Brown adipocytes are novel sites of expression and regulation of adiponectin and resistin

Say Viengchareun, Maria-Christina Zennaro, Laurent Pascual-Le Tallec, Marc Lombes*

INSERM U478, Institut Fédératif de Recherche Claude Bernard, Faculté de Médecine Xavier Bichat, 16 rue Henri Huchard, 75018 Paris, France

Received 17 September 2002; revised 24 October 2002; accepted 30 October 2002

First published online 19 November 2002

Edited by Veli-Pekka Lehto

Abstract **Adiponectin and resistin, two recently identified adipocyte-specific secretory factors, are able to modulate insulin actions in target tissues. To investigate their expression and hormonal regulation in brown adipocytes, we used the brown adipocyte cell line T37i, which, beside uncoupling protein expression, secretes leptin. Adiponectin and resistin mRNA were detected as a function of cell differentiation. Both transcripts were expressed at relatively high levels in differentiated T37i cells, reaching maximal levels on day 7, while resistin expression drastically fell afterwards. These stable transcripts ($t_{1/2} > 8$ h) were differentially regulated by factors involved in insulin responsiveness. Insulin and thiazolidinedione, a peroxisome proliferator-activated receptor γ agonist, stimulated resistin expression two- to four-fold in differentiated T37i cells, whereas adiponectin mRNA levels increased 1.5–2-fold. In contrast, dexamethasone and isoproterenol reduced by two-fold the level of adiponectin and resistin transcripts in differentiated T37i cells. This study provides the first direct evidence that differentiated brown adipocytes are endocrine cells capable of expressing adiponectin and resistin. The complex hormonal regulation of their expression in brown adipocytes clearly differs from that reported in white adipose tissue, pointing to differential physiological and pathophysiological implications of brown fat in energy homeostasis.**

Key words: Brown adipose tissue; Resistin; Adiponectin; Insulin sensitivity; Thiazolidinedione; Glucocorticoid

1. Introduction

For a long time white adipose tissue (WAT) was commonly viewed exclusively as an inert storage depot for triglycerides but this consideration is no longer true. Adipocytes have proved to be highly active cells with potent autocrine, paracrine and endocrine functions because they are able to secrete a wide range of protein factors including leptin [1], tumor necrosis factor [2], interleukin-6 [3], plasminogen activator inhibitor-1 [4], angiotensinogen, and acylation-stimulating protein [5].

Since the discovery of leptin, there has been great interest in these adipose tissue-derived secretory factors because they are believed to play an important role, particularly in situations of overloaded adipose tissue, in which they participate in the development of metabolic complications observed in obesity, diabetes, or insulin resistance.

Recently, two novel factors have been identified in white adipocytes. The first one, called adipocyte complement-related protein of 30 kDa, adipoQ or adiponectin, was discovered first [6,7] and was described as an important insulin-sensitizing hormone which is down-regulated in insulin resistance and obesity and replenishment of which in adiponectin-deficient states improves insulin sensitivity. A recent search for genes that are down-regulated by thiazolidinedione (TZD) antidiabetic drugs in mouse adipocytes led to the discovery of a second factor called resistin [8]. This latter circulates in the mouse, with increased levels in obesity, and has effects on glucose homeostasis that oppose those of insulin, suggesting that resistin is a potential link between obesity and insulin resistance.

Further studies investigated adiponectin and resistin gene expression only in white adipose cells and indicated that expression of these genes is under complex hormonal regulation, raising sometimes contradictory conclusions, especially concerning the biological role of resistin.

Beside the energy-storing WAT, mammals possess a specialized type of fat, the brown adipose tissue (BAT). This adipose tissue has absolutely separate physiological functions. Indeed, BAT is characterized by its thermogenic functions because it has the ability to dissipate energy and to provide heat. This results from the activity of the uncoupling proteins (UCP) which translocate protons through the inner membrane of mitochondria, uncouple oxidative phosphorylation and bypass the last enzymatic step of the respiratory chain, ATP synthase [9]. Therefore, BAT plays a major role in the regulation of body temperature, especially in small animals such as mice or rats. In human infants, BAT may provide a similar role, however the importance of BAT in adults remains to be elucidated.

