Differential Insulin Receptor Substrate-1 (IRS1)-Related Modulation of Neuropeptide Y and Proopiomelanocortin Expression in Nondiabetic and Diabetic IRS2<sup>-/-</sup> Mice

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Insulin resistance and type 2 diabetes correlate with impaired leptin and insulin signaling. Insulin receptor substrate-2 deficient (IRS2<sup>-/-</sup>) mice are an accepted model for the exploration of alterations in these signaling pathways and their relationship with diabetes; however, disturbances in hypothalamic signaling and the effect on neuropeptides controlling food intake remain unclear. Our aim was to analyze how leptin and insulin signaling may differentially affect the expression of hypothalamic neuropeptides regulating food intake and hypothalamic inflammation in diabetic (D) and nondiabetic (ND) IRS2<sup>-/-</sup> mice. We analyzed the activation of leptin and insulin targets by Western blotting and their association by immunoprecipitation, as well as the mRNA levels of neuropeptide Y (NPY), proopiomelanocortin, and inflammatory markers by real-time PCR and colocalization of forkhead box protein O1 (FOXO1) and NPY by double immunohistochemistry in the hypothalamus. Serum leptin and insulin levels and hypothalamic Janus kinase 2 and signal transducer and activator of transcription factor 3 activation were increased in ND IRS2<sup>-/-</sup> mice. IRS1 levels and its association with Janus kinase 2 and p85 and protein kinase B activation were increased in ND IRS2<sup>-/-</sup>. Increased FOXO1 positively correlated with NPY mRNA levels in D IRS2<sup>-/-</sup> mice, with FOXO1 showing mainly nuclear localization in D IRS2<sup>-/-</sup> and cytoplasmic in ND IRS2<sup>-/-</sup> mice. D IRS2<sup>-/-</sup> mice exhibited higher hypothalamic inflammation markers than ND IRS2<sup>-/-</sup> mice. In conclusion, differential activation of these pathways and changes in the expression of NPY and inflammation may exert a protective effect against hypothalamic deregulation of appetite, suggesting that manipulation of these targets could be of interest in the treatment of insulin resistance and type 2 diabetes. (Endocrinology 153: 0000–0000, 2012)

Abbreviations: Akt, Protein kinase B; CD, Cluster of differentiation; D, diabetic; FOXO1, forkhead box protein O1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; JAK, Janus kinase; ND, nondiabetic; IRS, insulin receptor substrate; JNK, c-Jun N-terminal kinase; NEFA, nonesterified fatty acids; NFκB, nuclear factor κB; NPY, neuropeptide Y; ObRb, long form of the leptin receptor; P3K, phosphatidylinositol 3-kinase; PDMC, proopiomelanocortin; PTEN, phosphatase and tensin homolog deleted on chromosome 10; PTP1B, tyrosine phosphatase type 1B; RT, reverse transcription; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription; WT, wild type.
The loss of expression of genes encoding insulin-signaling mediators provokes defects similar to those reported in insulin resistance and type 2 diabetes (1). Most studies have analyzed the molecular mechanisms of insulin resistance in peripheral tissues, with few reports showing the effects on hormone signaling in the brain (2). The leptin and insulin signaling pathways are interconnected at different levels, including insulin receptor substrates (IRS) and phosphatidylinositol 3-kinase (PI3K) (3). Leptin mimics the ability of insulin to promote glucose uptake through the activation of Janus kinase 2 (JAK2) that phosphorylates IRS2, activating PI3K. In addition, administration of PI3K inhibitors blocks the ability of leptin and insulin to reduce food intake (4), suggesting that PI3K signaling is implicated in the central actions of both hormones.

Although insulin’s actions are primarily mediated through IRS2, some data suggest that IRS1 is pivotal in mediating insulin’s actions in peripheral tissues when IRS2 is absent (5, 6). PI3K is especially activated in insulin target cells that express IRS (7), but it can also be activated in response to different hormones acting through the JAK2 pathway (8), as does leptin, further reinforcing the link between leptin and insulin signaling pathways.

Leptin and insulin regulate food intake by increasing anorexigenic and diminishing orexigenic pathways in the hypothalamus. Activation of insulin signaling in the hypothalamus reduces food intake by negatively regulating the forkhead box protein O1 (FOXO1), a transcription factor that stimulates the expression of neuropeptide Y (NPY) and inhibits the expression of proopiomelanocortin (POMC) (9), whereas leptin-stimulated signal transducer and activator of transcription factor 3 (STAT3) activation in POMC neurons also plays a key role in the maintenance of energy balance (10). A significant proportion of IRS2-deficient mice develop diabetes abruptly at 12–16 wk of age, whereas others present less deterioration of glucose homeostasis yet not leading to diabetes at this age. Increased food intake has been reported in diabetic IRS2-deficient (IRS2−/−) mice (11), as well as in mildly glucose-intolerant IRS2−/− females (12). Food intake regulates nucleus-cytoplasmic translocation of FOXO1 in hypothalamic neurons (13); therefore, it is likely that disturbances in insulin signaling may be related to high levels and nuclear localization of this transcription factor.

