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The expression of rat resistin isoforms is differentially regulated in visceral adipose tissues: effects of aging and food restriction

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Abstract

Two variants of the adipose hormone resistin are generated by alternative splicing in Wistar rats. Here we analyzed the expression of these resistin variants in 2 main visceral adipose depots, epididymal and retroperitoneal, as well as the resistin serum concentration during aging and food restriction. Total protein levels of resistin were also analyzed in extracts from both visceral adipose depots. Resistin variants show similar patterns of relative expression in visceral adipose tissues in 3-month–old rats, representing the short variant, s-resistin, which is 15% of the full-length transcript. However, only epididymal, but not retroperitoneal, fat pad shows a decrease in both messenger RNA and protein levels of resistin isoforms with aging. Food restriction decreases adiposity index in 8- and 24-month–old animals to values even lower than those of 3-month–old animals. Food restriction decreases resistin expression in both adipose tissues in 8-month–old but not in 24-month–old rats. Interestingly, concomitant with the improvement of insulin sensitivity asserted by homeostasis model assessment, resistin serum levels decrease only in food-restricted 8-month–old animals. In contrast, food restriction up-regulates s-resistin messenger RNA in epididymal adipose tissue, whereas no significant changes are appreciated in retroperitoneal adipose tissue. These data indicate that both forms of resistin are differentially regulated by fat depot location, aging, and even nutritional status, suggesting that alternative splicing plays a key role in this differential regulation.

1. Introduction

Resistin is a cysteine-rich hormone secreted by adipose tissue that belongs to the resistin-like molecules family [1]. Early studies showed that in vivo administration of recombinant resistin impairs glucose tolerance and insulin actions in normal mice, whereas administration of anti–resistin antibody enhances the insulin sensitivity of insulin-resistant and obese mice [2]. This initial observation led to the hypothesis that resistin could act in promoting insulin resistance in different tissues, linking obesity and type 2 diabetes mellitus [2]. It has been reported that mice lacking resistin display low blood glucose levels after fasting and decreased expression of gluconeogenic enzymes, suggesting a role for resistin in mediating hyperglycemia associated with obesity [3]. In agreement with these proposals, several reports seem to confirm that chronic hyperresistinemia impairs glucose metabolism, leading to hyperinsulinemia; hypertriglyceridemia; insulin resistance in skeletal muscle, liver, and adipose tissue; and glucose intolerance [4,5].

Visceral fat has been identified as a risk factor for the development of insulin resistance and type 2 diabetes mellitus. Increased levels of visceral adipose tissue (VAT) are correlated with decreased sensitivity to insulin [6,7]. Visceral adipose tissue is a major source of resistin [8], and increased adiposity correlates well with increased resistin plasma levels [9]. In agreement to this, Gabriely and coworkers [6] demonstrated the improvement of peripheral and hepatic insulin sensitivity in aged rats by removing the retroperitoneal and epididymal fat pads. These results

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support a potential role of resistin in insulin resistance development during aging that is characterized by an increased visceral adiposity [6]. Nevertheless, the relationship established between resistin and insulin resistance has not always been clear, leading to some controversy regarding the role of resistin as an insulin resistance mediator [10].

In Wistar rats, we have previously characterized an alternatively spliced resistin isoform, s-resistin, which is expressed mainly in VAT. This short form of resistin lacks the secretion domain and shows nuclear location in transfected cells [11], but its physiologic relevance remains to be established.

Wistar rats, like humans and other rodent models, have been shown to develop insulin resistance during aging [7,12,13]. Thus, in an attempt to analyze the possible relationship of both s-resistin and resistin (nonsecretable and secretable) isoforms with the already described [7,12,13] development of insulin resistance during aging, we have investigated their messenger RNA (mRNA) expression and total protein resistin isoforms levels in the 2 main visceral fat pads, epididymal and retroperitoneal, from 3-, 8-, and 24-month–old Wistar rats. On the other hand, as it is well known that nutritional status can modify the expression and serum levels of adipose-secreted factors [14-16], we also have used this animal model of aging and insulin resistance to study the effects of food restriction (FR) on the expression pattern of both resistin isoforms.

