EXPERIMENTAL STUDY

Differential expression of adrenomedullin and resistin in 3T3-L1 adipocytes treated with tumor necrosis factor- α

Yin Li, Kazuhito Totsune¹, Kazuhisa Takeda, Kazumichi Furuyama, Shigeki Shibahara and Kazuhiro Takahashi

Department of Molecular Biology and Applied Physiology and ¹Department of Medicine, Tohoku University School of Medicine, 2-1 Seiryō-machi, Aoba-ku, Sendai, Miyagi 980-8575, Japan

(Correspondence should be addressed to K Takahashi; Email: ktaka-md@mail.cc.tohoku.ac.jp)

Abstract

Design: It has recently been shown that deficiency of adrenomedullin (AM), a potent vasodilator peptide, leads to insulin resistance. We studied expression of AM in NIH 3T3-L1 adipocytes and compared it with expression of resistin, an adipocyte-derived peptide hormone that is proposed to cause insulin resistance. Moreover, we studied the effects of tumor necrosis factor- α (TNF- α), a known mediator of insulin resistance, on the expression of AM and resistin in 3T3-L1 adipocytes.

Methods: 3T3-L1 cells were induced to differentiate to adipocytes by insulin, dexamethasone and 3-isobutyl-1-methylxanthine. Expression of AM mRNA and resistin mRNA was examined by Northern blot analysis. Immunoreactive AM in the medium was measured by RIA.

Results: AM mRNA was expressed in preadipocytes, but barely detectable in adipocytes. Immunoreactive AM was detected in the medium of both preadipocytes and adipocytes, with about 2.5 times higher levels found in preadipocytes. In contrast, resistin mRNA was expressed in adipocytes, whereas it was not detected in preadipocytes. Treatment with TNF- α increased AM expression in both adipocytes and preadipocytes, whereas it decreased resistin mRNA levels in adipocytes.

Conclusions: The present study has shown that AM expression was down-regulated and resistin expression was up-regulated during adipocyte differentiation of 3T3-L1 cells. TNF- α acted as a potent negative regulator of resistin expression and a potent positive regulator of AM expression in adipocytes, raising the possibility that in addition to its known actions in causing insulin resistance, TNF- α may also have actions against insulin resistance through AM and resistin.

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Introduction

Adipose tissue has recently been considered to play not only a role in lipid metabolism, but also active endocrine roles in secreting a variety of bioactive molecules, such as leptin (1), adiponectin (2, 3) and tumor necrosis factor- α (TNF- α) (4). Adrenomedullin (AM) is a potent vasodilator peptide consisting of 52 amino acids, which was originally isolated from human pheochromocytoma (5). Shimomura *et al.* have recently reported that insulin resistance had developed in 55-week-old heterozygous AM knockout mice (6), indicating that AM deficiency leads to insulin resistance in aged mice. Plasma levels of AM were elevated in patients with diabetes mellitus (7, 8). AM is expressed in the pancreatic islets and inhibits insulin secretion in a dose-dependent manner (9, 10). AM appears to be ubiquitously expressed in many types of cells (11), such as vascular endothelial cells (12), vascular smooth muscle cells (13), fibroblasts (14) and neurons

(15). There have been no reports, however, on the expression of AM in adipocytes.

Resistin is a recently discovered peptide hormone specifically secreted by adipose tissue, and has been suggested to be involved in the pathogenesis of insulin resistance, which may link obesity to diabetes (16). Resistin is a 94-amino acid polypeptide containing 11 Cys residues, and secreted as a disulfide-linked dimer, with a single Cys residue being required for dimerization (17). Insulin-sensitizing thiazolidinediones decrease resistin gene expression through the pathway of peroxisome proliferator-activated receptor- γ (PPAR- γ) (16, 18, 19). The administration of resistin impaired glucose tolerance and insulin action in mice, whereas neutralization of resistin by anti-resistin antibody improved hyperglycemia and insulin resistance in mice fed a high-fat diet. Resistin antagonized insulin-stimulated glucose transport in cultured NIH 3T3-L1 adipocytes and pretreatment of 3T3-L1 adipocytes with anti-resistin antibody augmented insulin-stimulated glucose

transport (16). On the other hand, some recent reports on human studies have cast doubt on the relevance of the rodent data on resistin to human obesity and studies of resistin gene expression have been inconsistent (20–22). It has been shown that insulin is a positive regulator of resistin gene expression (23, 24), whereas a β -adrenergic receptor agonist or TNF- α suppresses resistin expression (25–27).