IRS2-deficient mice have been widely considered as an excellent model to study the development of diabetes because they present a sudden increase in blood glucose similar to that observed in fulminant diabetes onset in humans. The appearance of diabetes seems to be due to insufficient β-cell proliferation in conjunction with hepatic insulin resistance (14), although the hypothalamus may play a key role for the early onset of diabetes as it is involved in the control of glucose homeostasis and energy balance (15, 16). Changes in food intake and obesity in IRS2−/− mice could be more closely related to central signaling defects, because insulin signaling is impaired in the hypothalamus of IRS2−/− mice (17). Thus, alterations of leptin/insulin signaling in the hypothalamus could be a cause of the obese phenotype reported in these mice (2).

It has been reported that hypothalamus is a key target for inflammation (18, 19), and this activation is mainly attributed to the release of cytokines (20). During the last decade, type 2 diabetes has been linked to a low grade of hypothalamic inflammation. A proinflammatory cytokine profile has been associated with increased insulin resistance often found with aging (21). Moreover, the intracerebroventricular injection of TNF-α or IL blunts the effects of anorexigenic hormones (22) and induces hypothalamic inflammation, reproducing some clinical features of this metabolic disease (23). Hence, a central injection of TNF-α also causes an increase in baseline plasma insulin levels and insulin secretion by the pancreas, leading to defective pancreatic islet function (24). In addition, hypothalamic inflammation can impair hepatic insulin signaling, a key factor involved in the appearance of type 2 diabetes (25), reinforcing again the possible role of the hypothalamus in the onset of diabetes. Activation of the PI3K pathway inhibits inflammatory processes induced in animal models (26), and antiinflammatory drugs increase protein kinase B (Akt) phosphorylation (27), whereas a burst of Akt activation has been shown to occur in neurons exposed to nuclear factor κB (NFκB) inhibitors (28). These findings suggest that modifications of both signaling pathways not only contribute to changes in food intake, but also to hypothalamic inflammation associated with insulin resistance.

The goal of this study was 2-fold. First, we have analyzed the association between the activation of the leptin and insulin signaling pathways and the hypothalamic expression of neuropeptides that regulate food intake in diabetic and nondiabetic IRS2-deficient mice. Second, we have evaluated changes in hypothalamic inflammation parameters and their possible relationship with these signaling pathways in both groups of IRS2-deficient mice.

Materials and Methods

All studies were approved by the local ethics committee and complied with the Royal Decree 1201/2005 (Boletín Oficial del Estado, BOE no. 252) pertaining to the protection of experi-

Animals  
Wild-type (WT) and IRS2<sup>−/−</sup> mice, maintained on a similar mixed genetic background (C57BL/6 × 129sv), were purchased from The Jackson Laboratory (Bar Harbor, ME). Thirty-six adult, 11- to 12-wk-old male mice were individually caged and maintained on a 12-h light, 12-h dark cycle and fed standard chow and water ad libitum. Three groups of mice were used: 12 WT mice as controls, 12 diabetic IRS2<sup>−/−</sup> (D IRS2<sup>−/−</sup>) with nonfasting glucose levels over 27.75 mmol/liter (determined by the glucose oxidase method by using the Precision G glucose meter; Abott Laboratories, North Chicago, IL), and 12agematched non-diabetic mice IRS2<sup>−/−</sup> (ND IRS2<sup>−/−</sup>) with glucose levels under 11.10 mmol/liter. Food intake and body weight were measured daily at the same hour. Six to seven days after the debut of diabetes, animals were killed by decapitation at 0010 h under nonfasting conditions. Mice from the control and ND IRS2<sup>−/−</sup> groups were killed at the same time as the D IRS2<sup>−/−</sup> mice. In eight animals per group, the hypothalamus was divided in two halves, with one hemihypothalamus being used for immunoprecipitation and Western blot or bead array assay and the other for RNA extraction. Four animals were perfused with 4% paraformaldehyde, and the hypothalamus was isolated for immunohistochemistry. Trunk blood was collected in cooled tubes and centrifuged at 3000 g until the hormone levels were measured.

Metabolic studies  
Energy expenditure was measured by using an indirect open-circuit calorimeter (Oxylet, Panlab, Barcelona, Spain). Six mice per group were housed in individual metabolic chambers with acclimatization for 24 h before data were registered for 3 min every 20 min for 48 h. Animals had free access to food and water. Locomotor activity was measured by continuous recording of spontaneous activity by extensiometric weight transducers placed below the cages, which allow detection of activity even without displacement. The number of rearings was monitored by means of an infrared frame beam-interruption method. Calculations were performed with the Metabolism 2.2.01 software (Panlab).

