Resistin is expressed in different rat tissues and is regulated in a tissue- and gender-specific manner

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Abstract **Resistin is a polypeptide hormone first reported from human and rodent adipocytes. In order to better define the potential biological role of resistin we undertook a detailed analysis of its expression in different rat tissues. We demonstrate by real-time reverse transcription polymerase chain reaction, Southern blotting and immunohistochemistry that resistin is expressed not only in brown and white adipose tissue, but also in the stomach, small and large intestines, adrenal gland, and skeletal muscle. Food deprivation led to a decrease in resistin mRNA expression only in adipose tissue, not in any of the other tissues studied. Furthermore, resistin mRNA expression is higher in males than in females in adipose tissue, not in any of the other tissues. Thus, our data suggest that resistin is not exclusively localized in adipocytes, and indicate that its expression is regulated in a tissue- and sex-specific manner.**

Key words: Resistin; Gender; Fasting; Obesity

1. Introduction

Resistin is a recently discovered 12.5-kDa cysteine-rich secreted polypeptide first reported from rodent adipocytes [1,2]. It belongs to a multigene family termed RELMs or FIZZ proteins [3,4]. Plasma resistin levels are significantly increased in both genetically susceptible and high-fat-diet-induced obese mice. Interestingly, immunoneutralization of resistin improves hyperglycemia and insulin resistance in high-fat-diet-induced obese mice, while administration of recombinant resistin impairs glucose tolerance and insulin action in normal mice [1]. More recently, it was demonstrated that increases in circulating resistin levels markedly stimulate glucose production in the presence of fixed physiological insulin levels [5], whereas insulin suppressed resistin expression [6]. Taken together, these results suggested that resistin could be a link between obesity and type 2 diabetes [7–9].

Initial studies on resistin regulation have indicated that its gene expression is reduced by fasting [1,2]. It has also been reported that circulating resistin levels are raised in genetically obese (ob/ob, db/db) mice [1]. In keeping with this, resistin mRNA levels were down-regulated by chronic food restriction in normal and pregnant female rats [10]. Thus, it appears that resistin levels are markedly influenced by nutritional status. In addition, resistin is regulated by testosterone [10,11], glucocorticoids [6,12], endothelin-1 [13], growth hormone [14], tumor necrosis factor-α [15], lipopolysaccharide [16], and thyroid hormones [10], which exert their effects in many different tissues. In this connection, it is well known that most of the bioactive peptides secreted by adipocytes, and notably leptin [17], are also synthesized in many other tissues, where they play a variety of different roles [18,19]. Like leptin [20], resistin is regulated by gonadal hormones. Some authors have reported that resistin levels are higher in fat from female than from male mice [1], but the opposite pattern has been reported for rats [10]. It is not clear whether this discrepancy reflects a genuine interspecies difference, or simply the fact that the mouse study did not confirm the statistical significance of the observed differences. Recently, Morash et al. [21] reported that resistin is not exclusively synthesized in adipocytes, since resistin mRNA is also observed in mouse brain and pituitary. In humans, in addition to white adipose tissue (WAT), resistin has also been found in monocytes [22,23] and placenta [24].

The aim of this work was to analyze the expression of resistin in different rat tissues, and to study the tissue-specific regulation of resistin mRNA levels in relation to sex and food deprivation.

2. Materials and methods

2.1. Animals

We used male and female Sprague–Dawley rats (200–250 g). The animals were housed at 23°C under a 12-h light–darkness cycle with free access to food (depending on the requirements of each experiment) and water. All experiments were carried out in accordance with standards approved by the Faculty Animal Committee.

2.2. Experimental setting 1

Resistin mRNA expression was analyzed in various peripheral tissues of adult male rats. Animals were killed by decapitation, and all tissues were rapidly explanted, snap-frozen on dry ice, and stored at −80°C until use.

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2.3. Experimental setting 2

To assess possible sex differences in resistin mRNA expression levels, adult male and female rats were used. Animals were killed by decapitation, and the different tissues were explanted, snap-frozen on dry ice, and stored at −80°C until use. To investigate the effect of fasting on resistin mRNA, two groups of adult male rats (one fed ad libitum, the other fasted for 72 h) were used.

