Deletion of Nck1 attenuates hepatic ER stress signaling and improves glucose tolerance and insulin signaling in liver of obese mice

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Latreille M, Latreille M, Laberge G, Yamani L, Larose L. Deletion of Nck1 attenuates hepatic ER stress signaling and improves glucose tolerance and insulin signaling in liver of obese mice. Am J Physiol Endocrinol Metab 300:E423–E434, 2011. First published June 29, 2010; doi:10.1152/ajpendo.00088.2010.—Obesity has been shown to create stress in the endoplasmic reticulum (ER), and that initiates the activation of the unfolded protein response (UPR). This has been reported to cause insulin resistance in selective tissues through activation of the insulin-requiring enzyme 1α (IRE1α)-c-Jun NH2-terminal kinase (JNK) pathway, which results in the phosphorylation of the insulin receptor substrate-1 (IRS-1) at an inhibitory site and blocks insulin receptor signaling. In this study, we report that the Src homology domain-containing adaptor protein Nck1, previously shown to modulate the UPR, is of functional importance in obesity-induced ER stress signaling and inhibition of insulin actions. We have examined obese Nck1+/− and Nck1−/− mice for glucose tolerance, insulin sensitivity, and signaling as well as for ER stress markers and IRS-1 phosphorylation at Ser307. Our findings show that obese Nck1-deficient mice display improved glucose disposal accompanied by enhanced insulin signaling in liver. This correlates with attenuated IRE1α and JNK activation and IRS-1 phosphorylation at Ser307 compared with obese wild-type mice. Consistent with our in vivo data, we report that downregulation of Nck1 using siRNA in HepG2 cells results in decreased thapsigargin-induced IRE1α activation and signaling and IRS-1 phosphorylation at Ser307, whereas it markedly enhances insulin signaling. Overall, in liver and in cultured cells, we show that depletion of Nck1 attenuates the UPR signal and its inhibitory action on insulin signaling. Taken together, our findings implicate Nck1 in regulating the UPR, which secondary to obesity impairs glucose homeostasis and insulin actions.

Noncatalytic region of tyrosine kinase; endoplasmic reticulum; adaptor proteins; insulin-requiring enzyme-1-c-Jun NH2-terminal kinase pathway; insulin receptor substrate-1; insulin resistance; diabetes

The unfolded protein response (UPR) is at the center of critical molecular mechanisms allowing cells to respond to stress imposed on the endoplasmic reticulum (ER). The UPR involves activation of three distinct ER resident transmembrane stress sensors: inositol-requiring enzyme-1α (IRE1α), activating transcription factor 6 (ATF6), and protein kinase R-like ER kinase (PERK). IRE1α, a Ser/Thr protein kinase with endonuclease activity, propagates stress signals from the ER by initiating the unconventional splicing of the X-box-binding protein-1 (XBP-1) mRNA to produce the active transcriptional regulator XBP-1s (8, 24, 43), degrading specific mRNAs (18) and in parallel activating the c-Jun NH2-terminal kinase (JNK) (40). ATF6, a transcriptional regulator, is activated following ER stress through its translocation and cleavage in the Golgi (36, 37). PERK, a Ser/Thr protein kinase, attenuates general translation through phosphorylation of the α-subunit of eukaryotic initiation factor 2 (eIF2α) at Ser51 and simultaneously activates a transcriptional program through enhanced translation of ATF4 (16). Altogether, through signaling pathways triggered following activation of these ER sensors, the UPR upregulates expression of ER chaperones and enzymes involved in protein folding, lipid biosynthesis, and misfolded protein degradation to increase the protein folding and processing capacity of the ER, thereby alleviating stress.

Recent studies have linked activation of the UPR with obesity-induced insulin resistance and type 2 diabetes (28, 33, 34, 44). In fact, in genetic and dietary mouse models of obesity characterized by glucose intolerance and peripheral insulin resistance, liver and adipose tissue display signs of ER stress (33). In these tissues, hyperactivation of IRE1α was found to impair insulin signaling and mediate insulin resistance through IRE1α-JNK-induced direct phosphorylation of the insulin receptor substrate-1 (IRS-1) at Ser307 (33). Interestingly, activation of ER stress pathways, including IRE1α-dependent JNK activation, has also been detected in selected tissues of obese human subjects (5, 14, 35). Strongly supporting a close relationship between the UPR and development of insulin resistance in humans, markers of ER stress were significantly reduced in insulin target tissues following weight loss, paralleling significant improvement in insulin sensitivity (14).

Nck (noncatalytic region of tyrosine kinase) adaptor proteins are known primarily to mediate signaling from plasma membrane-activated receptors to cytosolic effectors regulating actin cytoskeleton remodeling (3). In mammals, Nck represents two highly related proteins encoded by independent genes (9, 41), Nck1 and Nck2, which are composed of three NH2-terminal Src homology 3 (SH3) domains followed by a COOH-terminal Src homology 2 (SH2) domain (7, 25). In mice, genetic deletion of individual Nck genes did not result in any significant phenotype, whereas simultaneous deletion of both was embryonically lethal on embryonic day 9.5 (4). These data strongly support the idea that, although these adaptors appear functionally redundant, Nck is essential for proper embryonic development.

In previous studies, we provided strong evidence of a functional role for Nck in stress-induced ER signaling. Indeed, we demonstrated that Nck adaptors are recovered in isolated ER stress fractions from FR3T fibroblasts (20, 29), colocalize with the cytoplasmic portion of calnexin at the ER in Hela cells (22), and participate in the UPR by modulating PERK- and IRE1α-dependent signaling using various cell lines, including rat FR3T fibroblasts, human Hela and human embryonic kidney (HEK)-293, and mouse embryonic fibroblasts lacking or not
Nck or rescued by overexpression of Nck1 (20, 22, 29). Given a close connection between hyperactivation of the IRE1-α-JNK pathway in the UPR and insulin resistance in vivo, we assessed in this study the functional relevance of Nck1 in obesity-induced ER stress-mediated glucose intolerance and insulin resistance in Nck1-deficient mice.