Immunoprecipitation  
The associations between targets were studied by immunoprecipitation. The half of each hypothalamus was homogenized on ice in 500 µl of lysis buffer (pH 7.6) containing 50 mM HEPES, 10 mM EDTA, 50 mM sodium pyrophosphate, 100 mM NaF, 10 mM Na<sub>3</sub>VO<sub>4</sub>, 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, and 10 µg/ml aprotinin. Protein (500 µg) was immunoprecipitated overnight at 4 C with the corresponding antibodies and then incubated with protein A-agarose beads (Roche, Mannheim, Germany) for 2 h at 4 C. Immunocomplexes were washed three times with lysis buffer, extracted for 5 min at 95 C in 4 × SDS-PAGE sample buffer (200 mM Tris-HCl, 12% sodium dodeyl sulfate, 4 mM EDTA, 8% 2-mercaptoethanol, 20% glycerol) and analyzed by Western blotting.

Western blotting  
The lysates were incubated overnight at −80 C and then clarified by centrifugation at 12,000 × g for 5 min at 4 C. The supernatants were transferred to fresh tubes and stored at −80 C until assayed. Total protein concentration was determined by the method of Bradford (Bio-Rad Laboratories, Hercules, CA). The proteins were resolved on a 10% SDS-PAGE and then transferred to polyvinyl difluoride membranes. Membranes were blocked with Tris-buffered saline containing 0.1% Tween 20 (TTBS) containing 5% (wt/vol) BSA during 2 h at 25 C. Primary antibodies included antiphosphorylated (p)-JAK2, p-Tyr705-STAT3, p-Thr308-Akt, p-Ser473-Akt, anti-FOXO1, anti-pSer468-RelA, IкB-α, RelA, and anti-suppressor of cytokine signaling 3 (SOCS3) from Cell Signaling Technology (Danvers, MA); anti-JAK2, anti-protein tyrosine phosphatase type 1B (PTP1B), and anti-p85 from Millipore Corp. (Billerica, MA); anti-Akt and anti-IRβ from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-STAT3 from R&D Systems (Minneapolis, MN); anti-phosphatase and tensin homolog deleted on chromosome 10 (PTEN) and anti-IRS-1 and -4 from Upstate Biotechnology (Lake Placid, NY). All membranes were incubated with the primary antibodies (diluted 1:1000) in blocking buffer at 4 C overnight. The membranes were washed three times with TTBS and incubated with the corresponding secondary antibody conjuga-
gated with peroxidase (Thermo Fisher Scientific, Inc., Waltham, MA) at a dilution of 1:2000 in nonfat milk during 90 min at 25 C. The proteins were detected by chemiluminescence using an ECL system (PerkinElmer Life Sciences, Boston, MA) and quantified by densitometry using a Kodak Gel Logic 1500 Image Analysis system and Molecular Imaging Software version 4.0 (Eastman Kodak, Rochester, NY). All blots were reblotted with anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; AnaSpec, San Jose, CA) to normalize each sample for gel-loading variability.

**Bead array assay**

The phosphorylated form of c-Jun N-terminal kinase (JNK) and total content were measured as previously described (29). Beads with fluorescent-labeled antigen conjugated to the antibody and tissue lysates (50 μl each) were incubated for 18 h at room temperature. Wells were washed, and antibody conjugated to biotin (25 μl) was added. After 30 min, beads were incubated during 30 min with 50 μl streptavidin conjugated to phycoerythrin (streptavidin- phycoerythrin, diluted 1:100). Beads were analyzed in a Bio-Plex suspension array system 200 (Bio-Rad Laboratories, Hercules, CA). Raw data (mean fluorescence intensity) were analyzed using the Bio-Plex Manager software 4.1 (Bio-Rad Laboratories).