2. Materials and methods

2.1. Animals

Male Wistar rats aged 3, 8, and 24 months were used throughout the study. The animals were obtained from the *Centro de Biología Molecular Severo Ochoa* (Madrid, Spain). Rats were fed and housed as described previously [17]. The animals were handled according to the laws of the European Union and the guidelines of the National Institutes of Health, and the experimental protocols were approved by the institutional committee of bioethics.

2.1.1. FR protocol

Five- and 21-month–old rats were randomly assigned to undergo an FR protocol as described earlier [16]. Animals were placed in individual cages and fed daily with 18 and 21 g of chow, respectively (equivalent to ≈80% of normal food intake). Usually, 2 months after starting the nutritional restriction, animals show a body weight equivalent to approximately 85% of ad libitum (AL)–fed age mates. Animals were weighed weekly, and the amount of food provided was adjusted individually to maintain their body weight during 1 additional month. Food-restricted animals were used at the age of 8 and 24 months, respectively.

Overnight-fasted rats were killed by decapitation; and epididymal and retroperitoneal adipose tissues were collected, rapidly frozen in liquid nitrogen, and then stored at −80°C until analysis. Serum samples were obtained by blood centrifugation and stored at −80°C until used.

2.2. RNA isolation

Total cellular RNA was extracted from frozen VAT using RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany) by following the manufacturer's instructions.

2.3. Complementary DNA synthesis and real-time reverse transcriptase polymerase chain reaction

Complementary DNA (cDNA) was synthesized from 1 μg of DNase-treated RNA as previously described [18]. Relative quantitation of resistin was measured by real-time polymerase chain reaction (PCR) on an ABI PRISM 7500 Fast Sequence Detection System instrument and software (Applied Biosystem, Foster City, CA) and using a Pre-Developed (Rn00595224_m1) TaqMan Assay Reagents (Applied Biosystem) that spanned the junction between exons 2 (spliced in s-resistin) and 3. Specific amplification of s-resistin cDNA (GenBank accession number: AJ555618) was achieved by SYBR Green dye using forward primer 5′-GAGCTCTCTGCCACGTGCCA-3′ that annealed to the exon 1/exon 3 junction and reverse primer 5′-AGTC-TATGCTTCCGCACTGGC-3′ flanking a fragment of 189 base pairs (bp).

When comparative expression analyses were performed, both isoforms were measured with SYBR Green real-time reverse transcriptase (RT)–PCR. In this case, resistin cDNA was achieved using forward primer 5′-ACTTAACAGGAT-GAAGAACCTTTC-3′ and reverse primer 5′-GTAGG-GAGCTGAAGTCTTGATTGAT-3′; both primers are located in exon 2 and flank a 140-bp fragment. Amplification of endogenous control 18S ribosomal RNA was included using VIC (TaqMan Assay) or SYBR Green as real-time reporter. With the latter, the primers used were 5′-CGGCTACCACATCCAAGGAA-3′ and 5′-GCTGGAAT-TACCGCGGCT-3′, rendering a fragment of 187 bp.

2.4. Tissue extracts preparation and total resistin isoforms quantification

Fat depots, epididymal and retroperitoneal, were homogenized in 2 vol of isolation medium (250 mmol/L sucrose, 10 mmol/L HEPES [pH 7.4], 1 mmol/L EDTA, 2 mmol/L EGTA, 5 mmol/L NaN_3, 5 mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride, 2 mmol/L Na3VO4, 10 μg/mL leupeptin, 10 μg/mL benzamidine, and 1 μg/mL pepstatin) using a manual Dounce homogenizer and centrifuged at 450*g* for 5 minutes at 4°C. Protein concentration of resulting extracts was determined by Bradford assay. The total amount of both resistin isoforms present was determined using a rat resistin enzyme-linked immunosorbent assay (ELISA) kit according with the directions of the manufacturer (Spibio; Bertin Group, Toulouse, France).