MATERIALS AND METHODS

Cell culture and treatment. HEK-293 cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% FBS, 0.75 mg/ml penicillin, and 0.1 mg/ml streptomycin at 37°C in a 5% CO2 environment. Human hepatocellular carcinoma (HepG2) cells were grown in the same conditions in minimum essential Eagle’s medium (MEM) containing 10% FBS. siRNAs against human Nck1 were purchased from Integrated DNA Technologies (IDT, Coralville, IA; 5'-AAACAUCAUAUCUCCUUUCAAG3') and Applied Biosystems/Ambion (Austin, TX; 5'-GGCCAAUUUUUACAAAGU-3'). Scrambled siRNA from IDT was used as control. HepG2 cells were transfected using Lipofectamine RNAiMAX reagent (Invitrogen, Burlington, ON, Canada) according to the manufacturer’s instructions, with a final siRNA concentration of 40 nM. Forty-eight to 72 hours following transfection, cells were treated for 30 or 120 min with 1 μM thapsigargin (Sigma) or treated for 5 min with 10 or 100 nM insulin. Cells were then washed with ice-cold phosphate-buffered saline and lysed using lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1 mM EDTA, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 1 mM sodium orthovanadate, 10 mM β-glycerophosphate, and protease inhibitors). Following centrifugation at 13,000 g, total cell lysates (30–50 μg protein) were resolved by SDS-PAGE, transferred onto a polyvinylidene difluoride membrane, and immunoblotted with indicated antibodies. Enhanced chemiluminescence was used according to the manufacturer’s specifications (Amersham GE Healthcare, Piscataway, NJ) to detect immunoreactive proteins.

Antibodies. Phospho-IRE1α-Ser172 and IRE1α antibodies were obtained from Novus Biologicals (Littleton, CO). Nck1 and Nck2 antibodies were generated in rabbits using glutathione S-transferase (GST) fusion proteins encoding human Nck isoform-specific amino acid sequences between the third SH3 and the SH2 domains (Nck1: QQNPLTSGLPPSSCDYRPSLGTGKAFGNP; Nck2: VLSGPA-LHPAHAPISQYTGPSGSRFGAIRE). Pan-Nck antibody has been described previously (21). PERK antibody is a rabbit polyclonal antibody raised against a GST-PERK fusion protein encoding mouse PERK COOH-terminal (amino acids 537–1,114). eIF2β antibody is a rabbit polyclonal antibody against a GST fusion protein encoding full-length mouse eIF2β. PECK antibody directed against the COOH-terminal region was purchased from Cell Applications (San Diego, CA). Phospho-tyrosine (PY99) and green fluorescent protein (B-2) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). β-Actin antibody (AC-74) was purchased from Sigma (Oakville, ON, Canada). JNK, phospho-JNK Thr183/Tyr185, v-akt murine thymoma viral oncogene (Akt), phospho-Ser473 Akt, and insulin receptor antibodies were obtained from New England Biolabs (Pickering, ON, Canada). IRS-1 and phospho-Ser212/216 IRS-1 antibodies were from Upstate Biotechnology (Lake Placid, NY). For HepG2 cell experiments, IRS-1 antibody was purchased from Sigma and Ras GTPIase-activating protein (RasGAP) (B-48) antibody from Santa Cruz Biotechnology. Glycogen synthase kinase-3β (GSK-3β) and phospho-GSK-3β (Ser21) were obtained from BD Biosciences (San Jose, CA) and Cell Signaling Technology (Danvers, MA) respectively.

Glycogen synthesis. HepG2 cells, transfected with siRNA as described earlier, were serum starved overnight in MEM containing 0.1% BSA. The next morning, [α-32P]glucose (0.68 μCi/well; GE Healthcare) was added prior to insulin stimulation (100 nM) for 6 h at 37°C. Reactions were stopped by washing the cells with ice-cold PBS, and the cells were lysed in NaOH (0.1 N). Cell lysates were recovered, and glycogen carrier (Sigma no. G-8876) was added at 2 mg/tube. Upon boiling for 30 min, ethanol (2 ml, 100%) was added, and the samples were stored at −20°C overnight for glycogen precipitation. The next morning, following centrifugation, glycogen pellets were recovered in water and then heated at 70°C for 5 min. Radioactivity incorporated into glycogen was evaluated using liquid scintillation (TriCarb 2800TR; PerkinElmer).

Nck1-knockout mice. Nck1-knockout mice from Dr. T. Pawson (Toronto, ON, Canada) were described previously (4). Offspring (wild-type as control littersmates and Nck1−/− mice) derived from heterozygous Nck1+/− mating pairs were kept in an animal room maintained at 21 ± 1°C with fixed 12:12-h dark-light cycles and free access to food and water. One-month-old male animals were housed pairwise and fed ad libitum a normal chow diet (NCD) containing 10% fat or a high-fat diet (HFD) containing 60% fat (Research Diets, New Brunswick, NJ) for 18–20 wk. All procedures were performed on unanesthetized animals, except for in vivo insulin signaling.