Brown adipocytes have also won the status of endocrine cells because it has been shown that they are also able to express and secrete leptin in their environment [10]. However, no information is available concerning the expression and the hormonal regulation of adiponectin and resistin transcripts in the BAT. We have recently established the T37i cell line, derived from a hibernoma developed in a transgenic mouse in which the expression of SV40 large T antigen was placed under the control of the proximal promoter of the human mineralocorticoid receptor gene [11]. T37i cells are capable of differentiating into mature brown adipocytes upon insulin and triiodothyronine exposure. During this differentiation process, adipogenic gene activation occurs including those of

*Corresponding author. Fax: (33)-1-42 29 16 44.
E-mail address: mlombes@bichat.inserm.fr (M. Lombes).

0014-5793/02/$22.00
PII: S0014-5793(02)03697-9

adipocyte-specific fatty acid binding protein 2 (aP2), lipoprotein lipase (LPL), and peroxisome proliferator-activated receptor γ2 (PPARγ2) [12]. More importantly, expression of UCP1, characteristic of brown adipocytes, but also UCP2 and UCP3 has been also demonstrated in this cell line [13]. Therefore, the T37i cell line represents a suitable model to study BAT physiology.

The aim of the present study was to examine whether adiponectin and resistin are expressed in brown adipocytes. We next investigated the hormonal regulation of both hormones in this cell line. Surprisingly, we report that insulin is the major stimulator of resistin expression and slightly increases the level of adiponectin transcripts. Moreover, we demonstrate that TZD is also a potent stimulator of adiponectin and resistin gene expression. In contrast, glucocorticoids and β-adrenergic ligands drastically decrease the levels of adiponectin and resistin transcripts. Taken together, our results indicate that both adipocyte-specific secretory factors are under complex hormonal regulations in the BAT, which are clearly distinct from those described in the WAT, especially regarding the effects of insulin and TZD.

2. Materials and methods

2.1. Cell culture

T37i cells were cultured in DMEM/Ham's F12 medium (Invitrogen, Pontoise, France) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 20 mM HEPES and grown at 37°C in a humidified atmosphere with 5% CO_2. Differentiation into mature brown adipocytes was achieved under standard conditions by incubating subconfluent undifferentiated T37i cells with 2 nM triiodothyronine (Sigma Chemical Co., St. Louis, MO, USA) and 20 nM insulin (Invitrogen) for 7 days [11]. Adiponectin and resistin expression were thereafter analyzed by ribonuclease protection assays (RPA). Cells were generally incubated for 8 h in serum-free medium before treatment with various hormones for 18 h. All experiments were performed with T37i cells between passages 10 and 20.

2.2. Subcloning of the mouse adiponectin and resistin gene by RT-PCR

Total RNA was extracted from differentiated T37i cells and from mouse BAT using the TRIZOL reagent (Invitrogen). Briefly, 2 μg of total RNA were reverse-transcribed with 200 units of reverse transcriptase using the Superscript™ II kit (Invitrogen) according to the manufacturer's recommendations. Resistin and adiponectin cDNA were then amplified for 30 cycles in a total volume of 25 μl containing 1×PCR buffer (50 mM KCl, 20 mM Tris–HCl, pH 8.4), 200 μM dNTPs, 1.5 mM $MgCl_2$, 10 pmol of sense and antisense primers, and 0.25 units of platinum Taq Polymerase (Invitrogen). Adiponectin cDNA (532 bp) was amplified using the following sense (5′-GACGTTACTACAACTGAAGAGC-3′) and antisense (5′-CATTCTTTTCCTGATACTGGTC-3′) oligonucleotide primers and resistin cDNA (386 bp) was amplified using the following sense (5′-ACTGAGTTGTGTCCTGCTAAG-3′) and antisense (5′-CCACGCTCACTTCCCCGACATC-3′) oligonucleotide primers. The thermal cycling program was 95°C for 45 s, following a hybridization step at 56°C (adiponectin and resistin) for 45 s, and an elongation step at 72°C for 45 s. Each cDNA was run on a 2% agarose gel and purified from the gel using the Qiaquick PCR Purification Kit Protocol (Qiagen, Courtaboeuf, France). Adiponectin and resistin cDNA were thereafter subcloned into the pGEM-T easy cloning plasmid (Promega, Madison, WI, USA). The sequencing analysis of these cDNA confirmed that the PCR products subcloned corresponded to adiponectin (GenBank™ accession number MMU37222) and resistin (GenBank™ accession number AF323080), respectively.

