Inhibition by insulin of resistin gene expression in 3T3-L1 adipocytes

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Received 18 September 2001; accepted 18 September 2001

First published online 5 October 2001

Edited by Jacques Hanoune

Abstract Expression of the gene encoding resistin, a low molecular weight protein secreted from adipose tissue postulated to link obesity and type II diabetes, was examined in 3T3-L1 adipocytes. Resistin mRNA was detected in 3T3-L1 cells by day 3 following induction of differentiation into adipocytes; by day 4 the level of resistin mRNA peaked and remained high. The PPARγ activators, rosiglitazone or darglitazone, reduced the level of resistin mRNA. Dexamethasone upregulated resistin mRNA level, but no effect was observed with the β_3-adrenoceptor agonist, BRL 37344. A substantial reduction in resistin mRNA level was observed with insulin, which induced decreases at physiological concentrations. Insulin may be a major inhibitor of resistin production, and this does not support a role for resistin in insulin resistance. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Adipocyte; β-Adrenoceptor agonist; Dexamethasone; Diabetes; Insulin; Resistin

1. Introduction

Recent studies have indicated that white adipose tissue is an endocrine and secretory organ, in addition to being the key site of lipid storage in mammals [1–3]. White fat is in particular the most important source of the cytokine-like hormone, leptin, there being a direct relationship between the amount of body fat and the circulating level of this protein [4–6]. A wide range of proteins factors are secreted from white adipose tissue, including tumor necrosis factor (TNF)α, interleukin-6, adiponectin, plasminogen activator inhibitor-1, angiotensinogen and acylation stimulating protein [1–3]. The most recent secretory proteins to be identified from white fat are metallothionein [7,8] and resistin [9]; both are low molecular weight cysteine-rich proteins and resistin has been proposed as an adipose-specific hormone linking insulin resistance to obesity [9].

Resistin was discovered as such during a screen for genes which are downregulated in adipocytes by activators of the PPARγ nuclear receptor [9]. The resistin gene is part of a family of resistin-like molecules (RELMs) [10] which were independently discovered as the FIZZ gene family, with FIZZ3 being similar to resistin [11]. Resistin is also the same as the cysteine-rich adipose tissue-specific secretory factor (ADSF) [12]. No function was ascribed to FIZZ3, but as ADSF it has been suggested that the protein acts as a feedback regulator of adipogenesis since it inhibits adipocyte differentiation [12]. Thus two quite separate functions have been proposed for the resistin/FIZZ3/ADSF protein (which we refer to for simplicity as resistin).

Initial studies on the regulation of resistin production have indicated that expression of the gene is reduced by fasting [9,12] and there is a rapid increase in expression on refeeding [12]. In addition, insulin has been reported to stimulate resistin gene expression in streptozotocin-diabetic mice [12] and in Zucker diabetic fatty rats [13]. It has also been reported that the circulating resistin level is elevated in genetically obese (*ob*/*ob*, *db*/*db*) and high fat-fed obese mice [9], but surprisingly resistin mRNA levels in adipose tissue of different models of obesity (*ob*/*ob*, *db*/*db*, *tub*/*tub*, KKA^y) appear to be lower than in lean littermates [13].

In the present study we have examined the regulation of resistin gene expression in 3T3-L1 cells differentiated into adipocytes. The effects of insulin, dexamethasone, a selective β_3-adrenoceptor agonist, forskolin and 8-bromo-cAMP have been investigated. The response to a β_3-agonist was of particular interest given the importance of the β_3-adrenoceptor in the control of lipolysis and of leptin production in rodent adipose tissue [14–17], and in view of the recent suggestion that isoprenaline inhibits resistin gene expression [18].

2. Materials and methods

2.1. Animals

Adult male mice (C57BL/6J-CBA, F2-hybrid) from the animal unit, Faculty of Medicine, University of Oslo (Oslo, Norway) were killed by cervical dislocation and the following tissues rapidly removed and frozen in liquid N_2: interscapular brown fat, white adipose tissue (epididymal, perirenal and subcutaneous depots), liver, heart, kidneys, lungs, skeletal muscle (gastrocnemius) and the brain. The tissues were stored at −70°C. The mice were fed Rat and Mouse standard diet No. 1 (B&K Universal AS, Nittedal, Norway) containing approximately 19% protein and 5% fat.