Measurement of blood glucose, serum insulin levels, in vivo glucose tolerance, and insulin sensitivity. Blood glucose levels were determined on briefly immobilized mice using the OneTouch Ultra Glucometer (LifeScan, Burnaby, BC, Canada) and tail vein snipping. Serum insulin levels were determined by saphenous vein bleeding and radioimmunoadsay (Linco Research, St. Charles, MO). Glucose tolerance tests were performed on overnight-fasted animals following an intraperitoneal (i.p) injection of d-glucose at 2 g/kg for mice on NCD and 1 g/kg for HFD mice. Insulin tolerance tests were performed on 5-h-fasted mice using an i.p injection of 0.75 IU/kg Humulin R (Eli Lilly, Toronto, ON, Canada). All procedures were conducted according to the guidelines of the McGill University Animal Care Committee (protocol no. 5069) and were approved by the committee.

Tissue preparation and immunoblotting. Age-matched mice were euthanized using a CO2 chamber, and tissues were rapidly collected, snap-frozen in liquid nitrogen, and kept at −80°C until further processing. For Nck isoform expression levels, liver, gastrocnemius muscles, epididymal gonadal adipose tissue, kidneys, and total pancreas were used. For assessing ER stress and insulin-signaling markers, we processed liver, epididymal gonadal adipose tissue, and gastrocnemius muscles as mentioned above. Tissue samples were homogenized using a Polytron at 20% (wt/vol) in 5 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 1 mM MgCl2, 1 mM dithiothreitol, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 100 mM sodium fluoride, and 17.5 mM β-glycerophosphate supplemented with protease inhibitors. Triton X-100 was added to a final concentration of 1%. Following incubation for 10 min at 4°C, samples were centrifuged at 13,000 g for 10 min at 4°C. Supernatants were further centrifuged at 200,000 g for 30 min at 4°C. Final supernatants were subjected to Bio-Rad protein assay (Bio-Rad, Mississauga, ON, Canada) for protein quantification. Immunoprecipitations were performed using 1 mg of protein, whereas 75 μg of protein was used for direct immunoblot analysis.

In vivo insulin signaling. Overnight-fasted mice were anesthetized with isoflurane, and insulin (3.8 IU/kg) was injected through the jugular vein. Three minutes later, liver and epididymal gonadal adipose tissue and gastrocnemius muscles were collected, frozen in liquid nitrogen, and kept at −80°C until further processing. Protein extraction, quantitation, and immunoblotting were performed as described above.

Semiquantitative reverse transcription-polymerase chain reaction. HepG2 cells or obese mice liver samples were lysed in TRIzol (Invitrogen, Carlsbad, CA) for total RNA isolation according to the manufacturer’s instructions. RNA was reverse transcribed to cDNA by using random hexamers and the Thermoscript RT-PCR system (Invitrogen). Ten percent of the cDNA synthesis reaction was submitted to semiquantitative PCR analysis for C/EBP homologous protein (CHOP), XBP-1, and GADD expression by using Platinum Taq DNA polymerase (Invitrogen). Specific oligonucleotides used...
were 5’-GGGCTTTGTATATGTTG-3’ and 5’-GCTGTAGTTGAGACCAG-3’ for XBP-1, 5’-CCTGCTTTTCACCTTG-3’ and 5’-CCGCTGTTCCTGCT-3’ for CHOP, and 5’- ACCAC- TGAGAAGGCTGG-3’ and 5’-CTCAGCTGACGCCAG-3’ for GAPDH. PCR products in their linear range were analyzed on agarose gels for CHOP and GAPDH, whereas they were analyzed on acrylamide gels for XBP-1.

*Statistical analyses.* Data were expressed as means ± SE. The total number of animals tested in each group is indicated by n. Statistical significance was determined according to Student’s t-test (unpaired) using SigmaStat software version 3.1 (Systat Software, Chicago, IL), with P ≤ 0.05 considered to be significant. In all tests, two groups with only one changed parameter were compared.

**RESULTS**

*Nck adapter protein expression in mice.* We examined Nck1 and Nck2 protein expression levels in various tissues of 20-wk-old C57BL/6 mice. For this, we developed rabbit polyclonal antibodies that recognized Nck1 or Nck2 with no cross reactivity in immunoblot assay (Fig. 1A). Using these antibodies to probe by immunoblotting an equivalent amount of tissue protein samples, we determined that Nck1 is highly expressed in kidneys, liver, skeletal muscle, and total pancreas, but to a lower extend in adipose tissue. Like Nck1, Nck2 is highly expressed in skeletal muscle and total pancreas (Nck2 short; Fig. 1B). However, in contrast to Nck1, Nck2 in kidneys is detected only following long exposure of the immunoblot and still in this condition is barely detected in liver and adipose tissue, suggesting very low levels of Nck2 protein in these tissues (Nck2 long; Fig. 1B). Interestingly, SDS-PAGE migration of Nck1 from skeletal muscle and adipose tissue appears retarded, and Nck2 from skeletal muscle often migrates as a doublet. The reason behind this doublet is yet to be determined, but it could suggest different levels of Nck phosphorylation in these tissues.

To gain insight into a role for Nck in glucose homeostasis, we assessed whether variations in Nck expression in insulin-sensitive peripheral tissues occur in parallel with insulin resistance associated with obesity. For this, we compared total Nck and Nck1 protein levels in liver, adipose tissue, and skeletal muscle in addition to Nck2 levels in skeletal muscle between wild-type mice fed a NCD or a HFD for 18–20 wk after weaning. Normalization of Nck protein expression levels according to loading controls appeared quite difficult because we noticed that several proteins that could serve as loading controls, including Akt, RasGAP, and actin, all show important variations in expression levels between the two diets in liver, adipose tissue, and skeletal muscle (Fig. 2). Therefore, Nck expression levels in each tissue were analyzed assuming equal protein loading as determined by Bio-Rad protein assay (Fig. 2). In this manner, we determined that total Nck and Nck1 in liver and adipose tissue did not vary between diets. In contrast, total Nck level in skeletal muscle was significantly decreased in mice fed a HFD compared with NCD-fed mice. This appears to result mainly from a marked reduction in levels of both Ncks, but this was clearly significant for Nck2. These results demonstrate that the development of diet-induced insulin resistance affects Nck expression only in skeletal muscle (gastrocnemius).

