

# Impaired Central Insulin Response in Aged Wistar Rats: Role of Adiposity

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Insulin, like leptin, is considered as a lipostatic signal acting at a central level. Aging and age-associated adiposity have been related to the development of leptin resistance in Wistar rats. In the present article, hypothalamic insulin response during aging has been studied in Wistar rats. Thus, the effects of intracerebroventricular infusion of insulin during a week on food intake and body weight as well as insulin signal transduction after acute intracerebroventricular insulin administration have been studied in 3-, 8-, and 24-month-old rats. To explore the possible role of age-associated adiposity, these experiments were also performed in 8- and 24-month-old rats after 3 months of food restriction to reduce visceral adiposity index to values below those of young animals. Intracerebroventricular administration of insulin during a week was more

efficient at reducing food intake and body weight in 3-month-old rats than in 8- and 24-month-old rats. Hypothalamic insulin-stimulated insulin receptor, GSK3, AKT, and p70S6K phosphorylation decreased with aging. Insulin receptor and IRS-2 phosphoserine was increased in 24-month-old rats. Food restriction improved both insulin responsiveness and insulin signaling. These data suggest that Wistar rats develop hypothalamic insulin resistance with aging. This can be explained by alterations of the signal transduction pathway. The fact that food restriction improves central insulin response and signal transduction points to the age-associated adiposity as a key player in the development of central insulin resistance. (*Endocrinology* 148: 5238–5247, 2007)

WOODS ET AL. (1) demonstrated the ability of insulin, acting at a central level, to decrease food intake in baboons and proposed a lipostatic role for this hormone. The expression of its receptor and the different elements of its signal transduction pathway in hypothalamic nuclei, the activation of this machinery by insulin and the effects of central administration of insulin on food intake and body weight in different animal models are data that now clearly support its lipostatic role acting at a central level (2). Insulin shares some of the hypothalamic targets of leptin (3, 4), and there is evidence concluding that these signals also share some signal transduction steps such as phosphatidylinositol 3-kinase (PI3K), suggesting the existence of cross talk between them (2, 4, 5). The effects of leptin at the central level on food intake and energy balance seem to be more efficient than those of insulin. Nevertheless, the data obtained in mice with a neuron-specific disruption of the IR gene, which present diet-sensitive obesity and

mild insulin resistance, among other alterations (6), clearly demonstrates the importance of insulin as an energy balance regulator, acting through the central nervous system. Thus, any alteration in any of the multiple steps involved in insulin signaling at the central level may have consequences in the control of both glucose homeostasis and energy balance.

Wistar rats, like humans and other rodent models, present a moderate increase in adiposity during aging and exhibit overall insulin resistance in the absence of changes in fasting plasma glucose and insulin concentrations (7, 8). Besides this peripheral insulin resistance, these animals also present age-associated central leptin resistance (9). A decrease in the expression of leptin receptor in hypothalamic nuclei together with an increase in suppressor of cytokine signaling-3 (SOCS-3) expression (9, 10) have been pointed out to be related with the above mentioned central leptin resistance associated with aging in these animals (9). In the present article, we have studied whether central insulin action is affected by aging in Wistar rats. The fact that central leptin resistance associated with aging in these animals is ameliorated by a food restriction protocol enough to decrease adiposity index to values below those of young animals (9) also led us to investigate the effects of the same food restriction protocol on central insulin action and the possible role of age-associated adiposity.

## Materials and Methods

### Experimental animals

The 3-, 8-, and 24-month-old male Wistar rats from our in-house colony (Centre of Molecular Biology, Madrid, Spain) were used through-

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Abbreviations: AKT, Protein kinase B/thymoma viral proto-oncogene; Foxo-1, forkhead transcription factor-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSK3, glycogen synthase kinase-3; icv, intracerebroventricular; HOMA-IR, homeostasis model assessment for insulin resistance; IR, insulin receptor; IRS-2, insulin receptor substrate-2; mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol 3-kinase; PIP3, phosphatidylinositol-3,4,5-trisphosphate; p70S6K, p70 ribosomal S6 kinase; PVDF, polyvinylidene difluoride; SOCS-3, suppressor of cytokine signaling-3.

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out this study. Rats were housed individually and placed in climate-controlled quarters with a 12-h light cycle and fed *ad libitum* standard laboratory chow and water. They were handled following the European Union laws and National Institutes of Health guidelines for animal care. Experimental procedures were approved by the Institutional Committee of Research Ethics.

### Food restriction

Five- and 21-month-old rats were randomly assigned to undergo a food restriction protocol as described earlier (11). Animals were placed in individual cages and fed daily an amount of chow equivalent to 80% of normal food intake. Usually, 2 months after starting the nutritional restriction, animals show a body weight equivalent to 85% of *ad libitum*-fed age-mates. Animals were weighed weekly, and the amount of food provided was adjusted individually to maintain their body weight during one additional month. Food-restricted animals were used at the age of 8 and 24 months, respectively.

### Materials

Leupeptin, aprotinin, phenylmethylsulfonyl fluoride, okadaic acid, benzamide, and protein A-agarose were purchased from Sigma Chemical Co. (St. Louis, MO). Antibodies directed toward insulin R $\beta$  (C-19) and Foxo-1 (H-128) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies toward IRS-2, phosphotyrosine clone 4610, were from Upstate Biotechnology, Inc. (Lake Placid, NY). Antibodies toward phospho-GSK3 $\beta$  (Ser 9), phospho-AKT (Ser 473), AKT, phospho-Foxo-1 (ser 256), p70S6K, and phospho-p70S6K (thr-389) were from Cell Signaling Technology (Beverly, MA). Antibodies toward GSK3 $\alpha/\beta$  were from Biosource International, Inc. (Camarillo, CA). Anti-PIP3 was from Echelon Biosciences (Salt Lake City, UT). Anti-phosphoserine was from Chemicon International, Inc. (Temecula, CA). Anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH) was from AbCam (Cambridge, UK). Antimouse and antirabbit alkaline phosphatase-linked antibodies were from Amersham Pharmacia Biotech (Buckinghamshire, UK). Polyvinylidene difluoride (PVDF) membranes were from Bio-Rad (Hercules, CA). The rests of the reagents were of analytical grade.

### Insulin administration

For central effects on food intake and body weight, intracerebroventricular (icv), insulin was administered using osmotic pumps as previously described (9). Rats were anesthetized by inhalation of a mixture of O<sub>2</sub>, NO<sub>2</sub>, and isoflurane and placed in the stereotaxic frame (David Kopf, Tujunga, CA). After an incision of the scalp, a unilateral opening of the skull was made with a dental drill at  $-1.6$  mm lateral to the midline and  $0.8$  mm anterior to bregma. Then a cannula ( $4 \times 0.36$  mm length  $\times$  diameter) connected to a mini-osmotic pump (model 2001; Alzet, Palo Alto, CA) was implanted in the right lateral cerebral ventricle. The cannula was fixed to the skull with dental cement. Osmotic pumps, with a releasing rate of  $1 \mu\text{l/h}$  were filled with human insulin. Insulin concentrations were adjusted to  $0.41$  mU/ $\mu\text{l}$  ( $10$  mU/d) or  $4.1$  mU/ $\mu\text{l}$  ( $100$  mU/d) with PBS. Control rats were implanted with pumps containing vehicle adjusted to the same osmolarity of the insulin dilution. Rats were killed a week after pump implantation. During this period of time, food intake and body weight were measured. Food-restricted rats under insulin or vehicle administration were allowed free access to food and water.

