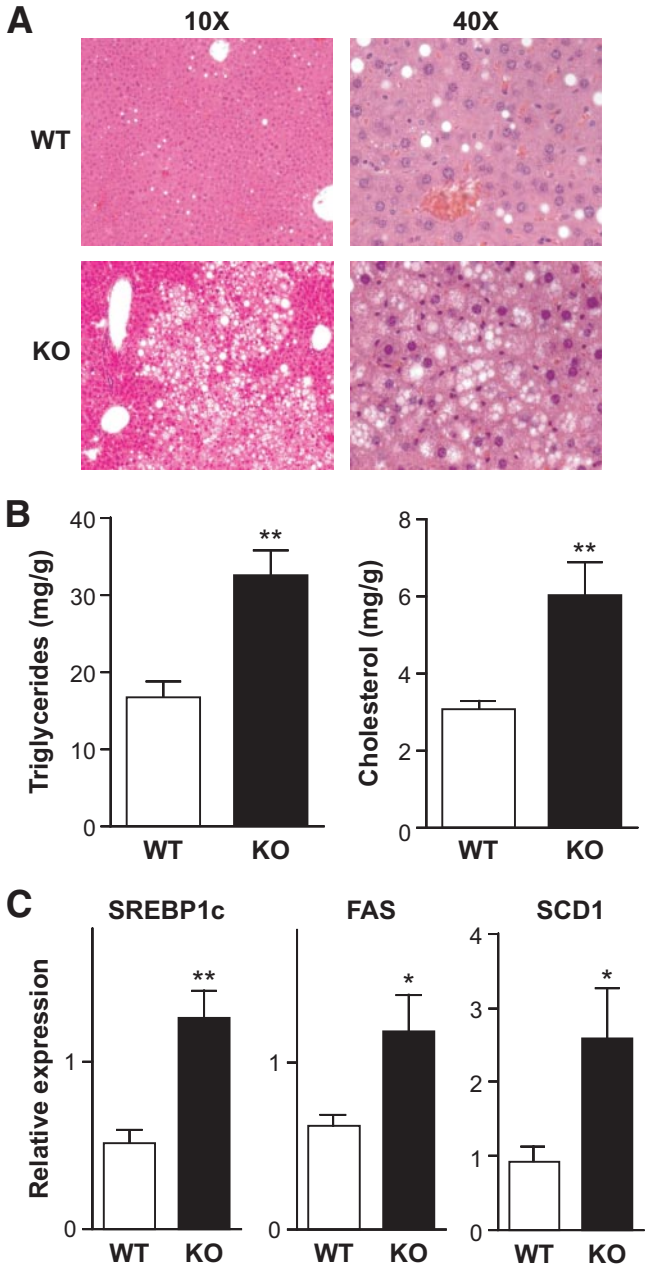

FIG. 6. Hepatic steatosis in high-fat–fed Nur77 null livers. *A*: Hematoxylin-eosin stain of wild-type (WT) and Nur77 knockout (KO) liver section. *B*: Liver lipid content. *C*: Lipogenic gene expression in liver. *$P < 0.05$, **$P < 0.01$. (A high-quality color digital representation of this figure is available in the online issue.)

induced obesity and insulin resistance (37). In contrast, Koves et al. (38) showed that chronic exposure of muscle to elevated lipids induced β-oxidation of fatty acids without concurrent upregulation of downstream metabolic pathways such as the trichloroacetic acid cycle and electron transport chain. This results in incomplete metabolism of fatty acids in the β-oxidation pathway and exacerbation of insulin resistance (39). Additional evidence supporting this argument includes the diabetic phenotype of muscle-specific PPARα transgenic mouse, which is reversed by pharmacologic inhibition of CPT-1 (40). In HFD-challenged Nur77 null mice, we showed that loss of Nur77 led to decreased glycolytic flux with reduced lactate and citrate levels, upregulation of lipoprotein lipase mRNA, downregulation of PDK4 and peroxisomal bifunctional β-oxidation enzyme, as well as increased intramuscular TAG and DAG. This constellation of findings is most consistent with a model in which decreased glucose metabolism leads to compensatory responses, including an attempt to increase glucose oxidation via PDK4 dowregulation, increased fatty acid uptake into skeletal muscle, diminished peroxisomal β-oxidation of long-chain fatty acids, and subsequent accumulation of TAG and DAG, which, if any of these events contributed to impaired insulin signaling in Nur77 null mice, remains to be investigated.