2.5. Serum analysis

Serum insulin and leptin were determined using specific rat radioimmunoassay kits (Linco Research, St Charles, MO). Resistin was assessed using a rat resistin ELISA kit

(BioVendor, Brno, Czech Republic). Blood glucose concentration was determined using a glucose analyzer (Accutrend; Roche, Basel, Switzerland).

2.6. Obesity Lee index and adiposity determination

Obesity Lee index was calculated as $10^4 \times \sqrt[3]{\text{body weight (in grams)}} \times \text{nasoanal length (in millimeters)}^{-1}$, as described previously [19]. Epididymal, perirenal, and retroperitoneal fat depots were carefully dissected and weighed. The sum of the weight of these fat pads (visceral fat) divided by body weight × 100 was used as an index of adiposity [19]. Homeostasis model assessment (HOMA) for insulin resistance was calculated as fasting insulin (in microunits per milliliter) × fasting glucose (in millimoles per liter/22.5) as described earlier [20], using the HOMA calculator program v2.2.2 in the Web site of Diabetes Trials Unit, The Oxford Center of Diabetes, Endocrinology & Metabolism (http://www.dtu.ox.ac.uk).

2.7. Statistical analysis

Statistical comparisons were performed using 1-way analysis of variance (GraphPad Prism 3.03 software, La Jolla, CA). When the main effect was significant, the Bonferroni post hoc test was applied to determine individual differences between means. For comparisons between FR and AL age mates, the unpaired Student *t* test was used. *P* values less than .05 were considered to be statistically significant.

3. Results

3.1. Characteristics of the animals

The data of Table 1 show that aging increases body weight and adiposity in Wistar rats. These animals do not present overt alterations of glucose homeostasis, as indicated by fasting serum glucose and insulin concentrations. However, when HOMA values are calculated (Table 1), an increase in this parameter can be observed in 8- and 24-month–old rats with respect to control 3-month–old rats, suggesting that insulin resistance develops with aging. Moreover, aged rats exhibit a progressive and marked increase in serum leptin concentrations (Table 1). The FR protocol used herein decreases serum leptin levels and adiposity index and allows to obtain aged rats with a level of adiposity even below that of young animals (Table 1).

3.2. Relative expression of resistin isoforms in VAT

To evaluate the relative expression of both resistin isoforms, we performed a real-time RT-PCR analysis in both tissues from control young mature 3-month–old rats. As shown in Fig. 1A, spliced s-resistin transcript shows lower relative levels than resistin in both adipose tissues. Thus, s-resistin levels in epididymal and retroperitoneal VAT represent, respectively, 13% and 15% of those found for the secretable resistin.

In addition, we compared the expression of each resistin isoform independently in both adipose depots. Our results indicate that resistin variants, analyzed in this manner, exhibit similar expression levels (Fig. 1B). Therefore, in control young rats, resistin and the nonsecretable isoform s-resistin are similarly expressed in epididymal and retroperitoneal VAT.

3.3. Effect of aging and FR on resistin isoform expression in VAT

To analyze changes in resistin expression with aging and nutritional status, real-time RT-PCR assays were performed using total RNA from epididymal and retroperitoneal VAT from 3-, 8-, and 24-month–old Wistar rats, as well as 8- and 24-month–old food-restricted animals. In rats fed AL, resistin mRNA levels decreased (40%) in epididymal VAT from 8-month–old with respect to 3-month–old rats. A more severe decrease (83%) was observed in 24-month–old rats (Fig. 2). In contrast, a different pattern of expression was observed in retroperitoneal VAT, where levels of resistin mRNA were similar in all ages tested (Fig. 2).