In the present study, we studied expression of AM during adipocyte differentiation of 3T3-L1 cells, and compared it with expression of resistin. Moreover, we studied the effects of TNF- α as well as two other cytokines, interleukin-1 β (IL-1 β) and interferon- γ (IFN- γ), on expression of AM and resistin in 3T3-L1 adipocytes. TNF- α is secreted not only by activated macrophages and lymphocytes, but also by adipocytes, and has been implicated as one of the factors responsible for insulin resistance (4).

Materials and methods

Materials

NIH 3T3-L1 cells (28, 29) were obtained from the Health Science Research Resource Bank, Osaka, Japan. Human TNF- α , human IL-1 β and recombinant murine resistin were obtained from PeptoTech EC Ltd (London, UK). Recombinant human IFN- γ (IFN- γ -1a, Imunomax- γ) was a gift from Shionogi Co. (Osaka, Japan). Human γ -insulin, dexamethasone and 3-isobutyl-1-methylxanthine were from Wako Chemical Co. (Osaka, Japan). Mouse AM was obtained from Phoenix Pharmaceuticals, Inc. (Belmont, CA, USA). Other peptides including human AM were obtained from Peptide Institute Co. (Osaka, Japan).

Cell culture and differentiation

3T3-L1 cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS). Confluent cells were induced to differentiate to adipocytes by culturing for 48 h in DMEM containing 10% FBS, 1 μ g/ml insulin, 1 μ mol/l dexamethasone and 0.5 mmol/l 3-isobutyl-1-methylxanthine. The cells were then cultured in DMEM containing 10% FBS for another 24 h. The medium was replaced by DMEM with 10% FBS, containing either TNF- α , IL-1 β or IFN- γ . All test reagents were diluted into culture medium. The 3T3-L1 cells cultured in parallel in DMEM with 10% FBS, but without insulin, dexamethasone and 3-isobutyl-1-methylxanthine, were used as preadipocytes. The cells were harvested for RNA extraction and the culture media were collected for the measurement of immunoreactive AM (IR-AM). The experiments were performed in five dishes per each treatment.

To examine the effects of exogenously added recombinant resistin or synthetic AM on expression of

resistin and AM in adipocytes, 3T3-L1 adipocytes were treated with recombinant murine resistin (10^{-10} , 10^{-9} and 10^{-8} mol/l) or mouse AM (10^{-9} , 10^{-8} and 10^{-7} mol/l). Expression of AM mRNA and resistin mRNA was examined by Northern blot analysis. IR-AM in the medium was measured by RIA.

RNA extraction and Northern blot analysis

Total cellular RNA was isolated from 3T3-L1 cells using TRIZOL reagent (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's instructions. For Northern blot analysis of resistin, AM and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (as an internal control), 15 μ g total RNA were electrophoresed on 1% agarose gels containing 16.6% formaldehyde. RNA was then transferred to nylon membranes (Zeta-Probe blotting membranes; Bio-Rad Lab, Hercules, CA, USA) and covalently bonded to the membrane by exposure to UV light.