Given that Nur77 deletion enhanced hepatic insulin sensitivity in diet-fed mice, it was somewhat surprising that Nur77 null mice challenged with a HFD actually developed exacerbated hepatic insulin sensitivity. This finding suggests that Nur77 deletion is insufficient to overcome the metabolic stress of lipid oversupply. It is conceivable that in response to muscle insulin resistance, nutritional or humoral factors could alter insulin action in other tissues, as secondary phenotypes were shown in muscle-specific GLUT4 knockout mice (41,42). Alternatively, if the hepatic steatosis observed in Nur77 null mice is a primary result of hepatic Nur77 deficiency, lipotoxicity may contribute to hepatic insulin resistance. Based on findings by Pols et al. (43) that Nur77 diminishes SREBP-1c activity, we investigated whether Nur77 directly suppresses hepatic lipogenesis. However, we were unable to demonstrate reduction of lipogenic gene expression when Nur77 was ectopically expressed in HepG2 cells and primary murine hepatocytes, nor when SREBP1c activity was measured in HEK293T cells, suggesting that the hepatic steatosis we observed may be secondary to the lipogenic effect of hyperinsulinemia.

One limitation of our analysis of the global Nur77 knockout mouse is the potential for additional metabolic effects of Nur77 in tissues not studied. We have determined that loss of hepatic Nur77 does not protect mice from diet-induced obesity and diabetes. However, the metabolic effect of Nur77 deletion on other tissues has not been explored. Our finding that Nur77 null mice have increased susceptibility for skeletal muscle insulin resistance is nevertheless consistent with the biologic pathways Nur77 regulates in muscle (17) and illustrates specifically the importance of muscle Nur77 in the regulation of whole-body glucose metabolism. The generation of tissue-specific Nur77 transgenic and knockout mouse models will be necessary to delineate the physiologic roles of Nur77 in metabolism.

RESEARCH DESIGN AND METHODS

Animal husbandry. Mice were fed ad libitum and maintained on a 12-h light-dark cycle and were age and sex matched for all experiments. Intraperitoneal glucose tolerance test was performed after a 6-h fast by intraperitoneal injection of 1 g/kg of dextrose. Animal studies were performed in accordance with University of California at Los Angeles animal research committees guidelines.

Tissue culture. Care of C2C12 cells, HEK293T cells, and primary murine hepatocytes has been previously described (16,17). HepG2 cells (ATCC, Rockville, MD) were maintained in 10% FBS/DMEM and incubated with 5% CO_2.

Plasma and tissue chemistry. Measurement of fasting mouse insulin, free fatty acid, triglyceride concentrations, and liver lipids were as described previously (44,45). Insulin during clamp experiments was determined by Linco-Millipore RIA kit (St. Charles, MO). Adiponectin concentration was measured by ELISA kit from B-Bridge International (Mountain View, CA). Concentrations of adipokines listed in supplementary Table 1 were measured by the Mouse Serum Adipokine Panel–7-plex kit (Millipore, MA). Total cholesterol was determined by Cholesterol E kit (Wako, VA). Muscle lipids,