2.4. RT-PCR and Southern blot analysis

Total RNA was extracted with the Trizol reagent (Invitrogen, Carlsbad, CA, USA) and 2 μg of this RNA was used to perform reverse transcription polymerase chain reaction (RT-PCR). Complementary DNA was synthesized using 200 U of Moloney murine leukemia reverse transcriptase (Invitrogen), 40 U of RNase Out (recombinant ribonuclease inhibitor; Invitrogen), 200 nM deoxy-NTP mixture, 15 mM $MgCl_2$, and 1 nM random hexamer primers, in a total volume of 30 μl. The reaction mixtures were incubated at 37°C for 1 h and at 42°C for 10 min. The RT reaction was terminated by heating at 95°C for 5 min. The amplification mixture was as follows: 1×PCR buffer [200 mM Tris–HCl pH 8.4 and 500 mM KCl (Invitrogen)], 50 mM $MgCl_2$, 200 nM deoxy-NTP mixture, 1 nM rat resistin upstream primer 5′-ACTTCAGCTCCCTACTGCCA-3′, 1 nM rat resistin downstream primer 5′-GCTCAGTTCTCAATCAACCGTCC-3′, and 1.25 U of Taq DNA polymerase (Invitrogen).

The primers used in the amplification of rat CD45 were 5′-TGTGAACATACGGATTGTGAA-3′ upstream primer and 5′-CTATGTCTGGTGTGCAGTTTG-3′ downstream primer.

The primers used in the amplification of rat aP2 were 5′-GACCTGGAAACTCGTCTCCA-3′ upstream primer and 5′-CATGACACATTCCACCACCA-3′ downstream primer.

PCR amplification cycle parameters were as follows: denaturation at 95°C for 30 s, annealing at 60°C for 1 min, and extension at 72°C for 1 min. The final step was extension at 72 °C for 10 min.

To confirm the quality of the mRNA in each sample, it was amplified together with β-actin (forward primer 5′-TACAACTCCTTGCAGCTCC-3′ and reverse primer 5′-ATCTTCATGAGGTAGTCAGTC-3′). The PCR amplification generated a single 273-bp rat resistin product, a 162-bp rat CD45 product, a 349-bp rat aP2 product, and a single 601-bp β-actin product.

PCR products were separated on 1.5% agarose gel, stained with ethidium bromide and examined with UV light using a Gel Doc 1000 Documentation System (Bio-Rad Laboratories, Hercules, CA, USA). To confirm authenticity of the amplimers, a Southern blot analysis was carried out. Hybridization of nylon-transferred resolved amplicons was performed using a ^{32}P cDNA probe specific for rat resistin [10]. Hybridization was carried out at 42°C for 18 h. After removing the excess labeled probe by washing, the hybridization patterns were revealed by autoradiography.

2.5. Analysis of resistin gene expression by real-time RT-PCR

Real-time RT-PCR analyses were performed in a fluorescent temperature cycler (LightCycler, Roche Molecular Biochemicals, Mannheim, Germany) as per the manufacturer's instructions. Two micrograms of total RNA were used for each RT reaction. The 20-μl amplification mixture contained 2 μl of RT reaction products plus 3 mM $MgCl_2$, 0.5 μM of each primer, and 1×LightCycler DNA Master SYBR Green I mix (Roche Molecular Biochemicals). After initial denaturation at 94°C for 30 s, reactions were cycled 34 times using the following parameters for resistin detection: denaturation at 95°C for 1 s, annealing at 60°C for 7 s, and extension at 72°C for 11 s. rHPRT cDNA was amplified under the same conditions. The amount of PCR products formed in each cycle was evaluated on the basis of SYBR Green I fluorescence. At the end of each run, melting curve profiles were produced (cooling the sample to 68°C and heating slowly to 95°C, with continuous measurement of fluorescence) to confirm amplification of specific transcripts (data not shown). The oligonucleotide primers specific for rat resistin are described above. Cycle-to-cycle fluorescence emission readings were monitored and quantified using the second derivative maximum method of the LightCycler software package (Roche Molecular Biochemicals). This method determines the crossing points of individual samples by an algorithm that identifies the first turning point of the fluorescence curve. This turning point corresponds to the first maximum of the second derivative curve and correlates inversely with the log of the initial template concentration. Resistin mRNA levels were normalized with respect to of rHPRT level in each sample.