**RNA extraction, reverse transcription (RT), and real-time PCR**

Total RNA was extracted according to the TRI-REAGENT protocol (Sigma, Saint Louis, MO). The RT reaction was performed on 2 μg of total RNA using the high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed in an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using TaqMan PCR Master Mix (Applied Biosystems) and the thermocycler parameters recommended by the manufacturer. PCR were performed in duplicate, in a total volume of 50 μl, containing 12.5 μl of the RT reaction. TaqMan gene expression assays were used for ObRb (long form of the leptin receptor), IRS1, NPY, and POMC (Mm00440181, Mm01278327, Mm00445771, and Rn00595020, respectively; Applied Biosystems). Relative TNF-α, IL-1β, IL-6, cluster of differentiation (CD) 11c, CD68, and IL-12p40 mRNA levels were measured with SYBR Green (Roche), and primers were from Sigma. The forward and reverse sequences were the following: 5’-CATCTTCTCAAAATTGCAGTA-3’ and 5’-GGGGTGAGAAGATTCAAC-3’; 5’-GCAACTTGGCTCTGAAATCACT-3’ and ATCTTCTGGGTGGTCGCTCAAC-3’; 5’-TAGTTCCCTTCTACCCCAATTTC and TTGGTCTCTTAGCCACTCCTTC-3’; 5’-GCCAGGATATGTTCACAGC-3’ and 5’-ACACAGTGTGCTCAGATGA-3’; 5’-CAAGTGTCCAGGGAGTGTG-3’ and 5’-CCAAAGGATAGCTGTCGCTAAGA-3’; and 5’-CTTGGCTCTTAGCAGCTCCTTC-3’ and 5’-GCAAGGAGAAGATTCAAC-3’, respectively. The PCR mixture contains 300 nM of each primer. Relative gene expression comparisons were carried out using an invariant endogenous control (GAPDH). According to the manufacturer’s guidelines, the ΔΔC<sub>T</sub> method was used for relative quantification.

**Immunohistochemistry**

Double-fluorescent immunohistochemistry for FOXO1 and NPY was carried out in free-floating vibratome sections (30 μm) in agitation.Sections were washed with Dulbecco’s PBS 1 × (Invitrogen, Auckland, New Zealand) and 0.1 m phosphate buffer (PB), pH 7.4, containing 0.3% BSA, 0.3% Triton X-100 (PBT) three times each. All subsequent washes were carried out with PBT three times. Sections were blocked for 90 min at room temperature in PB with 0.3% Triton X-100 and 3% BSA and incubated o/n at 4 C with anti-FOXO1 (1:250) and anti-NPY (1:100, Peninsula Laboratories, San Carlos, CA) with blocking buffer. Sections were then washed and incubated for 90 min at room temperature with antigen IgG biotin (1:1000) with blocking buffer. Afterward sections were washed and incubated under dark conditions with streptavidin, Alexa Fluor 488 (1:1000, Molecular Probes, Leiden, The Netherlands), and Alexa Fluor 633 antirabbit IgG (1:1000, Molecular Probes) in blocking buffer for 90 min at room temperature and washed three times with PB. Sections were mounted and cover slipped with Clear Mount (Electronic Microscopy Sciences, Hatfield, PA). Immunofluorescence was visualized directly by using a DM IRB confocal microscope (Leica, Wetzlar, Germany).
Serum leptin and insulin levels were measured with ELISA kits from Millipore according to the manufacturer’s instructions. The sensitivity was 0.04 ng/ml for leptin and 0.2 ng/ml for insulin. The intra- and interassay variations for leptin were 2.3% and 3.5%, respectively, and for insulin 1.9% and 7.6%, respectively.

** ELISA **

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** Colorimetric methods **

Quantitative determinations of triglycerides and nonesterified fatty acids (NEFA) in serum were performed by enzymatic colorimetric method assays from Randox (Ardmore, UK) and Wako (Neuss, Germany), respectively.

** Statistical analysis **

All results are presented as mean ± SEM. Statistical analysis of all data was carried out by one-way ANOVA followed by a Bonferroni’s test. The values were considered significantly different when P < 0.05. Statistical analyses were performed using Prisma software 4.0 (GraphPad, San Diego, CA).

** Results **

** Food intake, body weight, and metabolic studies **

Food and water intake were increased in D IRS2−/− mice after the debut of diabetes, compared with WT and ND IRS2−/− mice (Fig. 1, A and B, respectively). The body weight of ND IRS2−/− mice was slightly, but significantly, higher than WT and D IRS2−/− mice at time of death (Fig. 1C). Mean energy expenditure was diminished in D IRS2−/− mice compared with WT (Fig. 1D). The locomotor activity (Fig. 1E) and number of rearings (Fig. 1F) were decreased in both groups of IRS2−/− mice.

** Plasma profiles differ in D and ND IRS2−/− mice **

Diabetic IRS2−/− mice had higher blood glucose levels than WT and ND IRS2−/− mice. Blood glucose levels were also higher in ND IRS2−/− mice compared with WT, but significantly lower than D IRS2−/− mice (Fig. 2A). Serum triglyceride levels were increased in D IRS2−/− mice compared with WT and ND IRS2−/− mice, (Fig. 2B), whereas NEFA were augmented in ND IRS2−/− mice with respect to D IRS2−/− mice (Fig. 2C).

Serum leptin levels were reduced in D IRS2−/− mice and increased in ND IRS2−/− mice (Fig. 2D), whereas insulin levels were increased in both IRS2−/− groups, with this increase being greater in ND IRS2−/− mice (Fig. 2E).