For insulin signal transduction studies, a single dose of insulin was administered icv. Rats were anesthetized with sodium pentobarbital ( $40$  mg/kg, ip) and used as soon as anesthesia was assured by the loss of pedal and corneal reflexes. Rats were icv injected ( $5 \mu\text{l}$  bolus injection) with either vehicle or  $10$  mU insulin. After the appropriate time interval ( $15$  min), rats were killed, the cranium was opened, and the basal diencephalon, including the preoptic area and the hypothalamus, was quickly removed and immediately frozen until use.

### Adiposity and metabolic measurements

After killing, visceral adipose tissue was removed and weighed, and visceral adiposity was expressed as a percentage with respect to total body weight. Blood glucose was determined immediately using an

Accutrend glucose analyzer (Roche, Mannheim, Germany). Blood samples were centrifuged and plasma was frozen at  $-70^\circ\text{C}$  until determination of insulin and leptin. Plasma insulin and leptin were determined using RIA kits (Linco Research, St. Charles, MO). Homeostasis model assessment for insulin resistance (HOMA-IR) was calculated as fasting insulin ( $\mu\text{U/ml}$ )  $\times$  fasting glucose ( $[\text{mmol/liter}]/22.5$ ) as described earlier (12).

### Western blot analysis

Frozen tissues were homogenized in the solubilization buffer containing  $50$  mM Tris-HCl (pH 7.5),  $1$  mM EDTA,  $1$  mM EGTA,  $0.1\%$  2-mercaptoethanol,  $5$  mM sodium pyrophosphate,  $1\%$  Triton X-100,  $0.1$  mM phenylmethylsulfonyl fluoride,  $1 \mu\text{g/ml}$  leupeptin,  $1 \mu\text{g/ml}$  aprotinin,  $1 \mu\text{g/ml}$  benzamide,  $50$  mM sodium fluoride,  $0.5$  mM sodium orthovanadate,  $10$  mM sodium  $\beta$ -glycerolphosphate, and  $0.1 \mu\text{M}$  okadaic acid. After lysis at  $4^\circ\text{C}$  for  $60$  min, insoluble materials were removed by centrifugation ( $20,000 \times g$  at  $4^\circ\text{C}$  for  $60$  min), and protein concentration of resulting lysates was determined by Bradford. Solubilized materials were either directly subjected to Western blotting or to immunoprecipitation before Western blotting. For direct application,  $10 \mu\text{g}$  protein was used as whole-tissue extract for glycogen synthase kinase-3 (GSK3) analysis;  $40 \mu\text{g}$  for insulin receptor (IR), IR substrate-2 (IRS-2), and protein kinase B/thymoma viral proto-oncogene (AKT) analysis; and  $100 \mu\text{g}$  for forkhead transcription factor-1 (Foxo-1) and p70 ribosomal S6 kinase (p70S6K). Equal amounts of protein were subjected to SDS-PAGE and transferred onto PVDF membranes. Immunoblots were then blocked with  $5\%$  membrane-blocking agent (Amersham). After incubation with appropriate primary and secondary antibodies, PVDF membranes were washed, and targeted proteins were detected using enhanced chemifluorescence reagent ECF (Amersham). Obtained bands were quantified using Scion Image software. For the quantifications of insulin-induced protein phosphorylation of GSK3 and AKT, membranes were reblotted with the corresponding antibodies against total GSK3 and AKT, and data were expressed as fold stimulation *vs.* saline. For the quantification of protein expression, membranes were reblotted with anti-GAPDH to normalize with respect to the total amount of protein, and the data were expressed as a percentage with respect to the data of 3-month-old animals. For immunoprecipitation,  $4 \mu\text{g}$  phosphoserine or phosphotyrosine were incubated with protein A-agarose for  $2$  h at  $4^\circ\text{C}$ . The complexes were washed with solubilization buffer and were incubated with  $0.5$ – $1$  mg protein overnight at  $4^\circ\text{C}$ . Immunocomplexes were washed extensively and then subjected to Western blotting and quantified as described above. For the quantification of IR and IRS-2 phosphorylation, immunoprecipitated bands were normalized to total amount of IR and IRS-2 detected by immunoblot. Data of serine phosphorylation of IR and IRS-2 were expressed as percentage *vs.* the data of 3-month-old animals. Data of insulin-induced IR and IRS-2 tyrosine phosphorylation were expressed as fold stimulation with respect to saline.

### Immunohistochemistry

Immunohistochemical detection of phosphatidylinositol-3,4,5-trisphosphate (PIP3) and phospho-AKT was performed as described earlier (9). Briefly,  $15$  min after icv administration of saline or insulin, animals were transcardially perfused with  $4\%$  paraformaldehyde. The brain was postfixed and cryoprotected, and  $40$ - $\mu\text{m}$  free-floating sections from the hypothalamus were processed for immunostaining with phospho-AKT (Cell Signaling) diluted  $1:200$  in PBS- $0.02\%$  Triton X-100 or anti-PIP3 antibody at  $1:800$  dilution (Echelon Biosciences, Salt Lake City, UT) following the ABC method (Vector Laboratories, Inc., Burlingame, CA) and stained with nickel-enhanced diaminobenzidine.

### Statistical analysis

Statistical comparisons to determine the effect of age and insulin treatment were done by one-way ANOVA using the Prophet software (BNN Systems and Technologies, Cambridge, MA). For comparisons between food-restricted and *ad libitum* age-mates and insulin *vs.* saline, the unpaired Student's *t* test was used.

## Results

In accord with previous reports (9), the data of Table 1 show that aging is associated with an increase of body weight and adiposity in Wistar rats. Food-restricted animals maintain body weight around 85% of the *ad libitum* age-mates and decrease visceral adiposity to values close to those of young animals. There are not significant differences in fasting plasma glucose among the different groups of animals. Plasma insulin concentration does not present significant changes among the different groups of animals, except in 8-month-old food-restricted animals, which present a significant decrease. When HOMA-IR was calculated, no significant differences were found among groups, except in the case of 8-month-old food-restricted animals, which, as in the case of fasting insulin levels, present a significant decrease in this parameter (Table 1). In accord with previous reports (9, 13), leptin levels were significantly increased at 8 months and remained high at 24 months of age. Food restriction significantly decreased leptin levels in comparison with their *ad libitum* age-mates.