2.6. Immunohistochemistry

Samples of normal brown (BAT) and WAT, skeletal muscle, adrenal gland, stomach (fundus and pylorus), small bowel (duodenum) and liver were immersion-fixed in 10% buffered formalin for 24 h, then dehydrated and embedded in paraffin by a standard procedure. Sections 5 μm thick were mounted on Histobond adhesion slides (Marienfeld, Lauda-Königshofen, Germany), then dewaxed and rehydrated. For immunohistochemical staining, two different antibodies were used: anti-mouse resistin raised in guinea pig (Linco, St. Charles, MO, USA) and anti-human resistin raised in rabbit (Phoenix Pharmaceuticals, Belmont, CA, USA). With the antiserum against anti-human resistin, the Envision dextran polymer method was employed as detection system. The sections were incubated in (1) rabbit anti-human resistin antibody diluted 1/2000 for 1 h at room temperature, (2) 3% hydrogen peroxide for 10 min, (3) Envision/horseradish peroxidase rabbit/mouse (Dako) for 30 min, and (4) diaminobenzidine. Negative controls were performed by replacing the anti-resistin antibody with Tris-buffered saline (TBS), or with normal rabbit serum at the same concentration as the primary anti-human resistin antibody. This antibody shows 0% cross-reactivity with insulin, leptin and ghrelin, and its specificity for resistin has been demonstrated previously [21,25,26].

2.7. Statistical analysis

Mean resistin mRNA levels were compared between groups by analysis of variance followed by a post-hoc multiple comparison test. P values <0.05 were considered statistically significant.

3. Results

3.1. Expression of resistin in different rat tissues

Resistin mRNA expression was analyzed in various peripheral tissues of adult male rats (Fig. 1). In addition to its expression in WAT, resistin-specific DNA fragments were amplified by RT-PCR from RNA in BAT, stomach, large and small intestine, adrenal gland, and skeletal muscle (Fig. 1A). These results were confirmed by Southern blot analysis (Fig. 1B). Quantification of resistin mRNA levels by real-time RT-PCR, in all cases normalized with respect to rHPRT levels, indicates that resistin mRNA expression levels were highest in WAT and BAT and lower in all other tissues studied (Fig. 1D) and the results show that, while WAT is the tissue with highest levels, BAT, stomach, duodenum, jejunum, ileum, colon, adrenal gland, and skeletal muscle also express resistin mRNA. In the remaining tissues, resistin mRNA was almost undetectable.

To verify that the presence of resistin in the different tissues was not a consequence of white blood cell contamination, we assessed resistin mRNA expression in rat blood, finding that is not present in this tissue (Fig. 2A). We also investigated whether CD45 (a marker of mononuclear cells) is expressed in the tissues in which resistin was located, finding that it is present only in blood, not in any other tissue (Fig. 2B). Likewise, to verify that the presence of resistin was not a consequence of adipocyte contamination, we tested for mRNA of aP2 (an adipocyte-specific mRNA), which we detected only in WAT and BAT (Fig. 2C) as previously reported [27,28].

To further explore resistin expression in these tissues, we carried out an immunohistochemical study with two specific antibodies (directed to mouse and human resistin, respectively). With human resistin antiserum, we observed an intense immunostaining for resistin in both BAT (Fig. 3a) and WAT (Fig. 3b). Weak immunoreactivity was observed in duodenum, mainly the epithelial lining and crypts (Fig. 3c). In the stomach (fundic and pyloric mucosa), intense resistin immunoreactivity was observed, primarily in oxyntic cells (Fig.