2.2. Cell culture

3T3-L1 cells (American Type Culture Collection) were cultured at 37°C in a humidified atmosphere of 5% CO_2/95% air. The cells were maintained in growth medium with the following constituents: Dulbecco's modified Eagle's medium with 25 mM glucose (Sigma) containing 10% heat inactivated foetal bovine serum (FBS) (Integro b.v., The Netherlands), 2 mM L-glutamine and 50 U/ml penicillin and 50 ng/ml streptomycin (Sigma). Differentiation of the cells was initiated 2 days after confluence by incubation for 3 days in growth medium containing an additional 10% FBS, 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methyl-xanthine (Sigma) and 2 μM insulin. This was followed by 3 days in growth medium containing 0.2 μM insulin (no insulin thereafter). Differentiated cells were given fresh growth medium daily.

The cells were either maintained for up to 14 days after the induction of differentiation, and sampled every 1–2 days, or for 8 days after which time specific agents were added and the cells harvested 48 h

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0014-5793/01/$20.00 © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
PII: S0014-5793(01)02968-4

later. The following agents were employed: 8-bromo-cAMP (Sigma), BRL 37344 (Tocris), dexamethasaone, forskolin (Sigma), insulin, darglitazone and rosiglitazone. The cells were lysed in Trizol reagent (Gibco BRL) and stored at −70°C until analysis.

2.3. RNA preparation and Northern blot analysis

Total RNA was extracted from tissues and 3T3-L1 cells with Trizol, as described by the manufacturer. Twenty μg of the RNA was run on 1.4% agarose gels, blotted onto nylon membranes (Roche) by vacuum blotting and hybridised, as previously described [19,20]. An antisense oligonucleotide (5′-CGAATGTCCCACGAGCCACAGGCAGAGCCA) based on the mouse resistin cDNA sequence [9] was synthesised as a hybridisation probe and end-labelled (5′-end) with a digoxigenin ligand (Oswel DNA Services). Hybridisation signals were detected by chemiluminescence using CDP-*Star* (Roche) as the substrate, with membranes being exposed to film for up to 60 min. Signals were analysed by densitometry using a Personal Densitometer scanner and ImageQuant software (Molecular Dynamics Inc.).

After probing for resistin mRNA, blots were stripped and reprobed for other mRNAs or for 18S rRNA. The sequence of the digoxigenin-labelled antisense oligonucleotides used to detect *ob*, adipsin and lipoprotein lipase mRNAs and 18S rRNA were as described previously [19–21].

2.4. Data analysis

The effect of different treatments was expressed relative to the controls, which were assigned an arbitrary value of 100. The statistical significance of differences between groups was assessed using Student's *t*-test, with a threshold of $P < 0.05$.

3. Results

In initial studies, the expression of the resistin gene was assessed in mouse tissues by Northern blotting using a specific antisense oligonucleotide probe for resistin mRNA. A strong signal was obtained in each of the three white adipose tissue sites examined (epididymal, perirenal and subcutaneous) and there was no major difference in the level of resistin mRNA between these depots (Fig. 1). A very faint signal was discernible with interscapular brown adipose tissue, but no signal was detected in liver, brain, skeletal muscle, heart, kidney or the lungs (Fig. 1). Strong expression was present in 3T3-L1 cells examined at 12 days after the induction of differentiation into adipocytes (Fig. 1). A similar pattern of expression was evident for *ob* mRNA, except that a stronger signal was present in brown fat and there was no signal with 3T3-L1 cells (Fig. 1).

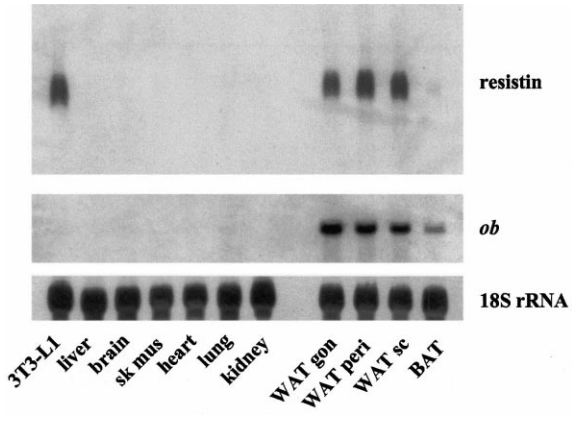

Fig. 1. Tissue specificity of resistin gene expression. Northern blot of total RNA from mouse tissues and 3T3-L1 cells were probed sequentially for resistin mRNA, *ob* mRNA and 18S rRNA, using specific antisense oligonucleotides. 20 μg of total RNA were added to each lane of the gel. The blots were stripped between hybridisations. The 3T3-L1 cells were harvested at 12 days after the induction of differentiation. WAT, white adipose tissue; BAT, interscapular brown adipose tissue; sk mus, skeletal muscle; gon, gonadal; peri, perirenal; sc, subcutaneous.