2.3. Analysis of transcripts by RPA

In vitro synthesis of riboprobes was achieved using the adiponectin-PGEM-T easy and resistin-PGEM-T easy plasmids linearized by *Bgl*II and *Cla*I, respectively. Adiponectin and resistin α-^{32}P-labeled antisense riboprobes were then synthesized with T7 RNA polymerase (Promega). The mouse β-actin riboprobe, used as an internal control, was synthesized with T7 RNA polymerase after digestion of the β-actin-pGEM3 plasmid by *Bsu*36I.

RPAs were performed as previously described [10]. Twenty micrograms of total RNA, isolated from T37i cells using Trizol (Invitrogen), were hybridized overnight at 50°C in a formamide/PIPES hybridization buffer with 4×10^5 cpm adiponectin riboprobe, 4×10^5 cpm resistin riboprobe and 2×10^5 cpm β-actin riboprobe. Non-hybridized RNA was digested at 30°C for 1 h with a ribonuclease A and T1 mixture followed by a 30 min proteinase K and sodium dodecyl sulfate treatment, at 37°C. After phenol–chloroform extraction and ethanol precipitation, protected fragments were electrophoresed on a 6% polyacrylamide/urea gel. Gels were thereafter dried and fixed in 10% acetic acid. Radioactivity was counted generally overnight with an InstantImager (Packard, Meriden, CT, USA) followed by autoradiography. Results are expressed in arbitrary units and corresponded to the ratio between adiponectin- or resistin-specific counts versus β-actin signal.

Unprotected adiponectin riboprobe migrated at 240 bases with a protected fragment migrating at 179 bases. Unprotected resistin riboprobe migrated at 335 bases with a protected fragment migrating at 280 bases. Unprotected β-actin riboprobe migrated at 158 bases with a protected fragment migrating at 137 bases.

2.4. Drugs

Dexamethasone and isoproterenol were purchased from Sigma whereas insulin was purchased from Invitrogen. TZD (rosiglitazone or BRL 49653) was a generous gift from Dr. R. Negrel (Sophia Antipolis, Nice, France).

2.5. Statistical analysis

Student's *t*-tests were performed to analyze the data by use of the computer software InStat version 2.01 for Macintosh (GraphPad Software, San Diego, CA, USA). Values were considered significantly different at $P<0.05$.

3. Results and discussion

3.1. Adiponectin and resistin genes are expressed in differentiated T37i cells

Expression of adiponectin and resistin mRNA was first examined by RT-PCR in the T37i cell line as a function of the differentiation state. As shown in Fig. 1A, a 532 bp product, 100% identical to the adiponectin gene transcript, as confirmed by cDNA sequencing, was detected in differentiated T37i cells as well as in mouse BAT. Moreover, these cells also expressed resistin mRNA since a 386 bp amplicon, 100% identical to the resistin gene transcript, was also observed (Fig. 1B). However, neither adiponectin nor resistin transcripts were detected in undifferentiated T37i cells suggesting that adiponectin and resistin gene activation occurs during the adipocyte differentiation process. These results are comparable to those obtained in the WAT since expression of both transcripts was reported only in fully differentiated murine and human white adipose cells [6,8,14,15].