### Effects of chronic central insulin administration on food intake and body weight

Figure 1 shows the effects of central insulin administration during a week on daily food intake in Wistar rats. Daily food intake was measured in each animal before and after pump implantation. No significant differences were found in daily food intake among days after pump implantation in each group of animals. As it can be observed, saline-infused animals present a decrease in daily food intake after surgery in comparison with the previous averages in all groups. This decrease is significantly higher in 24-month-old in comparison with 3-month-old rats, suggesting a poorer recovery from surgery in old animals. Saline-infused food-restricted animals, despite the surgery, show an increase in food intake in comparison with previous records in both the 8- and 24-month-old animals. Central insulin administration decreases daily food intake in all age groups of animals in a dose-dependent manner. Nevertheless, only in 3-month-old animals is there a significant effect with both doses of insulin used, with the magnitude of the decrease, after subtracting saline, being bigger in 3- than in 8- and 24-month-old rats (Fig. 1B). Food restriction improves insulin response in both 8- and 24-month-old animals, and the lower of the above used doses is enough to bring about higher and significant decreases of food intake compared with those observed in their *ad libitum* age-mates (Fig. 1B).

**TABLE 1.** Characteristics of the rats

	3 months	8 months	8 months FR	24 months	24 months FR
Body weight (g)	355.5 ± 5.8	493.1 ± 11.5 <sup>a</sup>	412.3 ± 7.11 <sup>a</sup>	662.3 ± 17.17 <sup>a,b</sup>	521.8 ± 10.6 <sup>a,c</sup>
Blood glucose (mg/dl)	140.5 ± 7.0	133.3 ± 6.1	132.3 ± 4.9	127.0 ± 6.2	130.1 ± 3.5
Insulin (ng/ml)	1.17 ± 0.14	1.16 ± 0.25	0.54 ± 0.08 <sup>a</sup>	1.57 ± 0.10	1.43 ± 0.28
Visceral adiposity (%)	3.1 ± 0.1	5.3 ± 0.3 <sup>a</sup>	1.4 ± 0.06 <sup>c</sup>	5.2 ± 0.4 <sup>a</sup>	1.8 ± 0.1 <sup>c</sup>
Leptin (ng/ml)	5.01 ± 0.1	16 ± 2.1 <sup>a</sup>	2.5 ± 0.2 <sup>c</sup>	20.3 ± 4.5 <sup>a</sup>	9 ± 1.5 <sup>c</sup>
HOMA index	7.5 ± 1.5	7.8 ± 1.9	2.3 ± 0.4 <sup>c</sup>	9.3 ± 2.4	8.4 ± 3.9

Data are means ± SEM of six to 12 animals per group. FR, Food-restricted animals.

<sup>a</sup>  $P < 0.05$  vs. 3 months.

<sup>b</sup>  $P < 0.05$  vs. 8 months.

<sup>c</sup>  $P < 0.05$  vs. same age fed *ad libitum*.

The effects of central insulin administration on body weight are shown in Fig. 2. In this case, saline-infused 3-month-old rats, but not 8- and 24-month-old animals, present an increase in body weight after pump implantation. In accord with the marked increase in food intake (Fig. 1), saline-infused 8- and 24-month-old food-restricted animals also present a significant increase in body weight. Similarly to the data of Fig. 1, insulin decreases body weight in all groups of animals in a dose-dependent manner, but this effect is more efficient in control young than in 8- and 24-month-old rats. After subtracting saline, the effect of insulin is clearly bigger in young than in 8- and 24-month-old animals (Fig. 2B). Moreover, the decrease in body weight induced by insulin reaches significance in young rats with both doses of insulin used, but it does not in 8- and 24-month-old rats when compared by ANOVA. In agreement with the data of Fig. 1, food restriction also improves the effects of insulin on body weight in 8- and 24-month-old rats, and a significant decrease in this parameter is appreciated in both food-restricted groups of animals.

### Hypothalamic insulin signaling

To study whether or not the above mentioned differences in central insulin response were due to differences in the function of the insulin signal transduction machinery, we have explored several steps of this pathway. Activation of PI3K represents a key step of insulin signaling that is involved in hypothalamic insulin regulation of food intake (14). In agreement with this report, insulin induced PIP3 generation in the arcuate nucleus but not in surrounding areas (supplemental Fig. 1, published as supplemental data on The Endocrine Society's Journals Online web site at <http://endo.endojournals.org>). Moreover, insulin also stimulated AKT phosphorylation within this nucleus (supplemental Fig. 2). Thus, the effects of icv insulin administration on hypothalamic AKT and GSK3 phosphorylation, two downstream steps of PI3K, were investigated. As shown in Fig. 3A, insulin stimulates the phosphorylation of AKT in all groups. Nevertheless, this effect decreases with aging, and insulin-stimulated AKT phosphorylation is significantly lower in 8- and 24-month-old rats than in 3-month-old animals. In agreement with the data of daily food intake, food restriction increases AKT phosphorylation in response to centrally administrated insulin. In food-restricted 24-month-old rats, AKT phosphorylation is significantly higher than in *ad libitum*-fed age-mates, and in 8-month-old food-restricted animals, its phosphorylation is similar to that observed in



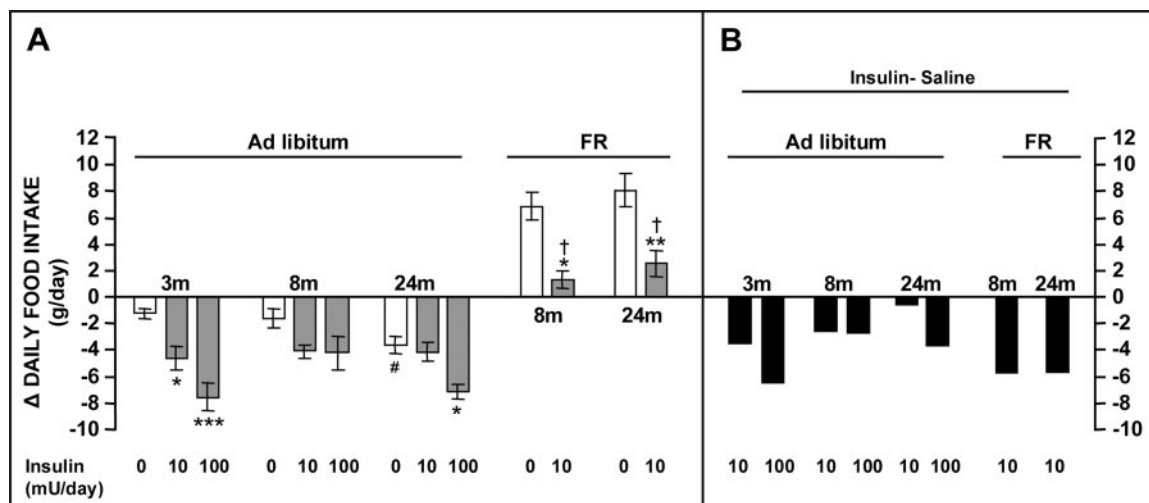


FIG. 1. Changes in daily food intake after central insulin administration for 7 d. Daily food intake in 3-, 8-, and 24-month-old (3m, 8m, and 24m) rats and 8- and 24-month-old food-restricted (FR) animals was measured individually before and during 7 d after pump implantation with either saline or the indicated doses of insulin as described in *Materials and Methods*. A, Bars represent mean values  $\pm$  SEM ( $n = 4-12$ ) of the changes observed in daily food intake in saline-infused (white bars) or insulin-infused (gray bars) animals. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  vs. saline; #,  $P < 0.05$  vs. 3m; †,  $P < 0.001$  vs. its *ad libitum*-fed age-mates. B, Black bars represent the net change in daily food intake after subtracting the mean value of changes in saline from the mean value of changes in insulin-treated animals.