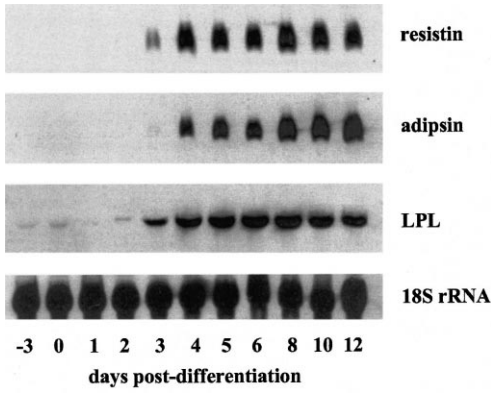

Fig. 2. Induction of resistin gene expression during differentiation of 3T3-L1 cells into adipocytes. Northern blot of total RNA from 3T3-L1 cells probed sequentially, before and after the induction of differentiation, for resistin mRNA, adipsin mRNA, lipoprotein lipase mRNA and 18S rRNA, using specific antisense oligonucleotides. 20 μg of total RNA were added to each lane of the gel. Differentiation was initiated on day 0 and the numbers indicate the days at which the cells were harvested.

Resistin mRNA was not detected in undifferentiated 3T3-L1 cells, but was evident by day 3 after the induction of differentiation into adipocytes (Fig. 2). A strong signal for resistin mRNA was present at day 4 post-differentiation and the signal remained high over the following 8 days. A similar pattern was evident with adipsin, a late marker of adipocyte differentiation, although at day 3 post-differentiation a signal for adipsin was barely detectable (Fig. 2). The level of lipoprotein lipase mRNA was high by day 3 post-differentiation and thereafter.

The effect of different agents on expression of the resistin

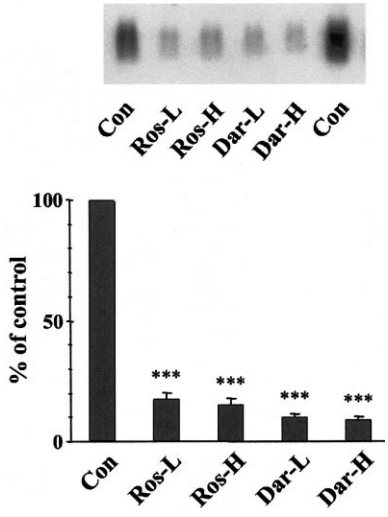

Fig. 3. Resistin mRNA levels in 3T3-L1 adipocytes incubated with PPARγ agonists. Cells were harvested at 8 days after the induction of differentiation and incubated for 48 h in media containing either 0.1 or 1 μM rosiglitazone (Ros-L and Ros-H, respectively) or darglitazone (Dar-L and Dar-H, respectively). 20 μg of total RNA were added to each lane of the gels. Upper panel shows a representative Northern blot. Lower panel gives mean values ± S.E.M. (bars) for groups of six. ***$P < 0.001$ compared with controls (Con).

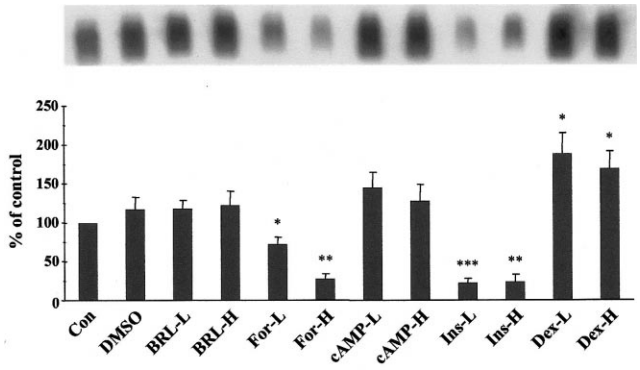

Fig. 4. Resistin mRNA levels in 3T3-L1 adipocytes incubated with various agents. Cells were harvested at 8 days after the induction of differentiation and incubated for 48 h in media containing either BRL 37344 (50 nM, BRL-L; 1 μM, BRL-H), forskolin (2 μM, For-L; 10 μM, For-H), 8-bromo-cAMP (cAMP-L, 2 μM; cAMP-H, 20 μM), insulin (1 μM, Ins-L; 10 μM, Ins-H) or dexamethasone (2 nM, Dex-L; 20 nM, Dex-H). 20 μg of total RNA were added to each lane of the gels. Upper panel shows a representative Northern blot. Lower panel gives mean values ± S.E.M. (bars) for groups of five. $*P<0.05$, $**P<0.01$, $***P<0.001$ compared with controls (Con or DMSO, according to whether DMSO was used as vehicle).