**Improved glucose homeostasis in obese mice lacking Nck1.** Having observed that, in contrast to Nck2, Nck1 is highly expressed in all insulin target tissues tested, we assessed whether genetic deficiency of Nck1 affects dietary influences on glucose disposal and insulin sensitivity by comparing Nck1-deficient mice (Nck1−/−) and wild-type littermates (Nck1+/+) fed a NCD or a HFD. On a HFD, Nck1−/− mice and wild-type (Nck1+/+) littermates showed no difference in weight gain, and both genotypes gained significantly more weight than their reciprocal counterparts kept on a NCD (Fig. 3A). However, we noticed that ≤12 wk after weaning, Nck1−/− mice maintained on a NCD weighed significantly less than their wild-type littermates.

On a NCD, Nck1−/− mice showed no particular change compared with wild-type Nck1+/+ mice regarding fed and overnight-fasted blood glucose levels at 18 wk after weaning and blood insulin levels at weaning (W0) and 18 wk after weaning (W18) (Fig. 3B). Glucose disposal in response to acute glucose challenge in typical glucose tolerance test revealed slight but significant glucose intolerance in lean Nck1−/− mice compared with their wild-type counterparts, as reflected by a significant increase in the calculated area under the curve (AUC0–120 min; Fig. 3C, inset). However, there was no change in insulin sensitivity in either mice genotype, as evaluated by insulin tolerance tests (Fig. 3D). On HFD, we observed comparable serum insulin levels at 4 wk of age before being fed on a HFD (W0) and 18 wk following HFD (W18) between wild-type and Nck1−/− mice (Fig. 3E). But for both
genotypes, insulin levels increased tremendously in mice fed a HFD compared with similar mice kept on a NCD (Fig. 3, B and E). This is in agreement with pancreatic β-cell compensation during HFD-induced insulin resistance. Likewise, after 18 wk on a HFD, fed blood glucose levels were similar between both groups (Fig. 3F) but elevated compared with reciprocal mice fed a NCD (Fig. 3B). In contrast, Nck1−/− mice displayed significantly lower blood glucose levels than wild-type littermates after an overnight fast (Fig. 3F). This suggests improved overall glucose homeostasis in obese Nck1−/− mice compared with obese wild-type mice, although overnight-fasted blood glucose levels in obese Nck1−/− mice are still higher than that in lean Nck1−/− mice (compare Fig. 3, B and F). To assess whether lower fasted glycemia observed in obese Nck1−/− mice resulted from changes in glucose disposal, we performed acute glucose and insulin tolerance tests in obese mice of both genotypes. Following glucose injection, we found that obese Nck1−/− mice clear glucose more efficiently than obese wild-type littermates (Fig. 3G). This difference is further established by a significant decrease in the AUC0–120 min in Nck1−/− mice (Fig. 3H). In agreement with improved glucose disposal, we observed persistently lower blood glucose levels during insulin tolerance test in obese Nck1−/− mice compared with obese wild-type littermates (Fig. 3I). However, when initial blood glucose values are taken into account, the difference between both obese mouse genotypes is significant only at 15 min following insulin injection (Fig. 3J). This might result mainly from abnormal increased blood glucose levels in obese wild-type mice at this specific time after insulin injection. Higher levels of stress in obese wild-type mice compared with Nck1−/− mice can be responsible for this abnormal elevated blood glucose following insulin injection. Alternatively, this could reflect improved insulin regulation of hepatic glucose output in obese Nck1−/− mice compared with obese wild-type littermates. In agreement with this concept, HFD-fed Nck1−/− mice displayed lower levels of hepatic phosphoenolpyruvate carboxykinase (PEPCK) protein compared
with wild-type mice kept on the same diet (Fig. 3K). Overall, our data reveal that deleting Nck1 is not detrimental for normal control of glucose homeostasis in lean mice, whereas in obese mice Nck1 contributes to impaired glucose homeostasis with only moderate effect on whole body insulin sensitivity.

Mice lacking Nck1 are protected against obesity-induced activation of ER stress signaling. We reported previously that Nck1 modulates ER stress signaling in cell culture (20, 22, 29). Knowing that excessive stress signaling from the ER has been associated with obesity (14, 33), we examined the relative