Aged Wistar rats have been shown to present peripheral insulin resistance without obvious alterations of glucose homeostasis; and FR reverts insulin resistance in 8-month–old rats, suggesting that adiposity rather than aging may be related to insulin resistance [7]. Thus, to discriminate

Table 1
Biological characteristics of the animals

	3 months AL	8 months AL	8 months FR	24 months AL	24 months FR
Body weight (g)	438 ± 17	562 ± 21*	422 ± 9‡	778 ± 22*,†	607 ± 13‡
Blood glucose (mg/dL)	123 ± 4	131 ± 5	109 ± 4	126 ± 4	125 ± 4
Serum insulin (ng/mL)	1.0 ± 0.1	1.0 ± 0.2	0.67 ± 0.03‡	1.2 ± 0.2	1.09 ± 0.03
Serum leptin (ng/mL)	4.9 ± 0.5	6.2 ± 0.4*	1.7 ± 0.1‡	19 ± 1*,†	7 ± 0.9‡
HOMA index	4.5 ± 0.21	4.2 ± 0.92	1.8 ± 0.22‡	5.6 ± 0.93	6.0 ± 0.54
Lee index	311 ± 3	315 ± 4	297 ± 3‡	319 ± 3	302 ± 4‡
Adiposity (%)	3.1 ± 0.1	5.3 ± 0.3*	1.4 ± 0.06‡	5.2 ± 0.4*	1.8 ± 0.1‡

Values are means ±SEM of 10 to 12 separate determinations per group of animals.

* $P < .05$ compared with 3-month–old rats.

† $P < .05$ compared with 8-month–old rats.

‡ $P < .05$ compared with AL age-mate rats.

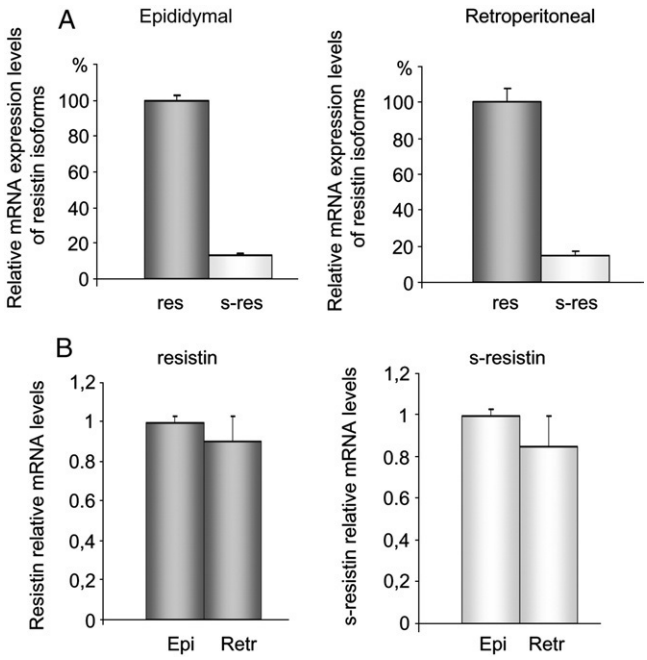

Fig. 1. Comparative analysis of relative expression of resistin isoforms in VAT. A, Relative quantitation of resistin and s-resistin mRNA expression was performed by SYBR Green real-time PCR as stated in "Materials and methods" in epididymal and retroperitoneal adipose tissue from 3-month–old rats. Values are means ± SEM of 5 to 7 separate determinations. Data were normalized to the resistin rate, which was assigned an arbitrary expression value of 100%. B, Comparative tissue analysis of the resistin isoforms expression. Both variants were specifically amplified ("Materials and methods"), and their expression was compared in each tissue. Values are means ± SEM of 5 to 7 separate determinations. Data were normalized to epididymal rate, which was assigned an arbitrary expression value of 1.

between the effects of age-associated adiposity from those of aging, we also analyzed resistin expression in both visceral fat depots in 8- and 24-month–old rats after 3 months of FR. Our results indicate that FR significantly decreases mRNA (70% in epididymal and 45% in retroperitoneal) in 8-month–old animals but fails to do so in 24-month–old rats (Fig. 2).