Northern probes for AM and resistin were prepared by RT-PCR. Total RNA (6 μ g) prepared from rat adrenal (for AM) or 3T3-L1 adipocytes (for resistin) was reverse transcribed as previously reported (30). One microliter of the reaction mixture was subjected to PCR. The sense primer for rat AM was 5'-TTTCGAGTCAAACGCTACCGC-3' (nucleotides 266–285) and the anti-sense primer was 5'-ATCAGGCGCTCTCCACCTTA-3' (complementary to nucleotides 1102–1121) (31). The sense primer for resistin was 5'-TTGTGTCCTGCTAAGTCCTC-3' (nucleotides 47–66) and the antisense primer was 5'-ACATCAGGAAGCGACCTGCA-3' (complementary to nucleotides 412–431) (Gene Bank accession No. AF323080). The PCR was performed as follows: AM, 40 cycles at 94 °C for 1 min, 60 °C for 1.5 min, 72 °C for 2 min; and resistin, 35 cycles at 96 °C for 30 s, 45 °C for 1 min, 72 °C for 2 min. The amplified cDNA fragments were inserted into the EcoRV site of the pBluescript I KS vector (Stratagene, La Jolla, CA, USA), yielding the subclones pBS-rAM10 and pBS-mRes respectively. The nucleotide sequence was determined using an autosequencer (Perkin-Elmer, Chiba, Japan) and was confirmed to be identical to the registered sequence of rat AM (Gene Bank accession No. AF323080) (31) and mouse resistin. The HindIII/NaeI fragment of pBS-rAM10 (vector/443) and the HindIII/EcoRI fragment of pBS-mRes (vector/vector) were labeled with [α - 32 P]dCTP with a BcaBest Labeling Kit (Takara Biomedicals, Shiga, Japan). The probe for G3PDH was the NcoI/PstI fragment derived from rat 720 bp G3PDH cDNA fragment (nucleotides 5–104 and 368–987) subcloned pGEM-T vector (Promega, Madison, WI, USA) (32).

Blots were prehybridized for 4 h at 42 °C in a solution containing 50% formamide, 5 $\times$ standard saline citrate (SSC), 5 $\times$ Denhardt's solution, 200 μ g/ml heat-denatured salmon sperm DNA and 1% SDS. Hybridization was carried out for at least 16 h at 42 °C in an

identical solution containing $1-3 \times 10^6$ d.p.m./ml of labeled probes. The blots were washed twice with $2 \times$ SSC and 0.1% SDS at 65°C for 5 min and twice with $0.1 \times$ SSC and 0.1% SDS at 65°C for 30 min. Radioactive signals were detected by exposing the filters to X-ray film (X-AR5; Eastman Kodak, Rochester, NY, USA) or with a Bioimage Analyzer (BAS1500; Fujifilm, Tokyo, Japan). The radioactivity corresponding to each band was determined using a Bioimage Analyzer.

Peptide extraction and RIA

Peptides in culture media were extracted using Sep-Pak C18 cartridges (Waters, Milford, MA, USA) as previously reported (30). The extract was reconstituted in assay buffer (0.1 mol/l phosphate buffer (pH 7.5) containing 0.1% (w/v) BSA, 0.2% (v/v) Triton X-100 and 0.1 (w/v) sodium azide) and assayed.

Mouse AM was used as a standard. ^{125}I -human AM prepared by the chloramine T method was used as a radioligand (33). The antiserum (No. 103) (15) against human AM, which cross-reacted with mouse AM, was used at a final dilution of 1:20 000. The sample or the standard peptide (200 μl) was incubated with the antiserum (200 μl) at 4°C for 48 h and then 100 μl ^{125}I -AM (approximately 4000 c.p.m./0.1 ml) were added to each sample. After a further 48 h incubation at 4°C , 100 μl 5% anti-rabbit IgG raised in goat were added. After 1 h incubation, the samples were centrifuged at 2300 g for at least 1 h and the supernatant was aspirated. The pellets were counted in a γ -counter. Intra-assay and interassay coefficients of variation were 5.6 and 5.8% respectively. The assay could detect changes of 10 ± 3.4 fmol/tube (mean $\pm$ s.d., $n = 5$) from zero with 95% confidence. There was no significant cross-reaction ($<0.01\%$) with other peptides including calcitonin gene-related peptide (CGRP), neuropeptide Y, somatostatin, endothelin-1, atrial natriuretic peptide (CGRP), corticotropin-releasing hormone and vasoactive intestinal polypeptide.