** Differential activation of leptin-related signaling pathways in hypothalamus of D and ND IRS2−/− mice **

The hypothalamic mRNA levels of ObRb were not different between the experimental groups (Fig. 3A). ND IRS2−/− mice presented higher levels of JAK2 (Fig. 3B) and STAT3 phosphorylation (Fig. 3C) than D IRS2−/− mice.

** FIG. 3. ** Leptin-related intracellular signaling components in the hypothalamus of WT, D IRS2−/−, and ND IRS2−/− mice. A, Relative levels of the mRNA for the ObRb. Relative protein levels of phosphorylated (p) JAK2 (B), STAT3 phosphorylated on tyrosine 705 (pTyr705-STAT3) (C), and SOCS3 (D). DU, Densitometry units; NS, nonsignificant P = 0.53; *, P < 0.05 by ANOVA.

** FIG. 4. ** Insulin receptor, IRS1, and activation of JNK in the hypothalamus of WT, D IRS2−/−, and ND IRS2−/− mice. A, Relative protein levels of the insulin receptor β chain (IRβ). B, Relative IRS1 protein levels. C, Relative IRS1 mRNA content. D, Relative phosphorylated (p) JNK protein levels. DU, Densitometry units; MFI, mean fluorescence intensity; NS, nonsignificant P = 0.68; *, P < 0.05 by ANOVA.
WT mice. However, levels of SOCS3 were lower in D IRS2\(^{-/-}\) mice compared with both WT and ND IRS2\(^{-/-}\) mice (Fig. 3D).

**Activation of the IRS1/PI3K pathway is higher in the hypothalamus of ND IRS2\(^{-/-}\) compared with D IRS2\(^{-/-}\) mice**

ND IRS2\(^{-/-}\) mice had lower concentrations of insulin receptor (IR)\(\beta\) compared with WT mice (Fig. 4A). However, IRS1 levels were higher in ND IRS2\(^{-/-}\) mice (Fig. 4B). To test whether this increase was due to an increase in protein synthesis and/or protein stability, mRNA levels were measured. No differences in these levels were found among the experimental groups (Fig. 4C). Because the activation of JNK decreases IRS1 stability promoting its degradation (30), we measured the phosphorylation of JNK as an index of its activation. Diabetic IRS2\(^{-/-}\) mice had a higher ratio of activation than WT and ND IRS2\(^{-/-}\) mice, with no differences between these groups (Fig. 4D). No changes in levels of IRS4 were detected among the experimental groups (WT, 100.0 \(\pm\) 14.3; D IRS2\(^{-/-}\), 92.7 \(\pm\) 21.8; and ND IRS2\(^{-/-}\), 81.4 \(\pm\) 10.7, expressed as percentage of densitometry units of WT).

PI3K is a point of convergence between the leptin and insulin pathways, and the association between JAK2 and IRS1 stimulates its kinase activity (31). Accordingly, we studied the association between JAK2 and IRS1. D IRS2\(^{-/-}\) mice presented lower association between JAK2 and IRS1 whereas ND IRS2\(^{-/-}\) mice exhibited higher association than WT mice (Fig. 5A). We then analyzed the association of the regulatory subunit of PI3K (p85) with IRS1. ND IRS2\(^{-/-}\) mice had higher association of these proteins than WT and D IRS2\(^{-/-}\) mice (Fig. 5B).

PTEN is known to be one of the major negative regulators of the PI3K pathway (32). Levels of this phosphatase were higher in D IRS2\(^{-/-}\) mice (Fig. 5C). Both groups of IRS2\(^{-/-}\) mice had increased pAkt-Thr308 levels (Fig. 5D), whereas pAkt-Ser473 was only significantly increased in ND IRS2\(^{-/-}\) mice (Fig. 5E).

PTP1B is a protein tyrosine phosphatase that negatively regulates leptin signaling, as well as insulin sensitivity, by directly interacting with the insulin receptor (33). No differences in PTP1B levels were found among the groups (WT, 100.0 \(\pm\) 6.0; D IRS2\(^{-/-}\), 98.5 \(\pm\) 21.1; and ND IRS2\(^{-/-}\), 97.4 \(\pm\) 5.6, expressed as percentage of densitometry units of WT).

**FOXO1 levels, cellular localization, and relative NPY and POMC mRNA levels differ in D IRS2\(^{-/-}\) and ND IRS2\(^{-/-}\) mice**

D IRS2\(^{-/-}\) mice had higher levels of FOXO1 than the other groups (Fig. 6A). Immunohistochemistry showed that FOXO1 is mainly expressed in the cell nuclei of D IRS2\(^{-/-}\) mice (Fig. 6C) in the arcuate nucleus, whereas it is mostly located in the cytoplasm of WT (Fig. 6B) and ND IRS2\(^{-/-}\) mice (Fig. 6D).