3.4. Effect of aging and FR on s-resistin isoform expression in VAT

Fig. 3 shows the expression pattern of the short variant, s-resistin, with aging and FR in epididymal and retroperitoneal VAT. In rats fed AL, s-resistin displays a different expression pattern with aging in the analyzed tissues. Thus, whereas in epididymal VAT there is no significant change in the expression of s-resistin at 8 months and a 60% decrease is appreciated in 24-month–old rats (Fig. 3), in retroperitoneal VAT, a significant 84% increase is observed in 8-month–old rats, with no significant change in 24-month–old rats (Fig. 3). In addition, a different response in the expression pattern is obtained for s-resistin under FR conditions. Thus, s-resistin expression is significantly induced in epididymal adipose tissue in 8- (67%) and 24-month–old rats (325%), with respect to their age mates fed AL; but FR does not modify s-resistin expression in retroperitoneal VAT from either 8- or 24-month–old rats (Fig. 3).

3.5. Effect of aging and FR on resistin protein levels in VAT and serum

In an attempt to evaluate the changes in total protein levels of resistin in VAT with aging and FR, we performed a specific rat resistin ELISA assay from total epididymal and retroperitoneal protein extract. Despite this assay not allowing discrimination between both resistin isoforms, the pattern observed for protein levels of resistin in each adipose tissue depot with aging is similar to that observed for resistin mRNA (Fig. 4). Moreover, although the total protein content of resistin in both tissues remain unaltered in restricted 8-month–old rats, a decrease was observed in 24-month–old animals (Fig. 4).

Because the Wistar rats used in this study present a moderate increase of adiposity with aging (Table 1) and the

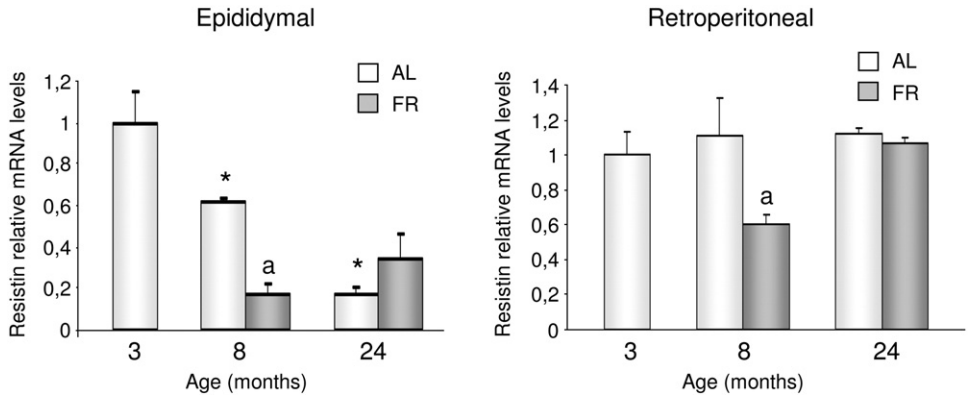

Fig. 2. Resistin mRNA expression in epididymal and retroperitoneal VAT from aged and FR rats. Messenger RNA resistin in epididymal and retroperitoneal adipose tissue from 3-, 8-, and 24-month–old rats fed AL (white bars), as well as their nutritionally restricted age mates (FR, gray bars), was determined as indicated in "Materials and methods." Data were normalized to the 3-months–old rats, which were assigned an arbitrary value of 1. *$P < .001$ vs other ages, [a]$P < .001$ vs same-age fed AL. Values are means ± SEM of 5 to 7 separate determinations per group of animals.