Fig. 3. Glucose homeostasis in obese Nck1<sup>−/−</sup> mice. A: body weight of Nck1<sup>+/+</sup> (• and ▽) and Nck1<sup>−/−</sup> (○ and △) male littermates maintained on NCD (• and ○) or HFD (○ and △) for 18 wk after weaning. Data are presented as means ± SE (n = 10). For both Nck1<sup>+/+</sup> and Nck1<sup>−/−</sup> mice, HFD vs. NCD (P < 0.001). *Significantly (P < 0.05) different between Nck1<sup>−/−</sup> and Nck1<sup>−/−</sup> on NCD ±12 wk postweaning. B: fed and overnight (O/N)-fasted blood glucose levels and fed insulin levels in Nck1<sup>−/−</sup> (black bars) and Nck1<sup>−/−</sup> mice (gray bars) at 1 mo of age and after 18 wk on NCD. Data are presented as means ± SE (n = 9). C: glucose tolerance tests performed following intraperitoneal (ip) injection with t-glucose (2 g/kg body wt) on O/N-fasted Nck1<sup>−/−</sup> (●) and Nck1<sup>−/−</sup> (○) mice kept on NCD for 18 wk. Data are presented as means ± SE (n = 8). C, inset: area under the curve between 0 and 120 min (AUC<sub>0–120 min</sub>) of the plasma glucose shown. Black and gray bars represent NCD-fed Nck1<sup>−/−</sup> and Nck1<sup>−/−</sup> mice, respectively. *Significantly different (P < 0.05) between Nck1<sup>−/−</sup> and Nck1<sup>−/−</sup>. D: insulin tolerance tests were performed following ip injection with insulin (0.75 IU/kg body wt) to 5-h-fasted Nck1<sup>−/−</sup> (●) and Nck1<sup>−/−</sup> (○) mice maintained on a NCD for 18 wk. Data are the mean ± SE of glucose levels normalized according to initial blood glucose for Nck1<sup>−/−</sup> (●) and Nck1<sup>−/−</sup> (○) mice (n = 10). E: blood glucose levels in Nck1<sup>−/−</sup> (black bars) and Nck1<sup>−/−</sup> (gray bars) at 1 mo of age and after 18 wk on a HFD. Data are presented as means ± SE (n = 10). F: blood glucose levels in Nck1<sup>−/−</sup> (black bars) and Nck1<sup>−/−</sup> (gray bars) mice fed a HFD for 18 wk and after an O/N fast period. Data are means ± SE (n = 10). G: glucose tolerance tests performed following ip injection with t-glucose (1 g/kg body wt) on O/N-fasted Nck1<sup>−/−</sup> (●) and Nck1<sup>−/−</sup> (○) mice kept on a HFD for 18 wk. Data are means ± SE (n = 10). *Significantly different (P < 0.05) between Nck1<sup>−/−</sup> and Nck1<sup>−/−</sup>. H: AUC<sub>0–120 min</sub> of the plasma glucose shown in G. Black and gray bars represent obese Nck1<sup>−/−</sup> and Nck1<sup>−/−</sup> mice, respectively. *Significantly different (P < 0.005) compared with Nck1<sup>+/+</sup> mice. I: insulin tolerance tests were performed following ip injection with insulin (0.75 IU/kg body wt) to 5-h-fasted Nck1<sup>−/−</sup> (●) and Nck1<sup>−/−</sup> (○) mice maintained on a HFD for 18 wk. Data are means ± SE (n = 10). *Significantly different (P < 0.005) between Nck1<sup>−/−</sup> and Nck1<sup>−/−</sup>. J: data from I normalized according to initial blood glucose for Nck1<sup>−/−</sup> (●) and Nck1<sup>−/−</sup> (○) mice. Data are means ± SE (n = 10). *Significantly different (P < 0.05) between Nck1<sup>−/−</sup> and Nck1<sup>−/−</sup>. K: protein expression levels of phosphoenuo2pyruvate carboxykinase (PEPCK) in liver in HFD-fed Nck1<sup>−/−</sup> and Nck1<sup>−/−</sup> mice. Equivalent amounts of protein (75 μg) were used to probe the expression of PEPCK in liver homogenates by direct immunoblotting. RasGAP immunoblotting was performed as loading control.
cerned levels of eIF2α compared with NCD-fed wild-type mice, we observed increased IRE1α phosphorylation at Thr183/Tyr185, recognized to parallel JNK activation, observed in liver of HFD-fed wild-type mice and wild-type littermates. As reported by others (33), we believe that PERK expression levels did not change in livers of wild-type mice between diets. Although it is yet to be demonstrated, we propose that the apparent decrease in PERK reflects rather lower affinity of the anti-PERK antibody used for the phosphorylated/active than for the unphosphorylated/inactive PERK. Nonetheless, increased phosphorylation of eIF2α and JNK, in agreement with activation of IRE1α, strongly suggests HFD-induced ER stress, resulting in hyper-activation of the UPR in liver. Phosphorylation of eIF2α and JNK activation were also markedly increased in adipose tissue of HFD-fed wild-type mice compared with NCD-fed mice, suggesting activation of ER stress signaling in this tissue as well (Fig. 4B). In contrast, although Nck1−/− mice fed on a HFD still displayed signs of diet-induced ER stress in liver and adipose tissue, this was significantly attenuated compared with HFD-fed wild-type mice, as established by significantly lower

Fig. 4. Endoplasmic reticulum (ER) stress signaling in Nck1−/− mice. A: direct immunoblotting with indicated antibodies of liver homogenates (75 μg) from Nck1−/− and Nck1−/− mice kept on NCD or HFD for 18 wk. Quantitative analysis of eIF2α Ser51 and JNK phosphorylation is expressed as the ratio of the phosphorylated form over total levels of the protein. To assemble the final representative figure, p-JNK and JNK WBs from Nck1−/− NCD and HFD samples are presented by spliced figures to delete an empty lane introduced between these samples during the loading of the gel. Data are means ± SE (n = 6). *Significantly different (P < 0.001) between Nck1−/− (gray bars) and Nck1−/− (black bars). C/EBP homologous protein (CHOP) mRNA levels in liver of Nck1−/− (black bar) and Nck1−/− (gray bar) mice kept on HFD for 18 wk as determined by semiquantitative RT-PCR. Data were normalized to GAPDH mRNA levels determined in parallel reactions and are means ± SE (n = 4). B: direct immunoblotting with indicated antibodies of adipose tissue homogenates (75 μg protein) from Nck1−/− and Nck1−/− mice fed a NCD or HFD. Quantitative analysis of eIF2α Ser51 and JNK phosphorylation is expressed as the ratio of the phosphorylated form over total levels of the protein. Data are means ± SE (n = 6). Refer to comment above in A regarding the use of spliced figures. *Significantly different (P < 0.001) between Nck1−/− (gray bars) and Nck1−/− (black bars). C: direct immunoblotting with indicated antibodies of skeletal muscle homogenates (75 μg protein) from Nck1−/− and Nck1−/− mice fed a NCD or HFD. Shown are results representative of 6 mice for each genotype and diet.
levels of phosphorylated eIF2α Ser51 and JNK phosphorylation/activation (Fig. 4, A and B). Further supporting attenuated UPR in liver of HFD-fed Nck1−/− mice, PERK levels as detected with our anti-PERK antibody were not changed, and phosphorylation of IRE1α Ser724 was importantly reduced. Although not statistically significant (P = 0.05), mRNA levels of CHOP, a transcription factor induced by various cellular stress, including ER stress (31), exhibited similar trends of being lower in liver of HFD-fed Nck1−/− mice (Fig. 4A).