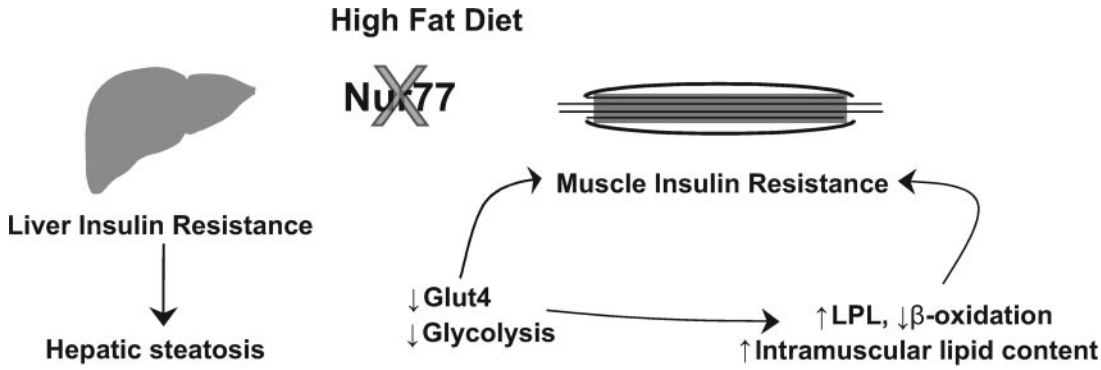

FIG. 7. Proposed mechanism of insulin resistance in Nur77 null mice. Nur77 deletion leads to reduced abundance of GLUT4 as well as diminished glycolysis in skeletal muscle. Reduced glucose utilization leads to compensatory increase in fatty acid uptake, which further exacerbates insulin resistance. Nur77 deletion in the setting of high-fat feeding also leads to hepatic insulin resistance, secondarily causing hepatic steatosis.

including DAG and ceramide, were extracted from skeletal muscle and assessed as previously described (46). TAGs were saponified, and glycerol was assessed (Free Glycerol Reagent; Sigma-Aldrich, MO). Determination of fatty-acylcarnitine and organic acid concentrations was described previously (39).

Indirect calorimetry. Mice were individually housed in the CLAMS and acclimated for 7 h in the cages. Data were collected for the next 48 h, spanning 2 day and 2 night periods. Effective mass coefficient was set at 0.75. Ambulatory activity level was measured as interruption of dual-axis infrared beams. Data were analyzed by Oxymax software (Columbus Instruments, OH).

Body composition. Body composition was determined by dual-energy X-ray absorptiometry densitometry (PIXImus, Madison, WI).

Insulin sensitivity in vivo. Hyperinsulinemic-euglycemic clamps using an insulin infusion of 12 mU $\cdot$ kg^{-1} $\cdot$ min^{-1} were performed as described previously (47). Differences in IS-GDR and HGP between treatment groups were detected using ANOVA, and significance was set a priori at $P < 0.05$.

Quantitative real-time PCR. Total RNA preparation and quantitative real-time PCR were performed as described (17). Expression was normalized to 36B4 expression. Primer sequences are listed in supplementary Table 2 and as previously described (16,17,48).

In vitro glycolysis. C2C12 myoblasts were plated in V7 plates (Seahorse Biosciences) 1 day before adenovirus infection. Adenovirus infection was performed as described (17). On the day of assay, media were refreshed with freshly made unbuffered Delbecco' modified Eagle's medium (plus glutamine 2 mmol/l, sodium pyruvate 1 mmol/l, glucose 25 mmol/l, NaCl 31.7 mmol/l, Phenol Red 15 mg/l, and pH 7.4); 2,4-dinitrophenol 1 mmol/l and 2-deoxyglucose 200 mmol/l were made in the same media. The XF24 Extracellular Flux Analyzer (Seahorse Bioscience) (49) was set up with measure:mix:wait cycles of 3:2:3 min. Protein quantitation was performed using the DC Protein Assay (BioRad, Richmond, CA).

Immunoblot. Monoclonal anti-GLUT4 antibody 1F8 29 was made in the Pilch lab, and affinity purified anti–insulin-regulated aminopeptidase polyclonal antibody was made by 21st Century Biochemical (Hopkinton, MA). Phospho-insulin receptor antibody was purchased from Cell Signaling (#3021, Beverly, MA). Other antibodies used were described previously (16,17,50). Mouse quadriceps muscle and liver were isolated and flash frozen. Tissue lysate and immunoblots were prepared as described (17).

Histology. Liver sections were fixed in 10% formalin and stained with hematoxylin and eosin by UCLA Translational Pathology Core Laboratory.

Statistics. Statistical analysis was performed by Student t test unless otherwise noted.

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