3-month-old rats. Figure 3B shows that the amount of hypothalamic AKT does not change nor with age or with food restriction.

Insulin stimulation of GSK3 phosphorylation is shown in Fig. 4. Insulin significantly increases GSK3 phosphorylation in 3- and 8-month-old rats, whereas in 24-month-old animals, the stimulation does not reach statistical significance. Food restriction increases this response and insulin stimulation reaches significance in both 8- and 24-month-old rats. Figure 4B shows quantification of total GSK3 by immunoblot. No change of total amount of GSK3 is appreciated at 8 months old, but a significant decrease is observed at 24 months old.

Insulin receptor phosphorylation is the first step in the

signal transduction pathway, and a decrease in insulin-induced phosphorylation has been described in different tissues associated with peripheral (15) and central (16) insulin resistance. As can be seen in Fig. 5A, insulin-stimulated receptor phosphorylation decreases in older animals, being appreciated at 8 months of age. Similarly to the above mentioned data, food restriction improves this response in older animals. Figure 5B shows that the amount of hypothalamic IR is not changed with aging or food restriction.

Although different IRS isoforms are expressed in the brain (16), IRS-2 has been identified as the isoform responsible to mediate central insulin effects on energy homeostasis (17), and mice lacking IRS-2 present hyperphagia (18). Its expres-

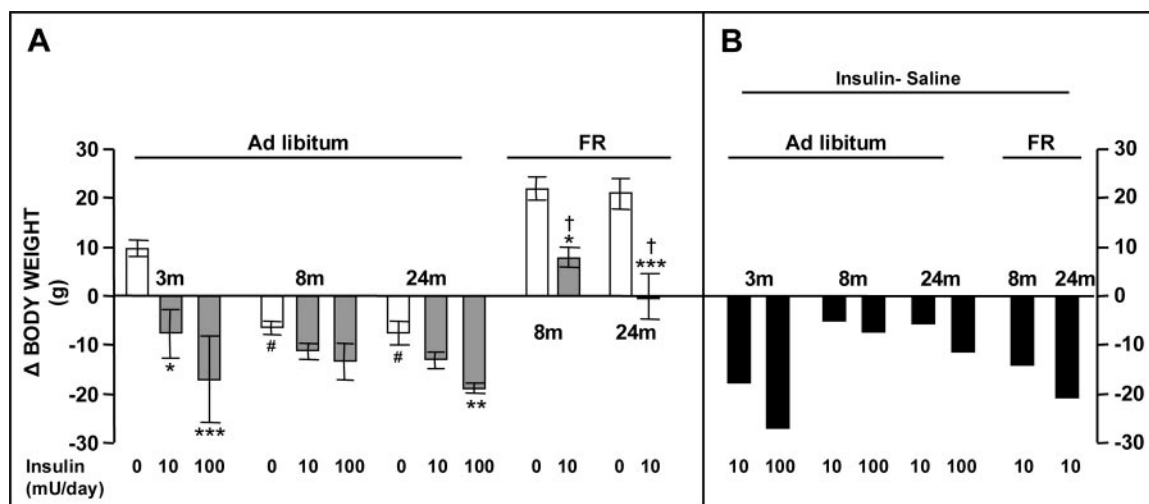


FIG. 2. Changes in body weight after central insulin administration for 7 d. Body weight of 3-, 8-, and 24-month-old (3m, 8m, and 24m) rats and food-restricted (FR) 8- and 24-month-old rats was measured before and 7 d after pump implantation with saline or the indicated doses of insulin as described in *Materials and Methods*. A, Bars represent the mean values  $\pm$  SEM of the observed changes in body weight of four to 12 animals per group after pump implantation with saline (white bars) or insulin (gray bars). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  vs. saline; #,  $P < 0.05$  vs. 3m; †,  $P < 0.001$  vs. its *ad libitum*-fed age-mates. B, Black bars represent the net change in body weight after subtracting the mean value of changes in saline from the mean value of changes in insulin.

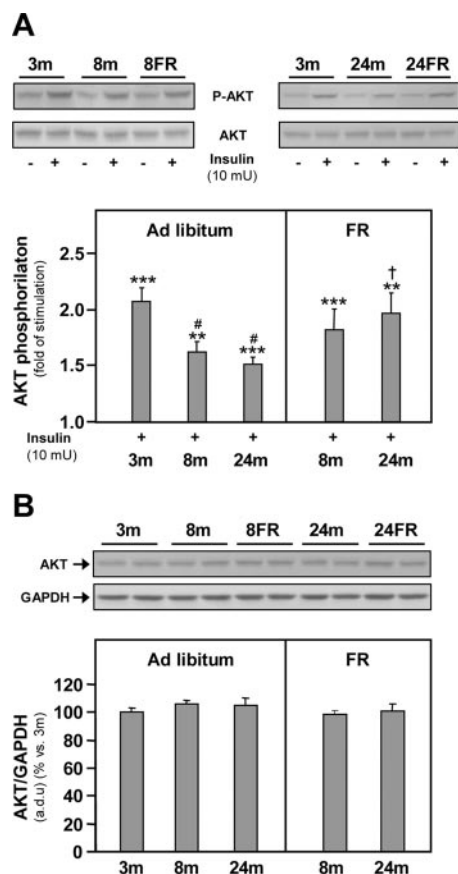


FIG. 3. Effect of aging and food restriction on hypothalamic insulin-induced AKT serine phosphorylation and AKT protein expression. A, Insulin-stimulated serine phosphorylation of AKT in hypothalamus from 3-, 8-, and 24-month-old (3m, 8m, and 24m) rats as well as their food-restricted (FR) age-mates was determined by Western blot as described in *Materials and Methods*. Upper panel, Representative Western blot. Bar graph represents quantification of phospho-AKT after correction for AKT by scanning densitometry. Data show fold stimulation with respect to saline. No significant differences in saline values were seen among the different groups of rats. B, AKT protein expression in hypothalamus from 3-, 8-, and 24-month-old rats as well as their food-restricted age-mates was determined by Western blot as described in *Materials and Methods*. Upper panel, Representative Western blot; lower panel, quantification by scanning densitometry of AKT after correction for GAPDH levels. OD units were adjusted so that the ratio obtained from hypothalamus of 3-month-old rats equaled 100. The values represent the mean  $\pm$  SEM ( $n = 4-10$ ). \*\*,  $P \leq 0.01$ ; \*\*\*,  $P < 0.001$  vs. saline; #,  $P < 0.05$  vs. 3m; †,  $P < 0.05$  vs. same age fed *ad libitum*. a.d.u., Arbitrary densitometric units.

sion in different hypothalamic nuclei has recently been detailed (14, 19) and Carvalheira *et al.* (16) have described a higher proportion of IRS-2 than IRS-1 in hypothalamus as well as a higher degree of colocalization of IRS-2 with IR. Therefore, we have explored IRS-2 phosphorylation in hypothalamus. Figure 6 shows that insulin-stimulated IRS-2 phosphorylation decreases with aging, whereas food restriction seems to improve it. As in the case of IR, we could not detect any significant changes in basal phosphorylation or in the amount of IRS-2 by immunoblot (Fig. 6B) among the different groups of animals.