In obese mice, hyperactivation of the IRE1α-JNK signaling pathway has been reported to create insulin resistance in liver by directly increasing phosphorylation of IRS-1 at its inhibitory site, Ser307 (33). Given that HFD-fed Nck1−/− mice presented lower levels of activation of the IRE1α-JNK pathway, we predicted that IRS-1 phosphorylation at Ser307 in liver of HFD-fed Nck1−/− mice would be decreased compared with HFD-fed wild-type mice. Indeed, we found lower levels of IRS-1 Ser307 phosphorylation in liver of obese Nck1−/− mice compared with obese wild-type littermates (Fig. 5A). Unexpectedly, we detected marked upregulation of IRS-1 protein levels in liver of obese Nck1−/− mice compared with obese wild-type mice (Fig. 5A). Consequently, IRS-1 Ser307 phosphorylation normalized for total IRS-1 levels was significantly reduced in obese Nck1−/− mice compared with obese wild-type mice (Fig. 5A), demonstrating that decreased IRE1α-JNK signaling in liver of obese Nck1−/− mice correlates with lower levels of IRS-1 phosphorylated at Ser307. IRS-1 expression levels did not differ between NCD-fed mice of both genotypes, and IRS-1 Ser307 phosphorylation was almost undetectable (Fig. 5B). Altogether, these findings establish that Nck1 deficiency in mice impairs obesity-induced signaling from the ER and correlates with lower levels of IRS-1 phosphorylated at Ser307, even though expression levels of IRS-1 are increased.

**Improved insulin signaling in liver of obese mice lacking Nck1.** To determine whether reduced IRE1α-JNK signaling in obese Nck1−/− mice attenuates insulin resistance in peripheral tissues, we compared insulin signaling in liver, adipose tissue, and skeletal muscle of obese wild-type and Nck1−/− mice. In liver, insulin signaling in obese Nck1−/− mice was improved as shown by significantly higher levels of insulin-induced Akt phosphorylation at Ser473 compared with obese wild-type mice (Fig. 6A). In contrast, insulin-stimulated Akt phosphorylation at Ser473 in adipose tissue and skeletal muscle in obese Nck1−/− mice, although they exhibited similar trends, were not found to differ significantly compared with wild-type mice (Fig. 6, B and C). As shown in Fig. 6, there was no difference in insulin-induced Akt phosphorylation at Ser473 in liver, adipose tissue, and skeletal muscle between NCD-fed mice of either genotype, revealing that, in physiological conditions, Nck1 is not critical in regulating insulin signaling. However, we regularly noticed increased levels of phosphorylated Akt at Ser473 in liver and adipose tissue in fasted NCD-fed Nck1−/− mice compared with NCD-fed wild-type mice in the same condition, suggesting that Nck1 might contribute to control basal levels of Akt activity in a subset of insulin-sensitive tissues. Finally, improved insulin signaling in liver of HFD-fed Nck1−/− mice occurs downstream of the insulin receptor since no difference in insulin-induced insulin receptor tyrosine phosphorylation could be observed between obese mice of both genotypes (Fig. 6A). These results strongly suggest that compared with obese wild-type mice, insulin signaling in liver is significantly improved in obese Nck1−/− mice, whereas it was not significantly changed in adipose tissue and skeletal muscle. Altogether, this argues in favor of a role for Nck1 in obesity-promoting ER stress signaling that impacts insulin signaling downstream of the insulin receptor in a subset of peripheral tissues. In addition, our study not only further supports an inverse correlation between abnormal activation of ER stress signaling and insulin signaling in liver but also suggests that the mechanism underlying insulin resistance in other tissues may differ compared with liver.

**Downregulation of Nck1 expression in HepG2 cells improved IRE1α-JNK signaling and reduced phosphorylation of IRS-1 at Ser307.** To establish whether the protective effect of deleting Nck1 in mice on obesity-induced ER stress signaling results from a defect in liver per se, we determined the consequences of downregulating expression levels of Nck1 on thapsigargin-induced IRE1α and JNK activation in the human hepatocellular carcinoma HepG2 cell line. Transfection of
Nck1 siRNA efficiently reduced Nck1 expression levels by >90% in HepG2 cells, whereas expression of RasGAP, another SH2/SH3 domain-containing protein, was not affected (Fig. 7A). Lowering Nck1 expression did not alter protein levels of either IRE1α or JNK but drastically reduced IRE1α and JNK phosphorylation in response to 30- and 120-min thapsigargin treatment (Fig. 7, A and B). Impaired activation of IRE1α in Nck1-depleted cells was also consistent with lower levels of XBP-1 splicing following 30 and 60 min of thapsigargin exposure (Fig. 7C). These effects were observed with three independent Nck1 siRNA oligonucleotides, indicating that this was unlikely to be due to nonspecific effects (data not shown). Moreover, lowering Nck1 levels by siRNA in HepG2 cells seems to attenuate absolute levels of thapsigargin-induced phosphorylation of IRS-1 at Ser307 (Fig. 7D). However, consistent with our findings in liver of obese Nck1−/− mice (Fig. 5A), total levels of IRS-1 protein increased significantly in cells treated with Nck1 siRNA (Fig. 7D). Therefore, increased expression of IRS-1 largely contributes to significantly reduce the pool of IRS-1 phosphorylated at Ser307, as demonstrated by a decreased ratio of p-IRS-1 Ser307 over total levels of IRS-1 in Nck1 siRNA-treated HepG2 cells (Fig. 7D). However, this does not exclude the possibility that, in the absence of Nck1, attenuation of JNK activity in an ER stress condition contributes to a decrease in phosphorylation of IRS-1 at Ser307 even if IRS-1 expression levels were upregulated. Taken together, these results indicate that in liver cells Nck1 contributes to ER stress-mediated activation of the IRE1α-JNK signaling pathway that generates an important pool of IRS-1 phosphorylated at its inhibitory site Ser307. In addition, our findings reveal that Nck1 is part of a mechanism that controls expression levels of IRS-1.