FOXO1 differentially modulates the neuropeptides regulating food intake, increasing the expression of orexigenic peptides and inhibiting anorexigenic factors (10). Levels of NPY mRNA were increased in D IRS2\(^{-/-}\) mice with respect to WT and ND IRS2\(^{-/-}\) mice (Fig. 6E), whereas POMC mRNA levels were lower in D IRS2\(^{-/-}\) mice compared with both WT and ND IRS2\(^{-/-}\) mice (Fig. 6D).
mice compared with WT (Fig. 6F). Double immunohistochemistry for FOXO1 in conjunction with NPY was performed to analyze their coexpression. Figure 6, panels G–O, illustrates that there is colocalization of NPY and FOXO1 in the cytoplasm of WT (Fig. 6, G–I) and ND IRS2−/− mice as compared with D IRS2−/− mice. The increase in both leptin and insulin serum levels in ND IRS2−/− mice could be responsible for the increased activation of their central signaling mechanisms. Another outstanding finding is the absence of changes in NPY and POMC in this group, probably related not only to FOXO1 levels but to its cytosolic location as well, which was similar to that of WT mice. In addition, the absence of a compensatory increase of IRS1 levels in D IRS2−/− mice, together with a lower leptin signaling compared with ND IRS2−/− mice, reduces the inhibitory effect of both signaling pathways on FOXO1-mediated transcription, allowing an orexigenic pattern of neuropeptide expression, concordant with hyperphagia in diabetic mice.

The increased food intake in D IRS2−/− mice is consistent with previous reports (11). Despite increased insulin levels, diabetic mice have low leptin levels, which could account for the increase in food intake (34). This increased food intake is in accordance with the changes in NPY and POMC observed here. Both leptin and insulin regulate the hypothalamic neuropeptides that control food intake (35), and the signaling pathways of these two hormones are interconnected at different levels, such as IRS and PI3K (36). Activation of PI3K in peripheral tissues by leptin has been reported to be dependent on IRS1 association with JAK2 that activates insulin signaling, which could be a protecting factor against diabetes in ND IRS2−/− mice. The increase in both leptin and insulin serum levels in ND IRS2−/− mice could be responsible for the increased activation of their central signaling mechanisms. Another outstanding finding is the absence of changes in NPY and POMC in this group, probably related not only to FOXO1 levels but to its cytosolic location as well, which was similar to that of WT mice. In addition, the absence of a compensatory increase of IRS1 levels in D IRS2−/− mice, together with a lower leptin signaling compared with ND IRS2−/− mice, reduces the inhibitory effect of both signaling pathways on FOXO1-mediated transcription, allowing an orexigenic pattern of neuropeptide expression, concordant with hyperphagia in diabetic mice.

In this study, we found an up-regulation of hypothalamic IRS1 together with an increased interaction between IRS1 and JAK2 that activates insulin signaling, which could be a protecting factor against diabetes in ND IRS2−/− mice. The increase in both leptin and insulin serum levels in ND IRS2−/− mice could be responsible for the increased activation of their central signaling mechanisms. Another outstanding finding is the absence of changes in NPY and POMC in this group, probably related not only to FOXO1 levels but to its cytosolic location as well, which was similar to that of WT mice. In addition, the absence of a compensatory increase of IRS1 levels in D IRS2−/− mice, together with a lower leptin signaling compared with ND IRS2−/− mice, reduces the inhibitory effect of both signaling pathways on FOXO1-mediated transcription, allowing an orexigenic pattern of neuropeptide expression, concordant with hyperphagia in diabetic mice.