Insulin induces phosphorylation of the transcriptional factor Foxo-1, promoting its exit from the nucleus, which leads

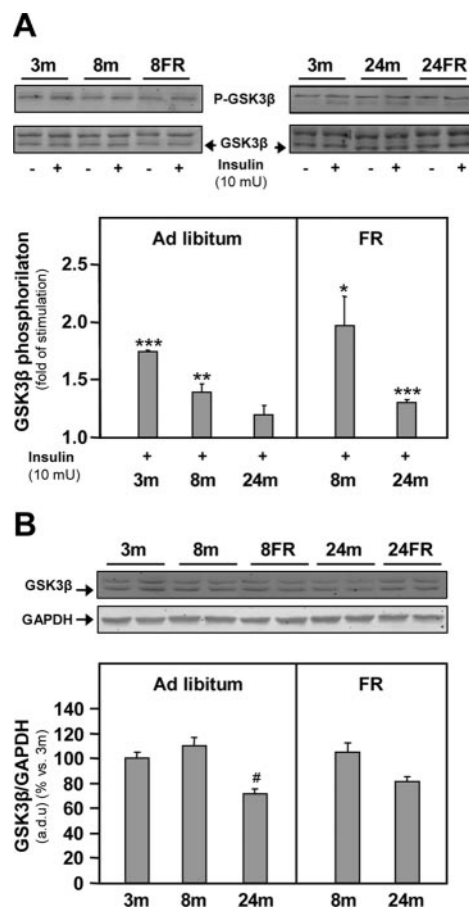
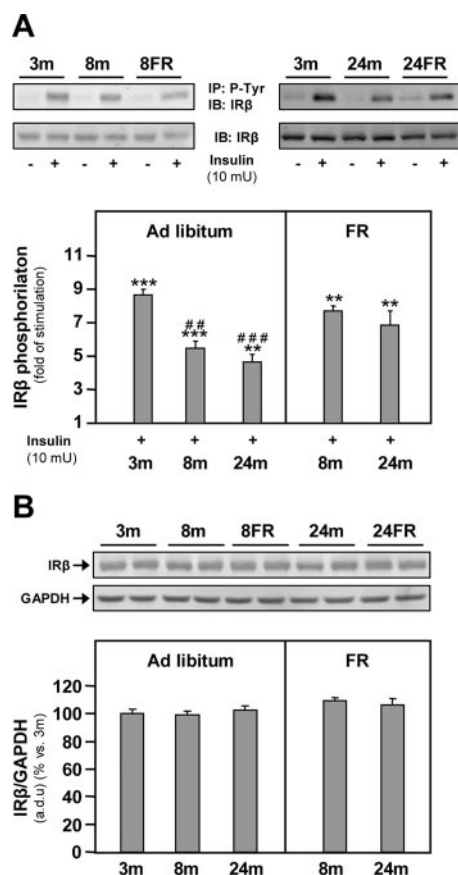


FIG. 4. Effect of aging and food restriction on hypothalamic insulin-induced GSK3β serine phosphorylation and GSK3β protein expression. A, Insulin-stimulated serine phosphorylation of GSK3β in hypothalamus from 3-, 8-, and 24-month-old (3m, 8m, and 24m) rats as well as their food-restricted (FR) age-mates was determined by Western blot as described in *Materials and Methods*. Upper panel, Representative Western blot. Bar graph represents quantification of phospho-GSK3β after correction by GSK3β by scanning densitometry. Data show fold stimulation with respect to saline. No significant differences in saline values were seen among the different groups of rats. B, GSK3β protein expression in hypothalamus from 3-, 8-, and 24-month-old rats as well as their food-restricted age-mates was determined by Western blot as described in *Materials and Methods*. Upper panel, Representative Western blot; lower panel, quantification by scanning densitometry of GSK3β after correction for GAPDH levels. OD units were adjusted so that the ratio obtained from hypothalamus of 3-month-old rats equaled 100. The values represent the mean  $\pm$  SEM ( $n = 4-10$ ). \*,  $P < 0.05$ ; \*\*,  $P \leq 0.01$ ; \*\*\*,  $P < 0.001$  vs. saline; #,  $P < 0.01$  vs. 3m. a.d.u., Arbitrary densitometric units.

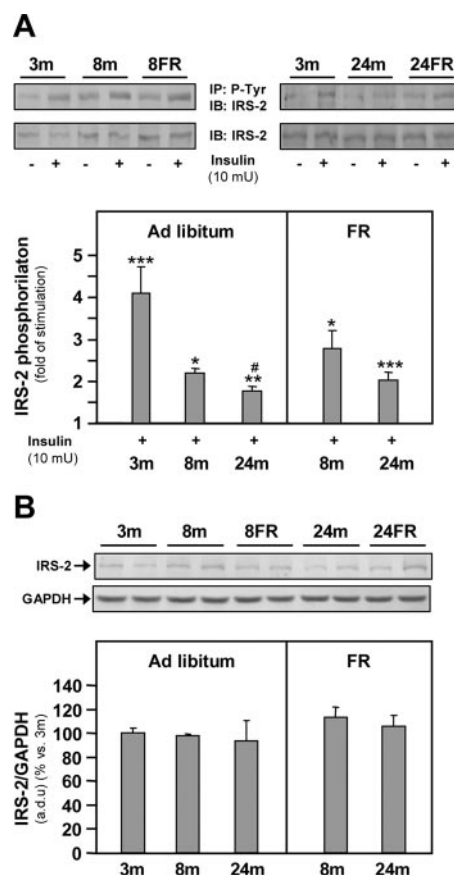
to several insulin responses (20). Precisely, hypothalamic insulin regulation of food intake has been described to be mediated by Foxo-1 (21–23). Therefore, we also have studied this transcription factor. As shown in Fig. 7, insulin significantly induced Foxo-1 phosphorylation in all groups. Although no significant differences are reached when the different groups were compared by ANOVA, similarly to that observed with other insulin signaling steps described herein (Figs. 3–6), this stimulation seems to decrease with aging, and food restriction improves this stimulation. No significant changes in basal levels of Foxo-1 protein or phospho-Foxo-1 in saline-treated animals among groups were detected, and