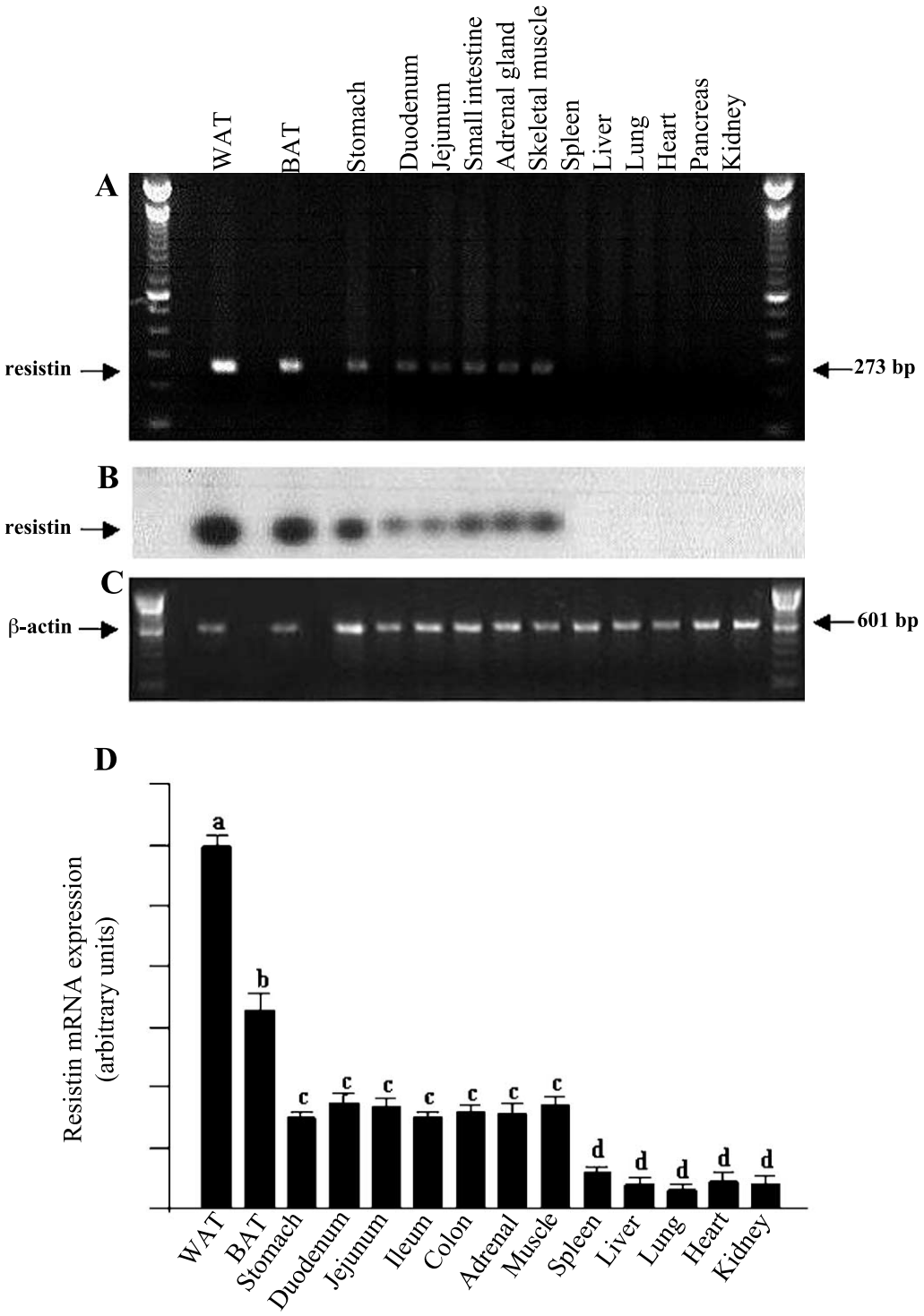

Fig. 1. Resistin mRNA expression in different rat tissues after 34 cycles of amplification: RT-PCR (A), Southern blot (B), β-actin (C). Semi-quantitative resistin mRNA levels in the different tissues, as measured by real-time RT-PCR over the same number of cycles, normalized with respect to endogenous rHPRT mRNA levels (D). Histograms with different superscript letters are statistically different (b = $P < 0.05$; c = $P < 0.01$ and d = $P < 0.001$).