Quantification of adiponectin and resistin mRNA levels by RPA revealed that expression of both hormones increased progressively during brown adipocyte differentiation progression and reached a maximal level on day 7 (Fig. 1C–F). This coincided with the cell morphological changes characteristic of brown adipocytes as well as the sequential activation of adipogenic gene expression (LPL, PPARγ2 and aP2), as already reported [12]. Interestingly, we noted that adiponectin mRNA levels remained relatively high even on day 11 of the differentiation process (Fig. 1E) whereas resistin mRNA levels dramatically decreased after day 7 (Fig. 1F), possibly due to an autocrine mechanism that down-regulates the level of re-

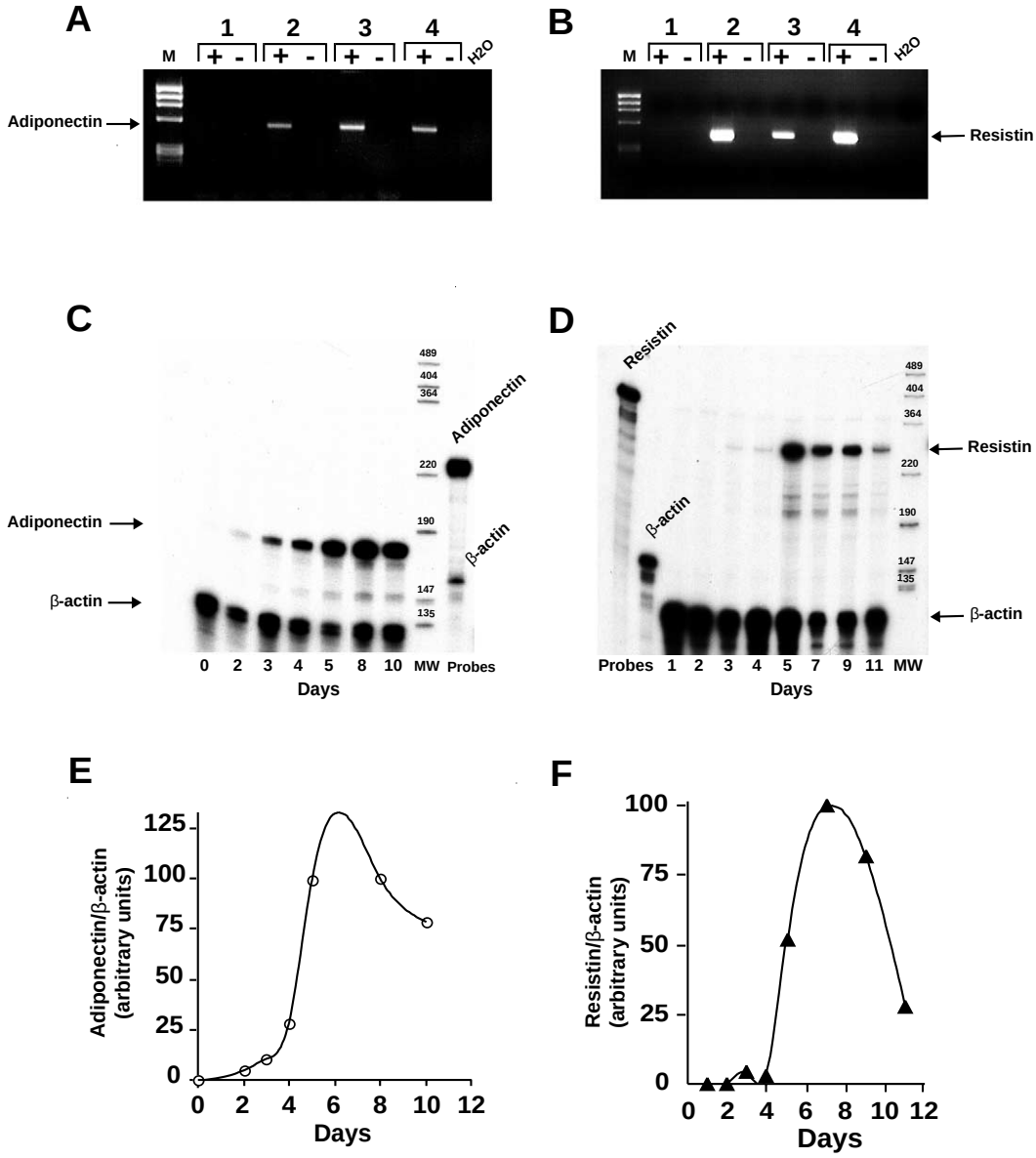

Fig. 1. Adiponectin and resistin genes are expressed in T37i cells. A,B: RT-PCR analyses of adiponectin (A) and resistin (B) gene expression. M: marker ladder, ΦX174 RF DNA/*Hae*III (Invitrogen); lane 1: undifferentiated T37i cells; lane 2: differentiated T37i cells on day 4 of the differentiation process; lane 3: differentiated T37i cells on day 8 of the differentiation process; lane 4: mouse interscapular BAT. Negative control was performed for each experimental condition by omitting the reverse transcriptase (− symbol). Arrows indicate the expected size of the PCR products. C,D: Time-dependent expression of adiponectin mRNA (C) and resistin mRNA (D) in T37i cells during differentiation. This illustrates one representative experiment. On different days of culture (from day 1 to day 11), total RNA was extracted and processed for RPA using specific probes for adiponectin or resistin together with β-actin. Signals were thereafter quantified by InstantImager and expressed as fold induction of adiponectin (E) or resistin (F) expression.

sistin mRNA. This latter result contrasts with that observed in 3T3-L1 cells, in which resistin expression peaked on day 4 and remained high even on day 12 [16], pointing to a tissue specificity of the BAT versus WAT regarding resistin expression. Of note, both adiponectin and resistin transcripts represented abundant messengers in the T37i cells, the level of adiponectin mRNA being always 10-fold higher than that of resistin, consistent with its status of most abundant gene transcript in adipose tissue [17]. These findings therefore suggest that adiponectin and resistin play important roles in brown adipocyte physiology.