**FIG. 5.** Effect of aging and food restriction on hypothalamic insulin-induced IR tyrosine phosphorylation and IR protein expression. **A**, Insulin-stimulated tyrosine phosphorylation of IR in hypothalamus from 3-, 8-, and 24-month-old (3m, 8m, and 24m) rats as well as their food-restricted (FR) age-mates was determined by immunoprecipitation (IP) with anti-phosphotyrosine (P-Tyr) antibodies and blotted (IB) with anti-IRβ antibody as described in *Materials and Methods*. *Upper panel*, Representative Western blot. *Bar graph* represents quantification of phospho-IR after correction for IR levels by scanning densitometry. Data show fold stimulation with respect to saline. No significant differences in saline values were appreciated among the different groups of rats. **B**, IR protein expression in hypothalamus from 3-, 8-, and 24-month-old rats as well as their nutritionally restricted age-mates was determined by Western blot as described in *Materials and Methods*. *Upper panel*, Representative Western blot. *Bar graph* represents quantification by scanning densitometry of IR after correction for GAPDH levels. OD units were adjusted so that the ratio obtained from hypothalamus of 3-month-old rats equaled 100. The values represent mean  $\pm$  SEM ( $n = 3-10$ ). \*\*,  $P \leq 0.01$ ; \*\*\*,  $P < 0.001$  vs. saline; ##,  $P < 0.01$ ; ###,  $P < 0.001$  vs. 3m. a.d.u., Arbitrary densitometric units.

the expression of hypothalamic Foxo-1, analyzed by quantitative RT-PCR analysis, did not show significant change among groups (data not shown).

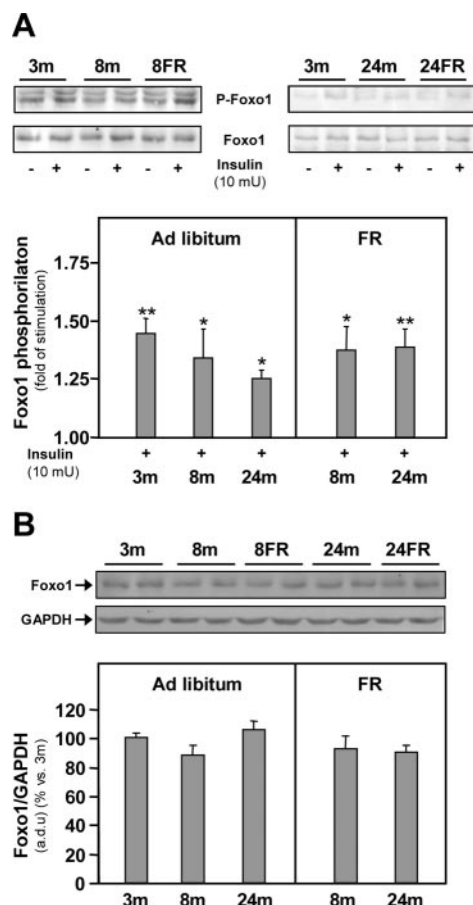
The mammalian target of rapamycin (mTOR) pathway is one of the branches under insulin action, and its chronic activation has been related to the development of insulin resistance (24). Thus, we also have tested this pathway by analyzing a downstream element of this pathway such as p70S6K. Figure 8 shows that insulin significantly stimulates p70S6K phosphorylation in all groups of animals. In this case, there is a significant decrease in the degree of stimulation induced by insulin with aging when compared by



**FIG. 6.** Effect of aging and food restriction on hypothalamic insulin-induced IRS-2 tyrosine phosphorylation and IRS-2 protein expression. **A**, Insulin-stimulated tyrosine phosphorylation of IRS-2 in hypothalamus from 3-, 8-, and 24-month-old (3m, 8m, and 24m) rats as well as their food-restricted (FR) age-mates was determined by immunoprecipitation (IP) with anti-phosphotyrosine (P-Tyr) antibodies and blotted (IB) with anti-IRS-2 antibody as described in *Materials and Methods*. *Upper panel*, Representative Western blot. *Bar graph* represents quantification of phospho-IRS-2 after correction for IRS-2 levels by scanning densitometry. Data show fold stimulation with respect to saline. No significant differences in saline values were appreciated among the different groups of rats. **B**, IRS-2 protein expression in hypothalamus from 3-, 8-, and 24-month-old rats as well as their food-restricted age-mates was determined by Western blot as described in *Materials and Methods*. *Upper panel*, Representative Western blot. *Bar graph* represents quantification by scanning densitometry of IRS-2 after correction for GAPDH levels. OD units were adjusted so that the ratio obtained from hypothalamus of 3-month-old rats equaled 100. The values represent the mean  $\pm$  SEM ( $n = 3-6$ ). \*,  $P < 0.05$ ; \*\*,  $P \leq 0.01$ ; \*\*\*,  $P < 0.001$  vs. saline; #,  $P < 0.05$  vs. 3m. a.d.u., Arbitrary densitometric units.

ANOVA, and food restriction improves this stimulation. On the other hand, no significant changes were detected among groups at the level of protein or phosphoprotein in basal, nonstimulated animals.

Serine phosphorylation of IR and IRS has been associated with decreased IR and IRS tyrosine phosphorylation and impairment of insulin signaling (25, 26). Moreover, an increase in serine phosphorylation of IR and IRS-2 has been described to be associated with an impairment of insulin signaling at the central level in obese Zucker rats (16). As shown in Fig. 9, serine phosphorylation of IR and IRS-2 seems to increase with aging, although this parameter

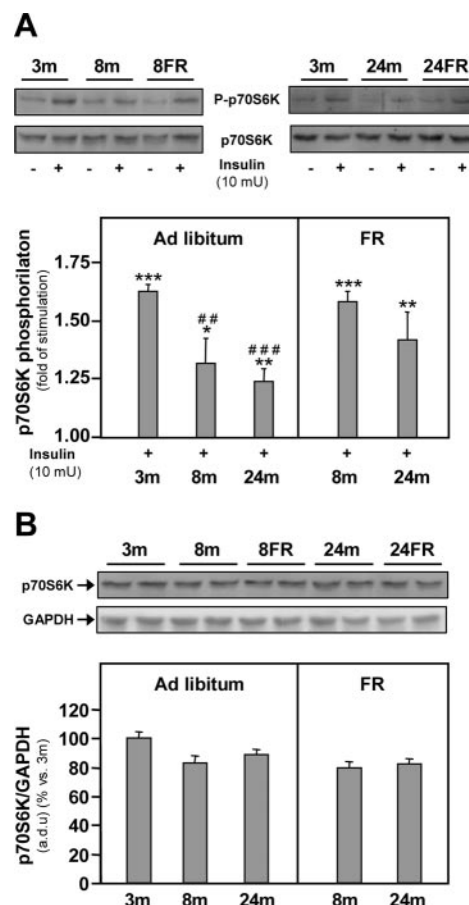


**FIG. 7.** Effect of aging and food restriction on hypothalamic insulin-induced Foxo-1 phosphorylation. **A**, Insulin-stimulated serine phosphorylation of Foxo-1 in hypothalamus from 3-, 8-, and 24-month-old (3m, 8m, and 24m) rats as well as their food-restricted (FR) age-mates was determined by Western blot as described in *Materials and Methods*. *Upper panel*, Representative Western blot. *Bar graph* represents quantification of phospho-Foxo-1 after correction for Foxo-1 by scanning densitometry. Data show fold stimulation with respect to saline. No significant differences in saline values were seen among the different groups of rats. **B**, Foxo-1 protein expression in hypothalamus from 3-, 8-, and 24-month-old rats as well as their nutritionally restricted age-mates was determined by Western blot as described in *Materials and Methods*. *Upper panel*, Representative Western blot; *lower panel*, quantification by scanning densitometry of Foxo-1 after correction for GAPDH levels. OD units were adjusted so that the ratio obtained from hypothalamus of 3-month-old rats equaled 100. The values represent the mean values  $\pm$  SEM ( $n = 3-10$ ). \*,  $P < 0.05$ ; \*\*,  $P \leq 0.01$  vs. saline. a.d.u., Arbitrary densitometric units.

reaches statistical significance only in 24-month-old animals when compared with young rats. Food restriction decreases serine phosphorylation in both IRS-1 and IRS-2 when compared with *ad libitum*-fed animals. Nevertheless, this decrease is statistically significant only for IRS-2 in the case of 8-month-old rats.