Downregulation of Nck1 expression in HepG2 cells improves insulin signaling. To determine whether Nck1 affects insulin signaling, we depleted HepG2 cells of Nck1 and monitored insulin-induced Akt phosphorylation at Ser473 by Western blotting. As shown in Fig. 8A, downregulation of Nck1 enhanced phosphorylation levels of Akt at Ser473 following insulin stimulation for 5 min at 10 and 100 nM. Similar effects were observed by monitoring phosphorylation of Akt at Thr308, further supporting the observation that decreased expression of Nck1 improves insulin-induced activation of Akt in hepatocytes. In agreement, we found higher levels of basal and insulin-induced tyrosine-phosphorylated IRS-1 in Nck1 siRNA-treated HepG2 cells compared with control (Fig. 8B). In contrast, insulin-induced tyrosine phosphorylation of the insulin receptor was comparable in both siRNA-treated HepG2 cells (Fig. 8B), ruling out that change in insulin receptor activation is the cause of enhanced insulin signaling observed in Nck1-depleted HepG2 cells. Confirming that IRS-1-dependent downstream signaling was enhanced in Nck1-depleted cells, insulin-induced GSK-3β phosphorylation was increased in Nck1 siRNA-treated cells compared with control cells (Fig. 8C), and this correlates with higher insulin-induced phosphorylation of IRS-1 at Ser307 even if IRS-1 expression levels were upregulated. Taken together, these results indicate that in liver cells Nck1 contributes to ER stress-mediated activation of the IRE1α-JNK signaling pathway that generates an important pool of IRS-1 phosphorylated at its inhibitory site Ser307. In addition, our findings reveal that Nck1 is part of a mechanism that controls expression levels of IRS-1.
glycogen synthase activity in HepG2 cells depleted from Nck1 (Fig. 8D). Interestingly, insulin-induced GSK-3β phosphorylation in liver of Nck1−/− mice appeared to be enhanced compared with control littermate mice (Fig. 8E), further supporting our data from HepG2 cells. Altogether, our findings reveal that HepG2 cells lacking Nck1 display enhanced insulin signaling due to increased IRS-1 tyrosine phosphorylation and enhanced Akt activation.

**DISCUSSION**

In the context of obesity, a central role for JNK in mediating insulin resistance has been reported previously (17, 39). More recently, a molecular link between obesity and JNK activation, where obesity-induced ER stress in metabolically active tissues results in hyperactivation of the UPR (33), has been identified. It then became clear that in the UPR signaling network, IRE1α plays a predominant role in mediating JNK activation that leads to phosphorylation of IRS-1 at Ser307 and impairs insulin signaling and actions (33). In the current study, using dietary-induced obese mice, we provided data indicating that Nck1 regulates the activation of IRE1α, which mediates JNK activation and leads to IRS-1 Ser307 phosphorylation. Indeed, we showed that, compared with obese Nck1+/− mice, obese Nck1−/− mice displayed attenuated levels of IRE1α and JNK activation and IRS-1 Ser307 phosphorylation in liver. In parallel, we discovered that obese Nck1−/− mice present improved insulin-induced Akt phosphorylation in liver, whereas insulin signaling in adipose tissues and skeletal muscle was not significantly affected. It is worth noting, however, that no sign of hyperactivation of the UPR was observed in skeletal muscle of obese mice (33), suggesting that, in contrast to what we found in liver, the mechanism underlying obesity-induced insulin resistance in this tissue is ER stress signaling independent. However, this concept is challenged by a recent study demonstrating that the UPR is activated in skeletal muscle by high-fat feeding (12). This difference could be attributed to the type of skeletal muscle analyzed, but additional investigations are required to establish this discrepancy.

Compelling evidence has shown that enhancing ER functions, thereby decreasing ER stress, by genetic approaches or...
chemical chaperones markedly improves insulin signaling and actions in obese animal models (32, 34, 38, 42). In agreement with this paradigm, attenuated UPR and improved insulin signaling in livers of obese Nck1/H11002/H11002 mice has a substantial impact on systemic glucose homeostasis, as shown by lower fasting blood glucose levels and higher rate of blood glucose disposal compared with obese Nck1/H11001/H11001 mice. However, whole body insulin sensitivity of obese Nck1/H11002/H11002 mice did not appear to be markedly different from obese Nck1/H11001/H11001 mice. This apparent discrepancy between overall glucose disposal and insulin sensitivity can be explained by the fact that insulin tolerance tests are primarily an indication of glucose disposal by skeletal muscle. Given that there is no indication of improved insulin signaling in this tissue in obese Nck1/−/− mice, it may not be surprising to find no change in whole body insulin sensitivity in the insulin tolerance test. Moreover, compromised stress-induced IRE1α-dependent JNK activation and IRS-1 Ser307 phosphorylation are observed in Nck1-depleted HepG2 cells in addition to Nck1-deficient liver tissue, but not in skeletal muscle. This suggests that the effect of Nck1 deletion in attenuating UPR signaling is cell autonomous and tissue specific. Taken altogether, our data further support a physiological role for Nck1 in the regulation of the UPR in liver by revealing that Nck1/−/− mice are protected against cellular ER stress, which induces defects in glucose disposal and insulin signaling secondary to obesity.