The increased food intake in D IRS2−/− mice is consistent with previous reports (11). Despite increased insulin levels, diabetic mice have low leptin levels, which could account for the increase in food intake (34). This increased food intake is in accordance with the changes in NPY and POMC observed here. Both leptin and insulin regulate the hypothalamic neuropeptides that control food intake (35), and the signaling pathways of these two hormones are interconnected at different levels, such as IRS and PI3K (36). Activation of PI3K in peripheral tissues by leptin has been reported to be dependent on IRS1 association with JAK2 and the 85-kDa subunit of PI3K (37). Here we found increased activation of JAK2, as well as higher associations of JAK2-IRS1 and p85-IRS1 in ND IRS2−/− compared with D IRS2−/− mice. Higher serum insulin con-
well (39). Therefore, higher JNK activation in IRS2 mice with mutations consisting in the loss of PTEN function may lead to higher Akt phosphorylation and lower nuclear FOXO1 than in D IRS2 mice. After Akt activation, FOXO1 is phosphorylated, exiting the nucleus (43) and, once in the cytoplasm, it is degraded by an ubiquitin proteasome-mediated system (44). Normal levels of FOXO1 and its cytoplasmic localization in ND IRS2 mice, as well as the inverse pattern found in both levels and localization in D IRS2 mice, correlate with the signaling of leptin and insulin and with the expression of hypothalamic neuropeptides involved in the regulation of food intake. Interestingly, it has been reported that phosphorylation of cytoplasmic FOXO1 at specific sites by JNK initiates the translocation into the nucleus (45). Thus, the higher activation of JNK in D IRS2 mice could also contribute to FOXO1 nuclear localization. D IRS2 mice presented hyperphagia and an orexigenic pattern of these neuropeptides that may be related with an altered signaling of leptin and insulin, as previously reported both in humans and animal models of resistance to both hormones (46, 47). In fact, animals with defective PI3K pathways or with a FOXO1 mutation resulting in constitutively nuclear location, show increased food intake and body weight accompanied by decreased expression of the POMC gene in the hypothalamus (48). On the contrary, inhibition of hypothalamic FOXO1 increases insulin sensitivity and reduces cumulative food intake (49). In turn, the activation of FOXO1 stimulates the transcription of NPY and increases body weight accompanied by decreased expression of the POMC gene in the hypothalamus (48). On the contrary, inhibition of hypothalamic FOXO1 increases insulin sensitivity and reduces cumulative food intake (49). In turn, the activation of FOXO1 stimulates the transcription of NPY and increases food intake (50), as we could also contribute to FOXO1 nuclear localization. D IRS2 mice, which has been shown to report in D IRS2 mice. Despite higher Akt phosphorylation in ND IRS2 mice, which has been shown to increase FOXO1 exportation to the cytoplasm and increase its degradation (51), the levels of FOXO1 found were similar to controls. It must be remembered that FOXO1 levels are regulated not only by phosphorylation, but also by other posttranslational modifications such as ubiquitination and acetylation that might change the stability of this factor (52).

IRS1-related signaling may confer resistance against hypothalamic inflammation in ND IRS2 mice. There was an increase in RelA phosphorylation at Ser-468 and Akt on Ser473 in diabetic mice with respect to ND mice. However, the increase in Akt phosphorylation in Thr308 in ND and D IRS2 mice may be related to glucose levels, because this phosphorylation is regulated by glycemia (42), and both groups were hyperglycemic compared with WT mice.

The pivotal role of increased IRS1 in ND IRS2 mice may lead to higher Akt phosphorylation and lower nuclear FOXO1 than in D IRS2 mice. After Akt activation, FOXO1 is phosphorylated, exiting the nucleus (43) and, once in the cytoplasm, it is degraded by an ubiquitin proteasome-mediated system (44). Normal levels of FOXO1 and its cytoplasmic localization in ND IRS2 mice, as well as the inverse pattern found in both levels and localization in D IRS2 mice, correlate with the signaling of leptin and insulin and with the expression of hypothalamic neuropeptides involved in the regulation of food intake. Interestingly, it has been reported that phosphorylation of cytoplasmic FOXO1 at specific sites by JNK initiates the translocation into the nucleus (45). Thus, the higher activation of JNK in D IRS2 mice could also contribute to FOXO1 nuclear localization. D IRS2 mice presented hyperphagia and an orexigenic pattern of these neuropeptides that may be related with an altered signaling of leptin and insulin, as previously reported both in humans and animal models of resistance to both hormones (46, 47). In fact, animals with defective PI3K pathways or with a FOXO1 mutation resulting in constitutively nuclear location, show increased food intake and body weight accompanied by decreased expression of the POMC gene in the hypothalamus (48). On the contrary, inhibition of hypothalamic FOXO1 increases insulin sensitivity and reduces cumulative food intake (49). In turn, the activation of FOXO1 stimulates the transcription of NPY and increases food intake (50), as we report in D IRS2 mice. Despite higher Akt phosphorylation in ND IRS2 mice, which has been shown to increase FOXO1 exportation to the cytoplasm and increase its degradation (51), the levels of FOXO1 found were similar to controls. It must be remembered that FOXO1 levels are regulated not only by phosphorylation, but also by other posttranslational modifications such as ubiquitination and acetylation that might change the stability of this factor (52).

IRS1-related signaling may confer resistance against hypothalamic inflammation in ND IRS2 mice. There was an increase in RelA phosphorylation at Ser-468 and
activation of Akt inactivates glycogen synthase kinase-3β by phosphorylation at Ser-9 (56), and it is known that glycogen synthase kinase-3β is involved in the regulation of NFκB activation and inflammatory response (57). Therefore, IRS1-mediated activation of Akt may reduce hypothalamic inflammation in these mice. In line with this, previous reports have shown that antiinflammatory and insulin-sensitizing drugs increase pAkt levels (26, 27).