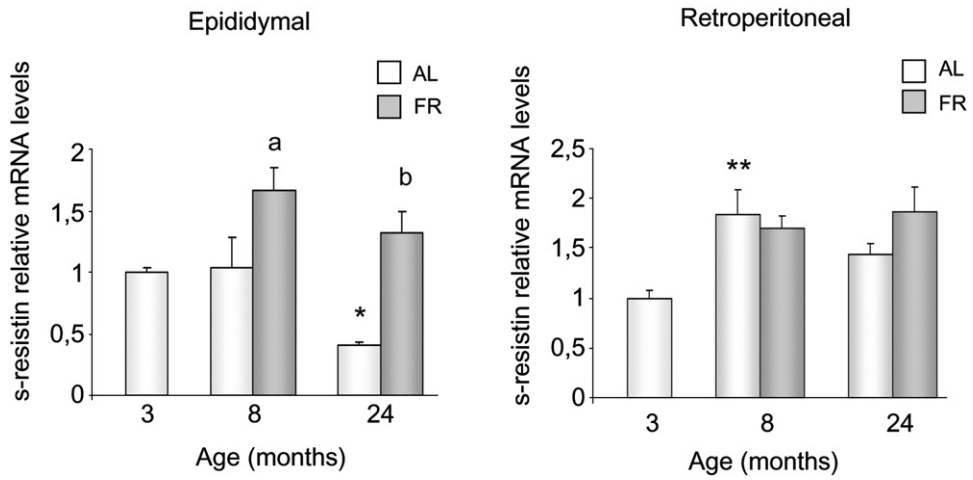

Fig. 3. The s-resistin mRNA expression in epididymal and retroperitoneal VAT from aged and FR rats. Expression of s-resistin in epididymal and retroperitoneal adipose tissues was analyzed from the same samples used in Fig. 2, as described in "Materials and methods." Data were normalized to the 3-months–old rats, which were assigned an arbitrary value of 1. *$P < .05$ vs other ages, **$P < .01$ vs 3-month–old animals, [a]$P < .05$ and [b]$P < .001$ compared with the same-age fed AL. Values are means ± SEM of 5 to 7 separate determinations per group of animals.

resistin serum levels are increased in some mice obesity models [2,9], we decided to investigate the possible correlation between resistin expression and protein content, in both visceral fat depots, with resistin serum levels during aging and FR conditions. As shown in Fig. 5, concomitant with the higher increase in adiposity during aging, 8-month–old rats exhibited a marked increase in resistin serum concentration. This parameter, however, decreased in 24-month–old rats but did not reach lower values than those of control young rats.

Moreover, as in the case of mRNA of resistin secretable isoform (Fig. 2), FR decreased resistin serum levels in 8-month–old animals, but failed to do so in 24-month–old rats (Fig. 5). Interestingly, it has been recently reported that the same FR protocol is not as effective in 24-month–old as in 8-month–old rats to revert insulin resistance [7].

4. Discussion

The data of Table 1 describing a moderate increase in adiposity as well as an increase of HOMA during aging agree well with our previous results [7,12] that, by euglycemic-hyperinsulinemic clamp techniques, demonstrated that aging is associated with the development of insulin resistance in Wistar rats.

The adipocyte-derived hormone resistin has been suggested to be involved in the development of obesity-associated diabetes and insulin resistance [2]. In the Wistar rat, 2 resistin variants originated by alternative splicing have been previously described [11]. Because of the given role of intraabdominal fat in the development of insulin resistance and taking into account that adipose tissue from different anatomical sites has different metabolic responses,