3d) and neuroendocrine cells (Fig. 3e). Skeletal muscle cells also showed intense immunostaining (Fig. 3f). Less intense immunoreactivity was observed in the adrenal cortex (Fig. 3g). Finally, no immunoreactivity was observed in the liver (Fig. 3h). The same localizations were observed with both antibodies used. Negative controls were performed by replacement of the primary antibody with TBS or normal serum: in no case was immunoreaction observed. Similar results were obtained using an anti-mouse resistin antiserum (data not shown).

3.2. *Influence of gender on resistin mRNA levels*

As shown in Fig. 4A, assessment of resistin mRNA levels in male and female rat tissues by real-time RT-PCR indicated

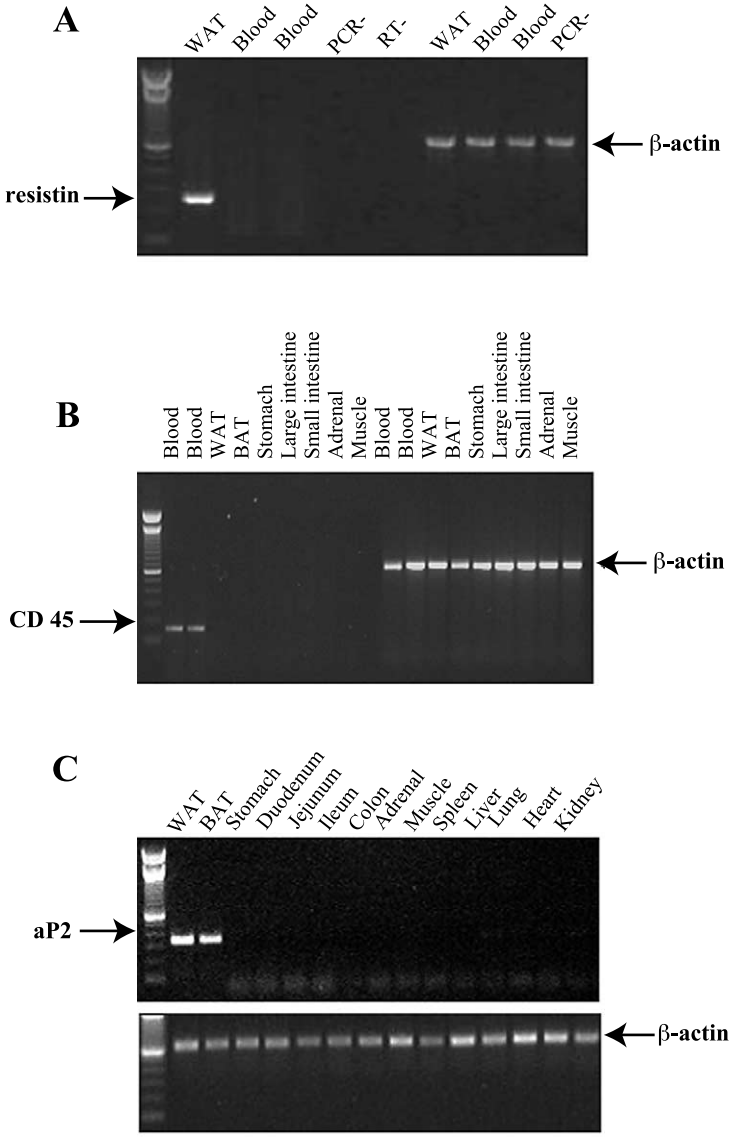

Fig. 2. Representative RT-PCR analysis showing absence of resistin mRNA expression in blood cells after 32 cycles (A). Representative RT-PCR analysis showing presence of CD45 mRNA in blood but not in any of the tissues in which resistin was detected (B). Representative RT-PCR analysis of aP2 mRNA expression in all the tissues in which resistin was detected (C). The quality of the mRNA was tested by parallel amplification of β-actin.

significantly higher levels in males than females in WAT and BAT, but no sex difference in any of the other tissues studied.