TNF-α exerts different actions in the pathogenesis of diabetes, and several functions related to the control of energy expenditure depend on its actions in the hypothalamus. In fact, this factor acts by producing a proinflammatory response through the induction of other cytokines and changing the expression of neurotransmitters implicated in the control of feeding (58). Intriguingly, D and ND IRS2−/− mice present similar increases in IL-6 mRNA levels. The increase in D IRS2−/− mice may be induced by TNF-α (58), whereas in ND IRS2−/− mice it could be due to increased pSTAT3 levels, as activated STAT directly binds to the IL-6 promoter, increasing its transcription (59). In both D and ND IRS2−/− mice IL-1β and CD11c, CD68, and F4/80, markers of activated microglia, did not differ from the WT group; thus there is only a low grade of hypothalamic inflammation at most. Considering that in the ND IRS2−/− mice only IL-6 was altered, and this cytokine can be either proinflammatory or antiinflammatory, the inflammation state in these animals is not clear.

Experimentally induced inflammation in the hypothalamus reduces oxygen consumption (23), which is concordant with data presented here, because energy expenditure is reduced in D IRS2−/− mice. In these animals there was an increase in hypothalamic mRNA levels of TNF-α, and it has been shown that this cytokine exerts a central anabolic effect at low concentrations, inhibiting the actions of leptin and insulin (60). Locomotor activity and the number of rearings are reduced in both IRS2−/− mice groups. This fact could be related to the neurological alterations described in these knockout
mice (61) and also with the role of IRS2 activation that is suggested to account for the increase in PI3K activity after exercise in controls (62).

Our results indicate that body weight may contribute to the differential signaling in D and ND IRS2−/− mice. D IRS2−/− mice present lower body weight that is associated with decreased leptin levels and signaling in these mice, whereas the higher weight of ND IRS2−/− mice is related to increased leptin and insulin levels and possibly the IRS1-associated effects. The contribution of the metabolic context cannot be excluded because we found higher levels of triglycerides in D IRS2−/−, mice and it has been shown that insulin resistance is also predicted by serum triglyceride concentrations (63).

Development of diabetes in IRS2−/− mice has been classically related to decreased β-cell function combined with blunted insulin signaling in the liver (17), although the hypothalamus could be also involved in the onset of the disease. On the one hand, the delay in the onset of diabetes could be due to the hyperinsulinemia in ND IRS2−/− mice by a compensatory effect of β-cell, and on the other hand, increased hypothalamic IRS1 signaling could exert a protective role by activating Akt, which reduces hypothalamic inflammation, as discussed above (26, 27). The hypothalamic inflammation reproduces some clinical features of type 2 diabetes and impairs pancreatic islet function (24) and hepatic insulin signaling (25). Alternatively, it is possible that the primary event in D IRS2−/− mice is the onset of severe diabetes that may lead to fat mass loss with a subsequent reduction of leptin levels and associated hyperphagia. In fact, replacement of leptin concentrations reestablishes adequate food intake and prevents insulin resistance induced by uncontrolled diabetes (64, 65); our results could indicate that the changes in leptin and insulin levels and IRS1-mediated signaling result in differential hypothalamic control of food intake in the two IRS2−/− groups, suggesting again a protective hypothalamic role against the development of diabetes in ND IRS2−/− mice. Thus, the use of diets (14) or pharmacological inhibition of specific targets in IRS2−/− mice to improve IRS1-mediated insulin signaling (66) may lead to new strategies for diabetes in the future.

A couple of caveats should be considered when evaluating these results. The hypothalamus is composed of diverse cell populations, and we have performed a semi-quantitative study of the entire hypothalamus for most of the analyzed parameters. Indeed, it is probable that distinct mechanisms are occurring in different cell types. The mechanism is complex as it implies not only modulation of neuropeptide expression through FOXO1 regulation and the modulation of hypothalamic fatty acid synthesis, but also IRS1 compensatory mechanisms that may be occurring in some, but not all, cells. However, the differences in the overall modifications in leptin and insulin signaling in the hypothalamus of D and ND IRS2−/− mice highlight the importance of this mechanism in determining the differential changes in metabolic neuropeptide expression and food intake. We also must consider that the similar signaling for leptin and insulin in WT and ND IRS2−/− mice does not entail an equal hypothalamic response to these hormones in ND mice. Thus, our results indicate that the higher weight and serum NEFA concentrations in ND IRS2−/− mice could increase hypothalamic resistance to leptin and insulin.

In conclusion, as summarized in Fig. 8, our data suggest that a compensatory increase of IRS1 and the activation of leptin- and insulin-signaling pathways in the hypothalamus may exert a protective effect against the hypothalamic defects inducing inflammation that lead to deregulation in the control of appetite and metabolism. These findings contribute to our understanding of the mechanisms involved in the pathogenesis of these disorders and seem to indicate that the manipulation of targets for both hormones in the hypothalamus could be promising for the treatment of insulin resistance and diabetes.

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