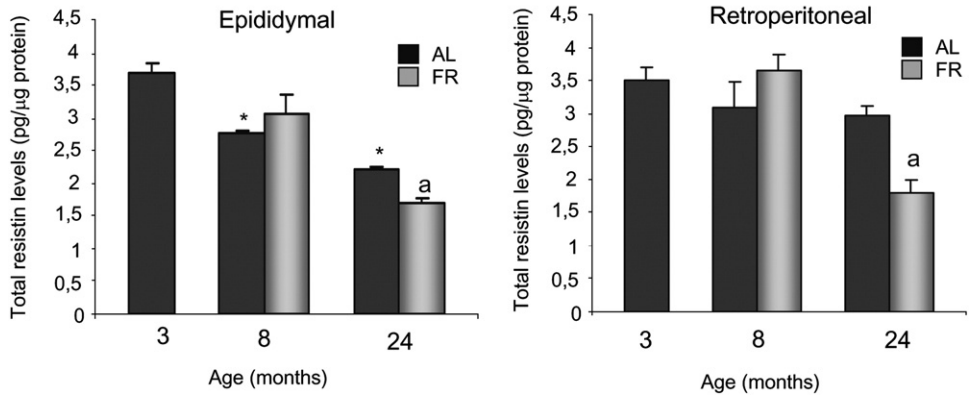

Fig. 4. Total protein levels of resistin in epididymal and retroperitoneal VAT from aged and FR rats. The total amount of resistin isoforms from total protein extracts of epididymal and retroperitoneal adipose tissues from the same animals described in Fig. 2 was determined by ELISA as described in "Materials and methods." Fed AL, dark bars; nutritionally restricted age mates (FR), gray bars. *$P < .01$ vs other ages, [a]$P < .001$ vs same-age fed AL. Values are means ± SEM of 3 to 4 separate determinations per group of animals.

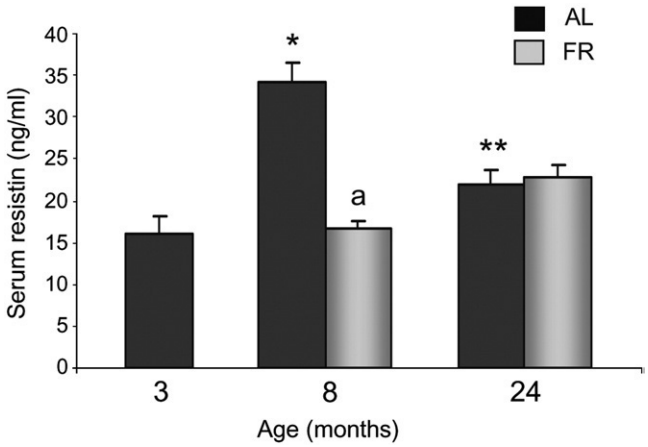

Fig. 5. Serum levels of the secretable resistin isoform from aged and FR rats. Serum resistin levels from the same animals described above were measured by rat resistin ELISA kit. *$P < .001$ vs 3-month–old animals, **$P < .001$ vs 8-month–old animals, [a]$P < .001$ vs same-age fed AL.

in the present work, we investigated the evolution of the expression of both resistin isoforms in the 2 main visceral fat depots, epididymal and retroperitoneal, during aging. Moreover, to gain insight on the role of both resistin isoforms, we analyzed their expression and protein content in adipose tissue from rats under a moderate FR protocol during 3 months, which brings about a significant decrease in visceral adiposity.

Our data presented herein demonstrate that aging has a differential effect on resistin expression in both adipose depots. Thus, whereas in epididymal fat a progressive decrease in both mRNA and protein levels is observed with aging, in retroperitoneal adipose tissue, these parameters remain unchanged. This pattern of resistin expression seems to contrast with the evolution of serum resistin concentration during aging. Nevertheless, it should be noted that the amount of retroperitoneal fat increases up to 4-fold during aging [7]. Therefore, the global contribution of this tissue to resistin production increases with aging. Epididymal fat also increases 3-fold in 8-month–old rats with respect to 3-month–old rats and remains constant up to the age of 24 months [7]. Thus, up to the age of 8 months, despite no increase in the relative expression of resistin, the accretion of both visceral fat pads would bring about a significant net increase in circulating resistin. The decline of serum resistin levels in 24-month–old animals with respect to 8-month–old animals could be explained by the decrease in the relative expression of resistin in epididymal fat.

Resistin expression has previously been analyzed in different genetic and nongenetic rodent models of obesity and diabetes. Thus, as described herein, elevated serum resistin levels have been detected despite a lower expression per cell or gram of tissue [2,9,21]. In agreement with the above-mentioned idea, the increase of serum resistin levels in these animal models has been explained by the accretion of fat in these animals [1,9]. Wistar rats show a progressive increase in serum leptin concentration with aging [16,22], and it has been described that resistin expression is down-regulated by subcutaneous infusion of leptin in *ob/ob* mice and Wistar rats [23,24]. Therefore, it is possible that the hyperleptinemia developed with aging could be responsible, at least in part, for the observed decrease of resistin expression in epididymal adipose tissue. Besides these considerations, resistin production by other adipose depots such as subcutaneous depot and its modulation may also contribute to serum resistin concentration.