Chromatographic characterization of IR-AM in the conditioned medium was performed by reverse-phase HPLC using $\mu\text{Bondapak C18}$ column (3.9×300 mm; Waters), as previously reported (30, 33). The conditioned medium was extracted with a Sep-Pak C18 cartridge, reconstituted in 0.1% (v/v) trifluoroacetic acid and loaded onto the column. HPLC was performed with a linear gradient of acetonitrile containing 0.1% (v/v) trifluoroacetic acid from 10 to 60% at a flow rate of 1 ml/min per fraction over 50 min. Each fraction (1 ml) was collected, air-dried and assayed.

Statistics

Data are expressed as means $\pm$ s.e.m., unless otherwise stated. Statistical analysis was performed by one-way

ANOVA followed by Fisher's protected least significant difference test.

Results

Expression of resistin and AM mRNAs in 3T3-L1 cells, and effects of $\text{TNF-}\alpha$, $\text{IFN-}\gamma$ and $\text{IL-1}\beta$

Microscopical examinations confirmed that 3T3-L1 cells showed morphological properties of adipocytes, such as accumulation of fat droplets, after the treatment with insulin, dexamethasone and 3-isobutyl-1-methylxanthine. Such changes were not observed in control preadipocytes.

AM mRNA was expressed in 3T3-L1 preadipocytes (control of preadipocytes in Fig. 1), whereas it was very weak or undetected in adipocytes (control of adipocytes in Fig. 1). 3T3-L1 adipocytes and preadipocytes were treated with $\text{TNF-}\alpha$ (10 ng/ml), $\text{IFN-}\gamma$ (100 U/ml) or $\text{IL-1}\beta$ (10 ng/ml) for 24 h. Treatment with $\text{TNF-}\alpha$ greatly augmented AM mRNA expression levels in both preadipocytes and adipocytes (Fig. 1). In preadipocytes, $\text{IL-1}\beta$ slightly augmented AM mRNA expression, whereas $\text{IFN-}\gamma$ did not affect AM mRNA expression.

On the other hand, resistin mRNA was detected in differentiated adipocytes but not in preadipocytes by Northern blot analysis. $\text{TNF-}\alpha$ treatment inhibited resistin mRNA level by about 60% compared with the untreated adipocyte control. In contrast, $\text{IFN-}\gamma$ or

Figure 1 Northern blot analysis of AM and resistin mRNAs in 3T3-L1 preadipocytes and adipocytes treated with $\text{IFN-}\gamma$ (100 U/ml), $\text{TNF-}\alpha$ (10 ng/ml) or $\text{IL-1}\beta$ (10 ng/ml) for 24 h. Each lane contains 15 μg total RNA. Bottom panel shows the expression of G3PDH mRNA as an internal control. Data are from one of three independent experiments with similar results.

IL-1 β did not significantly influence resistin mRNA expression in 3T3-L1 adipocytes (Fig. 1).

Effects of TNF- α , IFN- γ and IL-1 β on IR-AM levels in the medium of 3T3-L1 cells

IR-AM was detected in the media of both preadipocytes and adipocytes, with higher levels found in preadipocytes (preadipocytes, 90.48 ± 8.80 fmol/ 10^5 cells per 24 h, vs adipocytes, 36.21 ± 1.88 fmol/ 10^5 cells per 24 h = 172.72 ± 8.97 pmol/l) (Fig. 2). On the other hand, IR-AM was not detected in unconditioned medium (<25 pmol/l), indicating the active secretion of IR-AM from 3T3-L1 preadipocytes and adipocytes.

IR-AM levels in the media were significantly elevated by 24 h treatment with 10 ng/ml TNF- α of both preadipocytes and adipocytes (Fig. 2), and this elevation was more obvious in preadipocytes than in adipocytes. IL-1 β increased IR-AM levels in the media of only preadipocytes. IFN- γ had no significant effects on IR-AM levels in the media of either 3T3-L1 preadipocytes or adipocytes.

IR-AM in the medium of adipocytes with or without TNF- α treatment was characterized by reverse phase HPLC. As shown in Fig. 3, two major peaks eluting in the positions of mouse AM and mouse AM with oxidized methionine were observed. In addition to these two peaks, several minor peaks were observed and their identity needs further investigations. A peak eluting

in the position of mouse AM was larger in 3T3-L1 adipocytes treated with TNF- α (Fig. 3B).