Adiponectin and resistin gene expression could also be considered as markers of differentiation, together with UCP1, in the T37i cell line, since these genes were expressed exclusively in fully differentiated cells. This expression of adiponectin and resistin transcripts in differentiated T37i cells strengthens the idea that brown adipocytes are endocrine cells since they are possibly able to secrete these hormonal factors as well as leptin, as previously reported [10]. Thus, our cell line appears to be an appropriate system to investigate the hormonal regulation of these novel adipocyte-specific secretory factors in brown adipocytes.

3.2. *Hormonal regulation of adiponectin gene expression in differentiated T37i cell*

With the aim of understanding how adiponectin and resistin

transcripts were regulated in the BAT, we treated differentiated T37i cells on day 7 of the differentiation process with different mediators known to affect insulin sensitivity, including insulin, dexamethasone, TZD and isoproterenol. Adiponectin and resistin expression was thereafter analyzed by RPA.

Preliminary results showed that the level of adiponectin and resistin mRNA remained high in differentiated T37i cells even after incubation with 0.4 μM actinomycin D (data not shown), indicating that these two transcripts are relatively stable with a half-life $t_{1/2}$ of >8 h. We thus decided to incubate differentiated T37i cells for 8 h in serum-free medium before an overnight treatment with 20 nM insulin, 100 nM dexamethasone, 100 nM TZD or with 1 μM isoproterenol.

Fig. 2 indicates that insulin slightly increased by 1.4-fold the level of adiponectin transcripts ($P<0.01$ vs. control). A similar increase in adiponectin transcripts was also obtained with 100 nM insulin (data not shown), indicating that the stimulation was already maximal at 20 nM insulin. This is consistent with the high sensitivity of T37i cells to insulin action with an ED_{50} of 0.1 nM insulin as measured by the stimulation of leptin release [10] or glucose transport in these cells (unpublished results). The stimulatory effect of insulin on adiponectin expression observed in T37i cells contrasts with that reported in the 3T3-L1 cells in which insulin rapidly induced a strong decrease in adiponectin transcripts [18]. The opposite effect of insulin on adiponectin gene expression