Herein, we have demonstrated that Nck1 deficiency exerts a marked inhibitory effect on IRE1α-JNK activation and signaling in liver secondary to obesity in mice and in response to thapsigargin in HepG2 cells. These findings strongly support a significant role for Nck1 in mediating the UPR. At the molecular level, different proteins have been shown to regulate IRE1α activation and signaling in mammalian cells (2, 26, 27, 30). Among these, Bax inhibitor 1 (BI-1), an ER membrane protein that interacts with and inhibits IRE1α (26), has recently been reported to protect mice from obesity-induced insulin resistance and glucose intolerance when overexpressed in liver (1). Like BI-1, Nck1 interacts with and modulates IRE1α (29). However, in contrast to BI-1, our study indicates that Nck1 contributes to IRE1α activation and sig-

Fig. 8. Insulin signaling in Nck1-downregulated HepG2 cells. A–C: HepG2 cells transiently transfected with indicated siRNA were treated with insulin at 10 or 100 nM for 5 min. Clarified cell lysates were analyzed for protein phosphorylation and expression levels using indicated specific antibodies. Results were expressed as the ratio of indicated protein phosphorylated over total levels. Bar charts represent quantitative analysis of the data from 3 independent experiments. Statistical analysis was performed by comparing data from Nck1 siRNA (gray bars) to control siRNA (black bars) in the same condition. *Significantly different (P < 0.01), Nck1 siRNA vs. control siRNA. D: insulin-induced (100 nM, 6 h) glycogen synthesis in HepG2 cells transiently transfected with indicated siRNA was measured as described previously. Statistical analysis was performed by comparing data from Nck1 siRNA (gray bars) with control siRNA (black bars). Bar chart represents a typical immunoblot of 4 experiments performed in triplicate. *Significantly different (P < 0.01), Nck1 siRNA (gray bars) vs control siRNA (black bars). E: insulin-induced phosphorylation of GSK-3β at Ser9 in liver of Nck1+/+ and Nck1−/− mice. Liver homogenates from insulin-stimulated mice were analyzed for GSK-3β Ser9 phosphorylation, as described in MATERIALS AND METHODS. Shown is a typical immunoblot. Bar chart represents the ratio of phosphorylated GSK-3β normalized to total content of GSK-3β from 3 independent experiments. Data are means ± SE. *Significantly different (P < 0.01), Nck1−/− vs. Nck1+/+.
naling associated with altered glucose metabolism secondary to obesity. On the other hand, IRE1α signaling is specifically potentiated by the ER-localized tyrosine phosphatase protein tyrosine phosphatase 1B (PTP1B) (13, 15), although PTP-1B interaction with IRE1α has not yet been established. Interestingly, Dock, the drosophila homolog of Nck, interacts via its SH3 domains with the tyrosine phosphatase dPTP61F, the drosophila homolog of PTP1B (10). Therefore, although additional experiments are required to test this hypothesis, it is possible that Nck1 modulates IRE1α activation and signaling in concert with PTP1B.

Our study is the first to reveal the interesting contribution of Nck1 in regulating IRS-1 protein expression levels. Combined with decreased IRE1α-induced JNK activation, this argues that increased levels of IRS-1 could improve insulin signaling in liver of obese Nck1+/− mice by increasing the pool of IRS-1 available for efficient phosphorylation by the insulin receptor. How Nck1 participates in the regulation of IRS-1 protein levels is still unknown. The simplest model that can be suggested is that Nck1, by promoting the PERK-eIF2α pathway, could contribute to attenuated IRS-1 translation under conditions that challenge the ER function, and this would be lost upon Nck1 deletion. Alternatively, Nck has been found to interact with IRS-1 (23) and the suppressor of cytokine signaling 1 (SOCS1) (11). Murine deletion of SOCS1, a regulator of IRS-1 ubiquitination and degradation, has been shown to enhance hepatic insulin signaling (19). Nck1 could regulate IRS-1 expression levels, stability, and signaling through its interaction with SOCS1, and this negative regulation would be lost upon Nck1 deletion. Future studies will help shed light on this potential regulatory mechanism.

Finally, using Nck isoform specific antibodies, we demonstrated that expression of both Nck proteins varies among different tissues, as proposed previously from indirect tissue staining analysis of Nck in Nck1+/− and Nck2+/− mice using a Nck antibody that recognizes both Nck proteins (4). Failure to detect Nck2 in liver and adipose tissue suggests that Nck1 is the predominant Nck form expressed in these tissues. Considering this, it will be interesting to establish whether Nck2+/− mice will be also protected against ER stress-induced impaired glucose disposal and insulin resistance secondary to obesity.

In summary, our study clearly establishes an important role for Nck1 in IRE1α activation and signaling in mice subjected to a physiological stress like a high-fat diet. Interestingly, other studies have reported changes in Nck expression in obese insulin-resistant KKA2 mice (6). The significance of such variations in Nck expression levels on glucose homeostasis still requires further investigation, but our study clearly indicates that Nck1 contributes to the development of impaired glucose tolerance and decreased hepatic insulin signaling secondary to obesity.

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REFERENCES


ER STRESS AND INSULIN SIGNALING