3.3. Influence of fasting on resistin mRNA levels

In order to investigate the possible influence of nutritional status, we determined resistin mRNA levels after complete food deprivation for 72 h, in all the tissues in which resistin had been detected. Our results (Fig. 4B) indicate that resistin mRNA levels in WAT of fasted rats were lower than in non-fasted rats. No effect of fasting was observed in any of the other tissues studied.

4. Discussion

Resistin is a signaling molecule that was first reported as a protein secreted specifically by adipocytes [1]. However, it has very recently been demonstrated that resistin mRNA was expressed in mouse brain and pituitary [21] and in the brown adipocyte cell line T37i [29]. Available data on resistin gene expression in humans are rather contradictory. Some authors have reported that mononuclear blood cells express resistin [22,30,31] and that increased resistin mRNA expression in adipose tissue biopsies from obese patients could be due to a greater abundance of mononuclear cells in adipose tissue from obese patients; however, other authors have reported that adipose-tissue-depot-specific resistin protein and mRNA expression is not due to the presence of peripheral blood mononuclear cells [26].

In order to gain further insight into the physiological role of resistin, we decided to carry out a detailed analysis of its expression in different rat tissues, using real-time RT-PCR, Southern blotting and immunohistochemistry. We observed that resistin is not secreted exclusively by adipose tissue, but is also expressed in the gastrointestinal tract, adrenal gland, and skeletal muscle. The expression of resistin in the gastrointestinal tract and the variation in resistin mRNA levels in