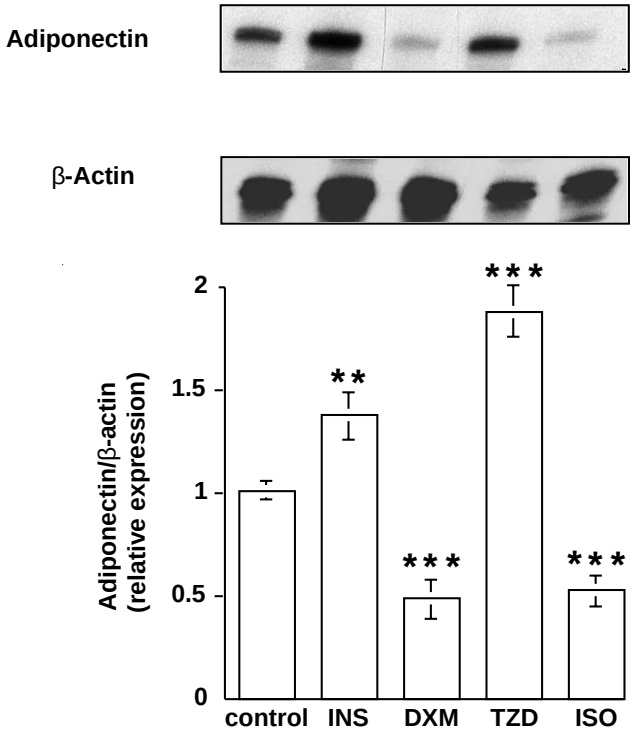

Fig. 2. Hormonal regulation of adiponectin gene expression in differentiated T37i cells. Differentiated T37i cells were incubated on day 7 of the differentiation state for 8 h in serum-free medium before treatment for 18 h with insulin 20 nM (INS), dexamethasone 100 nM (DXM), TZD 100 nM, or isoproterenol 1 μM (ISO). Total RNA was extracted and processed for RPA using specific probes for adiponectin and β-actin. Signals were quantified by Instant-Imager and expressed as relative expression of adiponectin. Results represent means ± S.E.M. for 9–18 independent determinations. $^{**}P<0.01$, $^{***}P<0.001$ vs. control.

provides additional support for important functional differences between the BAT versus the WAT.

Compared to insulin, TZD was a more potent stimulator of adiponectin expression, increasing adiponectin transcripts by two-fold ($P<0.001$ vs. control). This result was comparable to those obtained in the 3T3-L1 cell model in which this antidiabetic drug enhances both mRNA expression and secretion of adiponectin [19,20]. It was also shown that PPARγ agonists were able to increase adiponectin plasma levels in obese and diabetic mouse models [19] as well as in humans [20], supporting the notion that adiponectin is a marker of PPARγ activation in vivo and plays a major role in the regulation of glucose metabolism and body weight.

In contrast to insulin and TZD, dexamethasone decreased the level of adiponectin mRNA by approximately 50% ($P<0.001$ vs. control). This result was in agreement with those obtained in 3T3-L1 cells [18] or in human adipose visceral tissue [21], where glucocorticoids were shown to be potent inhibitors of adiponectin expression. We also observed a two-fold decrease in the level of adiponectin transcripts in T37i cells treated with isoproterenol, a result consistent with the 75% inhibition of adiponectin expression reported in murine white adipocytes [22]. This effect was mediated via the activation of the β3-adrenergic receptor and protein kinase A (PKA)-dependent pathway, supporting a role of decreased adiponectin in catecholamine-induced insulin resistance [22].

Altogether, we have demonstrated that the adiponectin gene is highly expressed in differentiated brown adipocytes and provided new insights into the regulation of this key hormone. Because adiponectin is able to affect insulin sensitivity most notably in the liver [23] and the muscle [24], any alteration of its expression may contribute to the development of insulin resistance [25]. Indeed, adiponectin plasma levels were shown to be decreased in obese [7,26] and diabetic patients [27,28], as well as in lipodystrophic patients [29], consistent with the involvement of adiponectin in insulin resistance and metabolic complications. Moreover, adiponectin was thought to be a physiological factor modulating endothelial functions thereby constituting a link to obesity and vascular diseases [30,31]. The exact contribution of the BAT-derived adiponectin in the development of such pathologies is likely to be less prominent than that of the WAT-secreted adiponectin. However, adiponectin locally secreted from the BAT may exert important metabolic effects through paracrine and/or autocrine mechanisms as recently proposed in bone marrow where this hormone had a major impact on lymphohematopoiesis and adipocyte differentiation [32].