gene was examined between 8 and 10 days after the induction of differentiation when resistin mRNA levels were both high and stable. Incubating 3T3-L1 adipocytes in media containing specific agonists of the PPARγ nuclear receptor resulted in a marked inhibition of resistin gene expression. At concentrations of 100 nM and 1 μM, rosiglitazone (BRL 49653) reduced the level of resistin mRNA by approximately 85%, while similar concentrations of darglitazone led to reductions of 90% (Fig. 3).

Resistin expression was not significantly affected by 48 h incubation with the selective β_3-adrenoceptor agonist, BRL 37344 (50 nM or 1 μM), nor with 8-bromo-cAMP (Fig. 4). The lack of effect of BRL 37344 was also seen after 24 h of treatment (results not shown). However, incubation with 2 or 10 μM forskolin significantly reduced resistin mRNA levels to 65 and 23% of control values, respectively (Fig. 4). In contrast, resistin mRNA levels were significantly increased, by 1.7- and 1.9-fold, after incubation with 2 and 20 nM dexamethasone, respectively (Fig. 4). Importantly, a substantial reduction (>4-fold) in resistin mRNA level was observed with the addition of insulin (Fig. 4).

The amounts of insulin (1 and 10 μM) used in the experiment in Fig. 4 were high. The effect of adding different concentrations of insulin (from 0.2 to 4000 nM) was therefore determined and the dose–response curve obtained is shown in Fig. 5. The level of resistin mRNA was reduced by even the lowest concentrations of insulin used, and fell by more than half with the addition of a level of 6 nM or more, before the effect plateaued at 24% of the control value for the two highest concentrations.

4. Discussion

The present study confirms, using a specific 30-mer antisense oligonucleotide, that the resistin gene is strongly expressed in white adipose tissue in mice. Expression was as high in subcutaneous white fat as in the perirenal and epididymal depots. This contrasts with the *ob* gene where there are large differences between depots in the level of *ob* mRNA with much higher expression occurring in the internal fat depots of adult mice than in the subcutaneous sites [22].

The main aim of the present study was to investigate some of the factors that regulate the expression of the resistin gene, using 3T3-L1 adipocytes. Resistin mRNA was not detectable by Northern blotting until 3 days after the induction of differentiation of 3T3-L1 cells into adipocytes. Adipsin mRNA appeared slightly later, indicating that expression of the resistin gene is an earlier marker of adipocyte differentiation than the adipsin gene. From day 4 onwards the level of resistin mRNA in the 3T3-L1 adipocytes remained high, as with adipsin. The high level of resistin mRNA in 3T3-L1 adipocytes means that this clonal cell line can be used to investigate the regulation of resistin gene expression.

Addition of the PPARγ activators, rosiglitazone and darglitazone, led to a marked reduction in the level of resistin mRNA in 3T3-L1 adipocytes, consistent with the initial observations which led to the identification of resistin [9]. However, a recent report has indicated that treatment with PPARγ agonists leads to an increase rather than a decrease in resistin mRNA in white adipose tissue of two types of obese animal, *ob*/*ob* mice and Zucker rats [13]. A further paradox is that while resistin mRNA is reported to be reduced in white fat of various types of obese model [13], serum resistin levels appear to be elevated [9].

Dexamethasone increased the level of resistin mRNA in 3T3-L1 adipocytes, indicating that glucocorticoids stimulate the expression of the resistin gene, similar to their effect on *ob* gene expression and leptin production [21,23]. This observation is consistent with the induction of insulin resistance by glucocorticoid excess [24]. The effect of dexamethasone was small, however, implying that glucocorticoids are not major regulators of resistin production; furthermore, a report which appeared during the preparation of the present paper found no effect of the synthetic glucocorticoid on resistin mRNA