Time-course effects of TNF- α on the expression of AM and resistin

AM mRNA expression was induced by 10 ng/ml TNF- α as early as 3 h, and remained to be increased up to 24 h in 3T3-L1 adipocytes (Fig. 4A). Fig. 4B shows time-course effects of TNF- α on IR-AM concentrations

Figure 2 IR-AM concentrations in the medium of 3T3-L1 preadipocytes and adipocytes treated with IFN- γ (100 U/ml), TNF- α (10 ng/ml), or IL-1 β (10 ng/ml) for 24 h. Data are means $\pm$ S.E.M. ($n = 5$). Statistical analysis was performed by one-way ANOVA followed by Fisher's protected least significant difference test. ** $P < 0.01$, *** $P < 0.0001$ (compared with untreated control within the same group).

Figure 3 Reverse phase HPLC of IR-AM in the medium of 3T3-L1 adipocytes. (A) Without TNF- α treatment and (B) with TNF- α treatment (10 ng/ml) for 24 h. The arrows indicate the elution position of mouse AM (mAM) and mouse AM with oxidized methionine (oxi mAM). Dotted lines show a gradient of acetonitrile (%).

Figure 4 Time-course effects of TNF- α (10 ng/ml) on AM and resistin expression in 3T3-L1 adipocytes. (A) Northern blot analysis. 3T3-L1 adipocytes were cultured with (+) or without (-) TNF- α (10 ng/ml). Each lane contains 15 μ g total RNA. Bottom panel shows the expression of G3PDH mRNA as an internal control. Data are from one of three independent experiments with similar results. (B) RIA of AM. 3T3-L1 adipocytes were cultured with (+) or without (-) TNF- α (10 ng/ml) for 6–24 h and IR-AM concentrations in the media were measured by RIA after extraction. Data are means $\pm$ S.E.M. ($n = 5$). Statistical analysis was performed by one-way ANOVA followed by Fisher's protected least significant difference test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$.

in the culture media of 3T3-L1 adipocytes. IR-AM concentrations in the media were significantly higher in TNF- α -treated 3T3-L1 adipocytes than in untreated 3T3-L1 adipocytes at all the three time points.

TNF- α suppressed resistin mRNA expression in a time-dependent manner. Significant inhibition was detectable from 6 h after 10 ng/ml TNF- α treatment and reached a maximum 24 h after the treatment (Fig. 4A).

Dose-response effects of TNF- α on the expression of AM and resistin

Figure 5A shows the dose-response effects of TNF- α on the expression of AM and resistin mRNAs. TNF- α increased AM mRNA expression dose-dependently in 3T3-L1 adipocytes (Fig. 5A). IR-AM levels in the medium were increased by the 24 h treatment with TNF- α dose-dependently (Fig. 5B). A significant increase in IR-AM was observed in the adipocytes treated with 1–10 ng/ml TNF- α .

On the other hand, TNF- α suppressed resistin mRNA expression at a concentration as low as 1 ng/ml and the maximal inhibition was found at a concentration of 10 ng/ml.

Effects of exogenously added synthetic mouse AM or recombinant murine resistin on expression of AM and resistin in 3T3-L1 adipocytes

Treatment of 3T3-L1 adipocytes with mouse AM (10^{-9} , 10^{-8} and 10^{-7} mol/l) had negligible effects on the expression of resistin mRNA and AM mRNA (data not shown). Treatment of 3T3-L1 adipocytes with recombinant murine resistin (10^{-10} , 10^{-9} and 10^{-8} mol/l) did not cause noticeable changes in the expression of resistin mRNA nor AM mRNA (data not shown). It had also no significant effects on IR-AM concentration in the medium of 3T3-L1 adipocytes. Thus, resistin and AM appear to have no significant effects on their own expression or on that of each other.