### Discussion

In agreement with previous studies (8), old Wistar rats do not present significant differences in fasting glucose and insulin levels by aging (Table 1), suggesting that they do not present overt alterations of glucose homeostasis. Moreover, when HOMA-IR is calculated, there is a slight increase at 24



**FIG. 8.** Effect of aging and food restriction on hypothalamic insulin-induced p70S6K phosphorylation and AKT protein expression. **A**, Insulin-stimulated serine phosphorylation of p70S6K in hypothalamus from 3-, 8-, and 24-month-old (3m, 8m, and 24m) rats as well as their food-restricted (FR) age-mates was determined by Western blot as described in *Materials and Methods*. *Upper panel*, Representative Western blot. *Bar graph* represents quantification of phospho-p70S6K after correction for p70S6K by scanning densitometry. Data show fold stimulation with respect to saline. No significant differences in saline values were seen among the different groups of rats. **B**, p70S6K protein expression in hypothalamus from 3-, 8-, and 24-month-old rats as well as their food-restricted age-mates was determined by Western blot as described in *Materials and Methods*. *Upper panel*, Representative Western blot; *lower panel*, quantification by scanning densitometry of p70S6K after correction for GAPDH levels. OD units were adjusted so that the ratio obtained from hypothalamus of 3-month-old rats equaled 100. The values represent the mean values  $\pm$  SEM ( $n = 3-10$ ). \*,  $P < 0.05$ ; \*\*,  $P \leq 0.01$ ; \*\*\*,  $P < 0.001$  vs. saline; ##,  $P < 0.01$ ; ###,  $P < 0.001$  vs. 3m. a.d.u., Arbitrary densitometric units.

months of age, but the difference does not reach statistical significance (Table 1). Nevertheless, previous studies (13) using oral glucose tolerance test and clamp techniques have demonstrated that these animals develop peripheral insulin resistance with aging. Food restriction is able to improve peripheral insulin resistance at 8 but not at 24 months of age (8, 13), suggesting that sustained adiposity during the life-span may lead to a state of insulin resistance difficult to reverse. In agreement with these reports, Table 1 shows that there are not significant differences in HOMA index during aging and that after food restriction, this parameter decreases



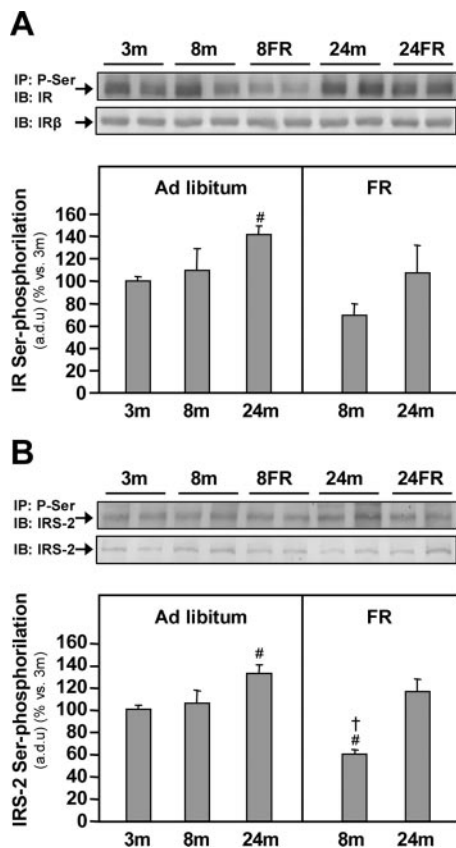


FIG. 9. Effect of aging and food restriction on hypothalamic IR and IRS-2 serine phosphorylation. A, Serine phosphorylation of IR in hypothalamus from 3-, 8-, and 24-month-old (3m, 8m, and 24m) rats as well as their food-restricted (FR) age-mates was determined by immunoprecipitation (IP) with anti-phosphoserine (P-Ser) antibodies and blotted (IB) with anti-IR $\beta$  antibody as described in *Materials and Methods*. Upper panel, Representative Western blot. Bar graph represents quantification of phospho-IR after correction for IR levels by scanning densitometry. OD units were adjusted so that the values obtained from hypothalamus of 3-month-old rats equaled 100. B, Serine phosphorylation of IRS-2 in hypothalamus from 3-, 8-, and 24-month-old rats as well as their nutritionally restricted age-mates was determined by immunoprecipitation (IP) with anti-phosphoserine (P-Ser) antibodies and blotted (IB) with anti-IRS-2 antibody as described in *Materials and Methods*. Upper panel, Representative Western blot. Bar graph represents quantification of phospho-IRS-2 after correction for IRS-2 levels by scanning densitometry. Optical density units were adjusted so that the values obtained from hypothalamus of 3-month-old rats equaled 100. The values represent the mean values  $\pm$  SEM ( $n = 3-6$ ). <sup>#</sup>,  $P < 0.05$  vs. 3m; <sup>†</sup>,  $P < 0.01$  vs. same age fed *ad libitum*. a.d.u., Arbitrary densitometric units.

significantly only in 8-month-old rats (Table 1). Besides this, 24-month-old Wistar rats also present central leptin resistance (9), and Table 1 shows that both 8- and 24-month-old rats present significantly higher values of plasma leptin than control young rats, which decrease with food restriction.

In the present report, we have studied the effects of aging and food restriction on centrally mediated insulin response and hypothalamic insulin signaling in these animals. The data presented herein demonstrate that a lower central response to insulin, in terms of change in both body weight and daily food intake (Figs. 1 and 2), is associated with aging in Wistar rats. The decrease in central insulin response can be

appreciated at 8 months of age and remains at 24 months. The fact that the beginning of the attenuation of central insulin response concurs with the major increase in adiposity index after sexual maturity suggests a role for adiposity in the deterioration of central insulin response. This idea is supported by the data provided by food-restricted animals, which, associated with a decreased adiposity, improve central insulin responsiveness in terms of changes in both body weight and food intake (Figs. 1 and 2). Because after food restriction the animals significantly increase their food intake, it might be possible that this physiological adaptation could participate in the improvement of central insulin response described herein. Nevertheless, the fact that food-restricted animals, which did not have the chance to experience the increase in food intake after the period of food restriction, present an improvement in central insulin signaling (Figs. 3–6 and 8) supports a role for food restriction and/or adiposity in the modulation of central insulin response.