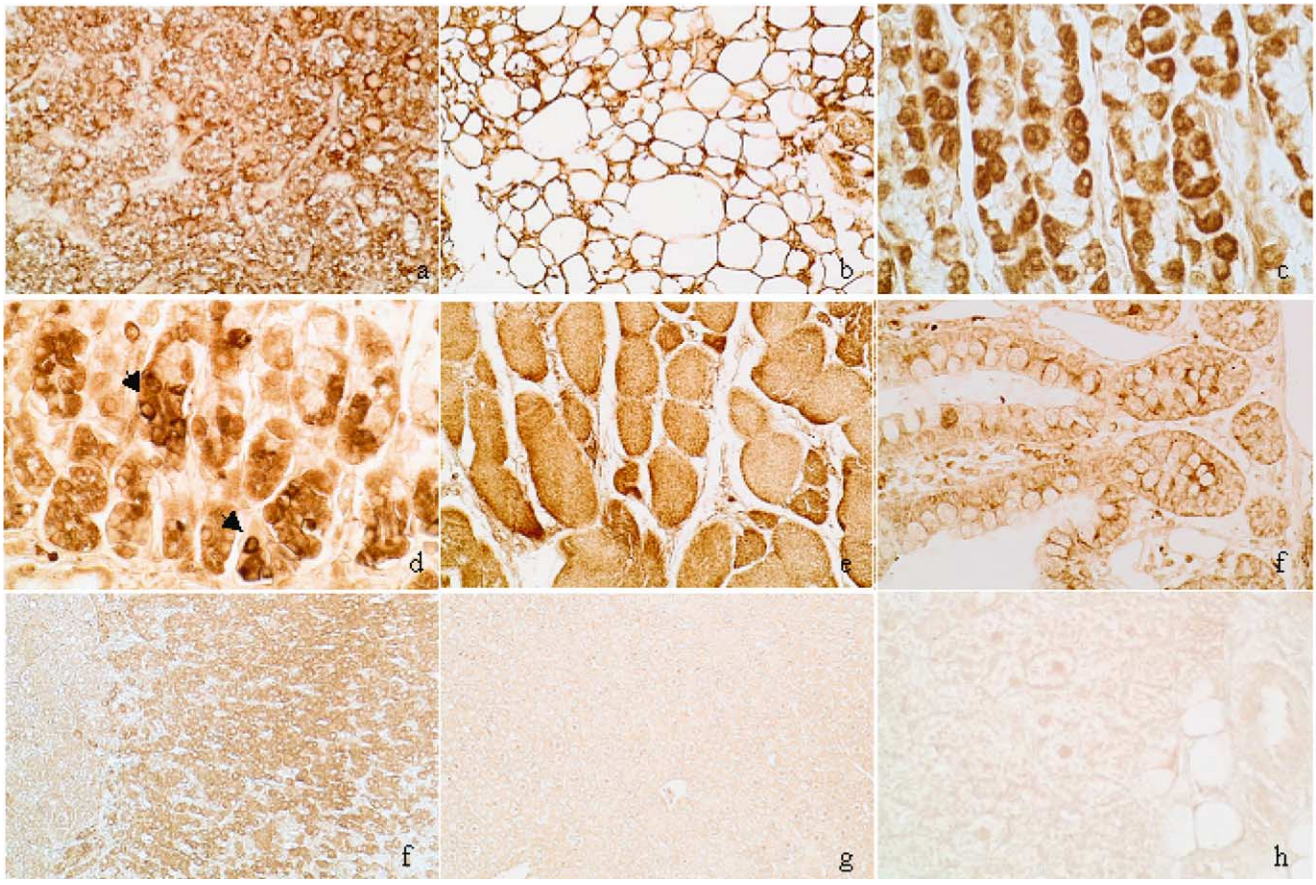

Fig. 3. Resistin immunoreactivity (human resistin antibody) was localized in: (a) BAT (objective magnification ×40); (b) WAT (×40); (c) epithelial lining of villus and crypts of the duodenum (×40); (d) oxyntic cells of gastric fundic glands (×20); (e) neuroendocrine cells (arrows) of the pyloric region (×40); (f) skeletal striated muscle fibers (×40); and (g) adrenal cortex (×20). No immunostaining was observed in (h) liver (×20).

adipose tissue depending on nutritional status (levels reduced by fasting, increased by refeeding) [1,2] suggest that this protein may play a role in the maintenance of metabolic homeostasis. The distribution of resistin mRNA in the gastrointestinal tract is similar to that of peroxisome proliferator-activated receptor-γ (PPAR-γ), a factor that clearly increases insulin sensitivity in vivo, and that markedly reduces resistin gene expression and protein secretion [32–34]. PPAR-γ has been reported to be expressed in the stomach, duodenum, jejunum and colon, but not in the liver [35]. In contrast, very low levels of expression of PPAR-γ were reported in skeletal muscle and the adrenal gland [36]. Our data do not support a clear relationship between PPAR-γ and resistin gene expression, although it would certainly be interesting to perform a colocalization analysis of resistin and PPAR-γ in the tissues studied; independently of this, the present study has identified cell populations expressing resistin in adult rats, which suggests possible future directions for investigating the functions of this protein. However, until the receptor for resistin is characterized, it will be difficult to clarify its physiological role(s) in the different tissues in which it is present.

The presence of resistin in human mononuclear cells raises the possibility that the wide tissue distribution of resistin mRNA could be due to the presence of blood cells. However, and in agreement with date obtained in mice [21], we failed to detect resistin mRNA in rat blood cells. Likewise, we verified that the mononuclear cell marker CD45 is not expressed in any of the tissues in which resistin was detected. So, we demonstrate by RT-PCR that resistin expression in different tissues is not due to contamination by monocytes. Another possibility is that the wide tissue distribution of resistin reflects the presence of adipocytes. To investigate this possibility, we tested for the adipose-specific marker aP2; however, we only detected mRNA of this protein in WAT and BAT, in which it has been previously reported [27,28], not in any of the other tissues in which resistin was detected. Taken together, these results indicate that the detection of resistin mRNA was not due to the presence of monocytes or adipocytes.