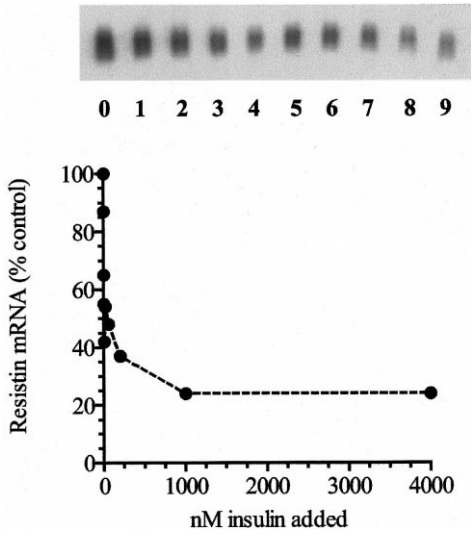

Fig. 5. Dose–response of the effect of insulin on resistin mRNA level in 3T3-L1 cells. Cells were harvested at 8 days after the induction of differentiation and incubated for 48 h in media to which different amounts of insulin were added. 0, no added insulin; 1, 0.2 nM; 2, 0.6 nM; 3, 2 nM; 4, 6 nM; 5, 20 nM; 6, 60 nM; 7, 200 nM; 8, 1000 nM; 9, 4000 nM insulin. 20 μg of total RNA were added to each lane of the gels. Values are the mean of two independent experiments.

level [18]. The same report suggested that the β-adrenoceptor agonist, isoprenaline, inhibits resistin gene expression [18]. In rodent adipose tissue, the dominant β-adrenoceptor in the stimulation of lipolysis is the β_3-subtype, and β_3-adrenoceptor agonists have a powerful inhibitory effect on *ob* gene expression and leptin production in the tissue [14–17]. Nevertheless, no response to the selective β_3-agonist, BRL 37344, in terms of resistin mRNA level, was found in 3T3-L1 adipocytes in the present work. This suggests that any regulatory effect of catecholamines on resistin production does not operate through β_3-adrenoceptors.

The lack of response to a β_3-adrenoceptor agonist implies that the reported inhibitory effect of isoprenaline on resistin expression occurs via β_1- or β_2-adrenoceptors. However, we have not found a significant effect of isoprenaline on resistin mRNA levels in 3T3-L1 adipocytes (control = 100; 1 μM isoprenaline = 79 ± 20; $n = 5$, $P > 0.1$); nor was there any significant response to another β_3-adrenoceptor agonist, CGP 12177. In these additional studies, 3T3-L1 adipocytes were incubated with the β-adrenoceptor agonists for 24 h, rather than for the 48 h used in the main experiments, and this is closer to the 16 h period employed in the earlier report [18]. We also found no effect on resistin mRNA levels of incubation with 8-bromo-cAMP, although there was a reduction with forskolin. Overall, our results do not suggest a significant role for catecholamines and the sympathetic system in the regulation of resistin gene expression [17].

A marked suppressive effect of insulin on resistin mRNA levels in 3T3-L1 adipocytes was observed in the present study, and this occurred with concentrations of insulin within and below the physiological range. Thus insulin may be a key regulator of resistin gene expression. In contrast to the direct inhibitory effect of insulin observed with 3T3-L1 cells, in vivo studies have reported that insulin stimulates resistin gene expression in Zucker diabetic fatty rats [13] and in streptozotocin-diabetic mice [12]. This apparent anomaly may reflect, of course, differences between an adipocyte clonal cell line and native white adipocytes, or between the direct and indirect effects of a hormone. Alternatively, pathological changes in streptozotocin-induced diabetes and in diabetic fatty animals may lead to abnormal responses to exogenous insulin.

Resistin has been independently discovered as FIZZ3 (a member of the FIZZ gene family) [11] and as ADSF [12]. As resistin it was proposed that the protein is involved in the induction of insulin resistance, linking obesity to diabetes [9], while ADSF was considered to inhibit adipocyte differentiation [12]. The inhibition by insulin of resistin gene expression in 3T3-L1 adipocytes is not consistent with a role for resistin in the induction of insulin resistance, since high insulin levels would be expected to stimulate production of a resistance factor. On the other hand, if resistin/ADSF inhibits adipocyte differentiation and insulin suppresses resistin gene expression, then rises in insulin would lead to the removal of a brake on the production of new adipocytes. This would be an appropriate response to the need to store excess nutrients, particularly during the development of obesity.

Acknowledgements: We are grateful for the financial support of the Johan Throne Holst Foundation, the Freia Medical Research Foundation and the Norwegian Cancer Research Society. We thank Anne Randi Alvestad for her assistance.

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