The decrease in central insulin response in aged animals can be explained, at least in part, by the impairment of several steps of the insulin signal transduction pathway. The effects of insulin on food intake have been linked to the activation of the PI3K branch of insulin signaling (14), and this pathway is clearly impaired in aged Wistar rats as can be deduced from the results of AKT and GSK3 phosphorylation presented herein (Figs. 3 and 4). In both cases, there is a progressive decline of insulin-stimulated phosphorylation with aging, and food restriction increases insulin-induced phosphorylation. The decrease in the expression of GSK3 at 24 months of age that is not seen in the case of AKT suggests a differential regulation of these two downstream steps of PI3K. This decrease in the expression of GSK3, which does not seem to be recovered by food restriction, can explain the poorer recovery of the stimulation of GSK3 in 24-month-old animals by food restriction when compared with 8-month-old animals or when compared with the recovery of AKT stimulation in food-restricted 24-month-old animals.

Others steps downstream of PI3K also seem to be altered. Thus, the mTOR pathway, as deduced by the data of insulin-induced p70S6K phosphorylation is clearly attenuated by aging, suggesting that this pathway may also be involved in the insulin control of energy homeostasis at the hypothalamic level.

The levels of Foxo-1 protein and its mRNA did not present any significant change among animal groups, suggesting that alteration of its expression is not the main cause of the altered hypothalamic insulin response during aging. In agreement with previous reports (21, 23), the data of Fig. 8 demonstrate that insulin stimulates Foxo-1 phosphorylation at the hypothalamic level in all groups of rats, suggesting a role for this transcription factor in the hypothalamic insulin response. In accord with this idea, despite that no significant differences were reached, insulin-induced phosphorylation of Foxo-1 seems to decrease with aging and to improve with food restriction.

The data of IR and IRS-2 phosphorylation demonstrate that the impairment in the insulin signal pathway also occurs in earlier steps in the cascade such as insulin-induced IR and IRS-2 phosphorylation. The decrease in insulin-stimulated IR



and IRS-2 tyrosine phosphorylation (Figs. 5 and 6) may, in turn, be explained by IR and IRS-2 serine phosphorylation (Fig. 9), at least in the case of 24-month-old animals where the increase in serine phosphorylation is significant. Serine phosphorylation of these proteins has been proposed, among others, as a mechanism involved in states of insulin resistance (25, 26). In agreement with this idea, a statistically significant reduction in serine phosphorylation is observed in IRS-2 in 8-month-old rats, and serine phosphorylation of both IR and IRS-2 remains lower in food-restricted rats as compared with *ad libitum*-fed animals.

Serine/threonine phosphorylation of IRS has been shown to be mediated by several kinases such as c-jun N-terminal kinase (27), ERK (28), or mTOR (29). Chronic activation of the mTOR pathway has been related to increases in IRS serine phosphorylation, which contributes to insulin resistance (30). As shown in Fig. 8, insulin stimulates p70S6K in the hypothalamus. Although in this case we could not detect any significant increase in basal p70S6K phosphorylation by aging, the role of other kinases and the persistent activation of this pathway during aging and/or obesity may lead to IRS serine phosphorylation and hence attenuate insulin action. Besides this, it has to be pointed out that the mTOR pathway can be also regulated by TNF- $\alpha$  (31) and signal transduction elements such as AMP-activated protein kinase, which plays an important role in hypothalamic regulation of energy balance and would deserve further investigation.

Although the present article does not delimitate the precise nuclei where the observed alterations in insulin signal transduction take place, icv insulin administration induced PIP3 generation and AKT phosphorylation in arcuate nucleus but not in surrounding areas (supplemental Figs. 1 and 2). Thus, in agreement with previous reports (14), these data suggest that this nucleus is a key target of insulin and plays an important role regulating food intake and energy homeostasis.

The improvement of central insulin response and signal transduction by caloric restriction at 24 months of age agrees well with the described improvement of central leptin response in food-restricted 24-month-old Wistar rats (9). These data seem to contrast with those obtained in peripheral tissues such as muscle and adipose tissue, which present more difficulty in recovering age-associated insulin resistance by the same caloric restriction protocol (13). Nevertheless, it might be possible that the increased adiposity, established in early adulthood and sustained along aging, may cause some irreversible harm, leading to a state of insulin resistance more difficult to recover in peripheral tissues. Although some role of aging by itself cannot be ruled out, the improvement in central insulin response by food restriction clearly points to age-associated adiposity as a key factor in the development of central insulin resistance as it does in central leptin resistance (9). Although, as mentioned above, these animals cannot be considered as obese, and there are clear differences with obesity models, the data presented herein are similar to those described in Zucker rats (16), which present an impairment of hypothalamic insulin signaling associated with obesity. In contrast to the data of Carvalheira *et al.* (16), we could not detect any significant decrease in the levels of IRS-2. Nevertheless, our data also report an impairment of

several signal transduction steps without changes in IR levels, associated with increased IR and IRS-2 serine phosphorylation in 24-month-old rats.

Leptin resistance in old Wistar rats has been explained, at least in part, by the decrease in the expression of leptin receptor and the increase of the expression of SOCS-3 in hypothalamus (9, 10, 32). Besides its inhibitory modulation of leptin action (33), SOCS-3 also has been shown to inhibit insulin signaling by interfering with tyrosine phosphorylation (34, 35) and/or by degradation of IRS-1 and IRS-2 (36). Although, as mentioned above, no significant changes have been seen in IRS-2 levels with aging or food restriction, the reported increase of SOCS-3 expression in aged rats (10) may well contribute to the impairment of insulin signal transduction described herein at the level of IR and IRS tyrosine phosphorylation. In agreement with this idea, food restriction, which has been shown to decrease hypothalamic SOCS-3 expression (10), improves insulin signaling (Figs. 3–6), and recovers central leptin responsiveness (9).

In conclusion, the data presented herein demonstrate that central insulin response is attenuated in aged Wistar rats, which can be explained by alterations of several steps in the insulin signal transduction cascade. The increase in serine phosphorylation of IR and IRS-2, clearly demonstrated at 24 months of age, suggest that serine phosphorylation may be one of the mechanisms involved in the alteration of some steps in the insulin signal pathway. The fact that food-restricted animals present an improvement in both central insulin response and signal transduction steps points to the increased adiposity associated with aging as a key factor in the development of the above mentioned impairment of central insulin signaling. Although more studies would be necessary to clarify how adiposity modulates the signals that regulate energy homeostasis at the central level, adipokines are good candidates to play this role. In this sense, it has been described that TNF- $\alpha$  modulates the expression of proinflammatory proteins and neurotransmitters involved in the regulation of food intake (37). On the other hand, because leptin, via phosphatidylinositol-3-OH kinase, has been shown to modulate global insulin sensitivity at the central level (38) and aged Wistar rats present central leptin resistance (9), it is possible that the attenuated permissive effect of leptin on insulin action, together with the decreased central insulin responsiveness described herein, may contribute to the development of global insulin resistance associated with aging and/or age-associated adiposity.

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