Data from different studies reported that resistin gene expression decreases by fasting and is restored by refeeding [1,9,25]. In agreement to that, we found a decrease of resistin expression in both visceral fat pads in 8-month–old food-restricted rats. Thus, unlike aging, FR affects resistin expression in the same manner in both epididymal and retroperitoneal fat. This decrease in resistin expression, together with the observed reduction in visceral adiposity, would explain the decrease of 53% of serum resistin in 8-month–old FR rats. Moreover, the fact that this FR protocol, concomitant with the decrease observed of HOMA in Table 1, is shown to revert insulin resistance in 8-month–old Wistar rats [6,7] supports a role for resistin in the impairment of insulin action. Another interesting datum to be considered is the evolution of adiponectin levels. Our previous data [7] demonstrate that this parameter does not present significant changes during aging. However, it should be pointed out that FR increased this parameter in 8-month–old but not in 24-month–old rats, suggesting a role for adiponectin in the recovery of insulin responsiveness in 8-month–old animals by FR.

In contrast to the changes observed in 8-month–old rats, FR has no effect on both resistin expression and serum resistin level in 24-month–old rats. Interestingly, these animals do not recover from insulin resistance after application of the same FR protocol as deduced by euglycemic-hyperinsulinemic clamp studies [7] or by the HOMA data presented herein (Table 1), supporting a clear relationship between resistin levels and insulin resistance. The reasons why 24-month–old FR rats do not show a decrease of resistin serum levels and do not recover insulin response as well as 8-month–old FR rats do still remain to be fully clarified. Nevertheless, it is possible that the sustained increase of adiposity and proinflammatory adipokines secretion for a long time, until the age of 21 months when caloric intervention takes place in these animals, may cause alterations more difficult to revert by caloric restriction. Alternatively, besides adiposity, aging by itself and/or other aging-associated factors might also regulate the serum levels and the expression of secretable resistin isoform in fat cells.

On the other hand, despite the decline in mRNA and serum resistin levels in 8-month–old FR rats, total resistin isoform levels in both tissues remained unchanged under FR conditions in these animals. Taking into account that the antibody used cannot discriminate between resistin isoforms (resistin from s-resistin proteins), these data could be due to

the increase of the expression of s-resistin in FR conditions (as discussed below). It would be also possible that, under FR, resistin could remain in the tissue by impairment of its secretion process. More studies could be necessary to investigate the cellular distribution of resistin isoforms under FR conditions.

The possible role for the intracellular resistin isoform s-resistin in the development of adipose tissue insulin resistance with aging remains to be elucidated. The results presented herein indicate that s-resistin levels are regulated by aging in a different way in both visceral adipose depots. Moreover, our results clearly show that the mRNA of intracellular s-resistin, unlike resistin, is up-regulated by FR only in epididymal fat at both ages analyzed and could contribute to keep up the protein levels detected in 8-month–old FR rats in this tissue. Thus, these data suggest that resistin and s-resistin isoforms could play a different role in nutritional adaptive responses in epididymal adipose tissue. The fact that s-resistin localizes in the nucleus in transformed cells [11] may suggest that it could play a role as an intracrine factor in the regulation of adipocyte gene expression. However, further studies are required to understand the function of this protein.

Previous studies reported physiologic differences between visceral and subcutaneous fat that correlated with differential responses to nutrients and changes in the gene expression for resistin and other cytokines [13,25,26] that could reflect a different expression of factors and/or receptors distribution among the different adipose depots. Here we have also observed evident differences between distinct visceral adipose depots in regard to the pattern of resistin isoforms expression. These data support the view that different fat depots may play different roles in adapting the organism to different physiologic circumstances. Besides, resistin isoform synthesis also responds to distinct factors and/or environmental changes, such as aging or nutritional status, in a tissue-specific manner, suggesting that regulation of alternative splicing could play a key role in this different pattern of resistin isoform expression.

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