3.3. *Hormonal regulation of resistin gene expression in differentiated T37i cell*

Fig. 3 presents the hormonal regulation of resistin transcripts by the same mediators. Our results indicated that resistin expression was strongly induced by insulin in a magnitude order of three- to four-fold ($P<0.001$ vs. control), suggesting that resistin gene transcription in T37i cells is tightly and rapidly regulated by insulin. This finding is reminiscent of the transcriptional control of *ob* gene expression by insulin observed in brown adipocytes [10], which involved activation of both phosphatidylinositol 3-kinase- and mitogen-activated protein kinase-dependent pathways. Unlike the stimulatory effect of insulin on resistin expression in the BAT, whether insulin is a positive or negative modulator of resistin

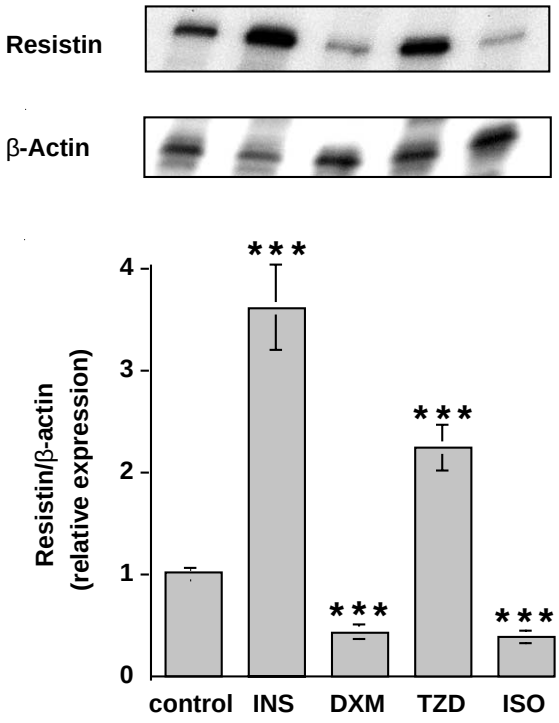

Fig. 3. Hormonal regulation of resistin gene expression in differentiated T37i cells. Differentiated T37i cells were incubated on day 7 of the differentiation state for 8 h in serum-free medium before treatment for 18 h with insulin 20 nM (INS), dexamethasone 100 nM (DXM), TZD 100 nM, or isoproterenol 1 μM (ISO). Total RNA was extracted and processed for RPA using specific probes for resistin and β-actin. Signals were quantified by InstantImager and expressed as relative expression of resistin. Results represent means ± S.E.M. for 9–20 independent determinations. ***$P < 0.001$ vs. control.

expression in the WAT is still a matter of controversy. Indeed, some authors reported that in vivo adipocyte resistin gene expression and protein were clearly increased during fasting/refeeding or by insulin administration [8,33] while others demonstrated a strong decrease in resistin expression in 3T3-L1 cells after incubation with insulin [16,34].

With respect to TZD, we showed that this antidiabetic drug was able to increase the level of resistin mRNA by two- to three-fold ($P < 0.001$ vs. control). This result was very surprising because resistin was initially identified as a gene down-regulated by TZD [8]. The inhibitory effect of TZD on resistin expression in 3T3-L1 cells was confirmed by subsequent studies [16,34,35]. However, the effects of TZD on resistin expression in WAT are also controversial since an increase in adipose tissue resistin mRNA levels was observed in *ob/ob* mice and Zucker rats treated with PPARγ agonists [36]. The important functional differences observed between the brown T37i and the white 3T3-L1 adipocyte cell model in terms of their differential responsiveness to TZD may actually rely on distinct mechanisms of gene transcriptional activation, most notably the function of PPARγ and C/EBPα, the two major adipogenic regulators. Indeed, at variance with the WAT, C/EBPα and PPARγ do not seem to be required for BAT differentiation and function [37,38].