Furthermore, immunohistochemistry performed with two specific antibodies (anti-human and anti-mouse resistin) demonstrated that resistin is localized in specific cells in each tissue (for example, oxyntic and neuroendocrine cells in the stomach; villi and crypts in the duodenum, cortical cells in adrenal gland); by contrast, no immunoreactivity was detected in mononuclear blood cells in any of the different tissues. Thus, whereas some authors have identified resistin in human blood mononuclear cells, in our experiments performed in rats, we were unable to corroborate this result. The reason for this could be the great variation in the organization of the resistin sequences in the different species [37], and this probably explains the functional differences between mouse and human resistin.

Having confirmed that resistin is widely expressed in different rat tissues, we decided to assess whether its levels are

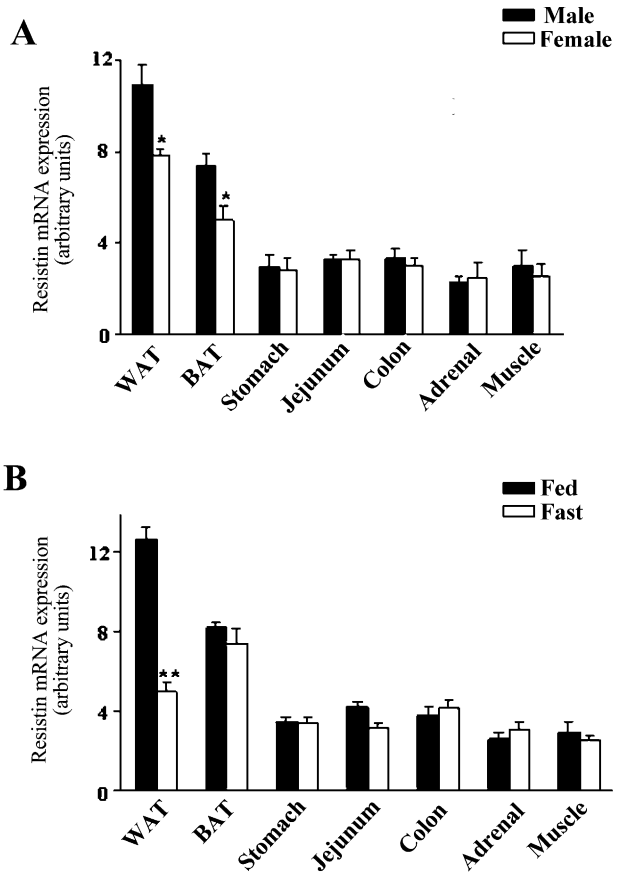

Fig. 4. Resistin mRNA levels in different tissues of male and female rats, as measured by real-time RT-PCR. Amplification of endogenous rHPRT mRNA was considered as control (A). Semi-quantitative real-time RT-PCR resistin mRNA of the expression levels in the different tissues of male rats fasted over 72 h. Amplification of endogenous HPRT mRNA was considered as control (B). The data are represented as mean ± S.E.M. ($n = 4$/group). $^{*}P < 0.05$, $^{**}P < 0.01$

regulated in a tissue-specific fashion. Specifically we assessed resistin mRNA levels in fed and fasted rats. In agreement with previous data [1,2], we found that resistin mRNA levels were decreased in WAT of fasted rats in comparison with ad-libitum-fed rats. Nutritional status appears to affect only WAT resistin levels but not resistin levels in any of the other tissues studied.

We then assessed the influence of gender on resistin mRNA levels in all the tissues in which this hormone was detected. Previously, our laboratory has demonstrated that WAT resistin mRNA expression is higher in males than in females at all ages studied [10]. In the present study we found that resistin is influenced by gender in a tissue-specific manner: its expression is higher in WAT and BAT of male rats than of female rats, but its levels do not differ between genders in the other tissues studied. This suggests that resistin may play different roles in males and females; however, the full significance of this result is unknown, and further studies must be performed to explore the physiological role of this hormone in the sexes.

In summary, this study provides the first evidence (a) that resistin is expressed in different rat tissues; (b) that in fasted rats, resistin mRNA expression decreases in WAT but not in other tissues; and (c) that resistin mRNA expression is higher in male than female rats in WAT and BAT, but not in other tissues.

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