As illustrated in Fig. 3, we next demonstrated that incubation of T37i cells by dexamethasone or isoproterenol decreased by 50% the level of resistin mRNA ($P < 0.001$ vs. control). The inhibitory effect of glucocorticoids on resistin expression resembles that previously reported for leptin expression in the T37i brown adipocyte model [10] but more work is needed to clearly define the molecular mechanisms restricting the expression of these adipocyte-specific genes. Along this line, catecholamines, through activation of the PKA cascade, were also potent negative regulators of resistin gene expression in 3T3-L1 cells [16,40].

Finally, to summarize our results, a schematic representation of the regulatory effects of various stimuli on resistin and adiponectin expression in brown adipocytes compared to that observed in white adipocytes is presented in Fig. 4. We provide the first direct evidence that differentiated brown adipocytes expressed both resistin and adiponectin genes which are under strikingly different hormonal regulation than that previously reported in the WAT. These findings bring additional support for major functional differences between these two types of adipose tissue and suggest their distinct involvement in various pathophysiological disorders. Indeed, given the major role played by resistin and adiponectin as factors regulating insulin sensitivity, BAT could be seen as a novel endocrine source of hormones, not only in rodents but also in humans [9,41,42]. The major impact of BAT in energy homeostasis has been highlighted by recent reports on animal models. A marked susceptibility to diet-induced obesity, diabetes and insulin resistance was observed in mice with ablation of BAT [43] and BAT-specific insulin receptor knockout mice unexpectedly presented with a defect in insulin secretion and impaired glucose tolerance [44] suggesting a functional link between brown fat and β cell function. In addition, it is more likely that brown adipocytes might locally secrete resistin and adiponectin resulting in important paracrine and autocrine actions within specific locations including white fat [39], bone marrow [32] or mammary glands [45]. Further studies are needed to precisely determine the complex regulatory cross-talk between adipocyte-specific secretory factors. It also remains to elucidate how the endocrine, paracrine or auto-

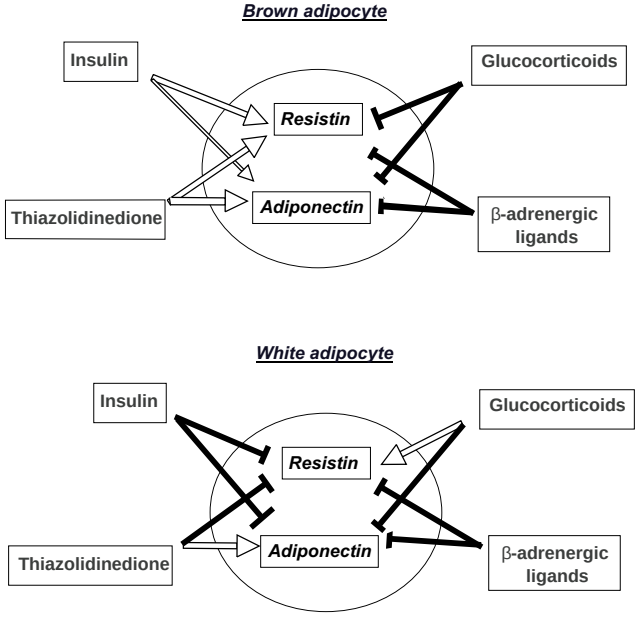

Fig. 4. Schematic models of hormonal regulation of adiponectin and resistin gene expression in brown and white adipocytes.

crine interplay among adipocytokine signaling pathways coordinately regulates insulin's actions and glucose and lipid metabolism to finally control adipose tissue mass and body weight.

Acknowledgements: We are indebted to Dr. R. Negrel (Sophia Antipolis, Nice, France) for providing us with the thiazolidinedione rosiglitazone (BRL 49653) and to Dr. D. Laouri for the β-actin-PGEMT-easy plasmid.

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