Discussion

Insulin resistance is frequently associated with obesity. However, the precise mechanisms linking obesity to insulin resistance are still unclear. Recent studies suggest that various adipocyte-derived factors regulate insulin sensitivity. Resistin has been identified as a new adipocyte-derived peptide hormone induced during adipogenesis, and implicated in causing insulin resistance (16, 23), although some recent reports have cast doubt on the relationship of resistin to insulin resistance (20–22). It has been shown that AM deficiency leads to insulin resistance using aged heterozygous AM knockout mice (6). Nishimatsu *et al.* reported that AM induced activation of the phosphatidylinositol 3-kinase/Akt pathway in the rat aortic endothelium (34). It is therefore plausible that AM acts on muscle or liver as a positive regulator in the insulin signaling pathway, such as the phosphatidylinositol 3-kinase/Akt pathway. The present study has shown that adipocyte differentiation is accompanied by down-regulation of AM expression and up-regulation of resistin expression, raising the possibility that low expression of AM and high expression of resistin in adipocytes may be related to the higher occurrence of insulin resistance in obese subjects.

TNF- α is secreted by adipocytes, and antagonizes the stimulation of glucose uptake by insulin. TNF- α inhibited insulin-mediated tyrosine phosphorylation of the insulin receptor and insulin receptor substrate 1 (35–37). TNF- α down-regulates glucose transporter GLUT4 gene expression in cultured adipocytes and myocytes (38–41). Thus, TNF- α is supposed to be one of the major causes of insulin resistance. We hypothesized that TNF- α is related to insulin resistance

Figure 5 Dose-response effects of TNF- α on AM and resistin expression in 3T3-L1 adipocytes. (A) Northern blot analysis. 3T3-L1 adipocytes were treated with TNF- α for 24 h at indicated concentrations. Each lane contains 15 μ g total RNA. Bottom panel shows the expression of G3PDH mRNA as an internal control. Data are from one of three independent experiments with similar results. (B) RIA of AM. Data are means $\pm$ S.E.M. ($n = 5$). Statistical analysis was performed by one-way ANOVA followed by Fisher's protected least significant difference test. ** $P < 0.01$, *** $P < 0.0001$ (compared with untreated controls).

also through its paracrine or autocrine actions on adipocytes, in addition to actions on other tissues such as muscle. Contrary to our expectation, TNF- α increased expression of AM but suppressed expression of resistin in 3T3-L1 adipocytes. The suppression of resistin mRNA expression by TNF- α is consistent with previous reports (26, 27). Thus, these paracrine or autocrine actions of TNF- α in adipocytes appear to be against insulin resistance.

The present study has shown for the first time the production and the secretion of AM by adipocytes, although its amount was smaller than that by preadipocytes. AM mRNA expression was very low or undetected in untreated 3T3-L1 adipocytes, whereas IR-AM was detected in the culture medium at significantly higher levels than in unconditioned medium. This may be due to a higher sensitivity of the RIA. HPLC analysis confirmed that IR-AM in the culture media mainly consisted of AM and AM with oxidized methionine, which may be generated during the extraction procedure. It was reported that TNF- α stimulated AM expression in cultured vascular smooth muscle cells (13), and

cultured human astrocytes (42), whereas it decreased the AM expression in T98G human glioblastoma cells (43), and had no noticeable effects in F0202 human retinal pigment epithelial cells (44). The effects of TNF- α on the AM expression in 3T3-L1 cells were similar to those in vascular smooth muscle cells (13) and human astrocytes (42).

It has been proposed that TNF- α expression is increased in adipose tissue of obese subjects. It is therefore possible that AM expression in adipose tissue of obese subjects is rather enhanced by TNF- α and other humoral factors. Furthermore, plasma concentrations of IR-AM were elevated in patients with diabetes mellitus (7, 8). Since obesity is common in patients with diabetes mellitus, our findings have raised the possibility that AM secreted by adipose tissue contributed to the elevated plasma IR-AM concentrations in diabetic patients. Further studies are therefore required to clarify whether or not AM expression in adipose tissue is enhanced in the obese state.

AM is a potent vasodilator peptide (5). AM secreted by adipocytes may act on vascular vessels and regulate a local circulation in adipose tissue. Receptor activity-modifying protein-1, -2 and -3, which form the AM receptor or the CGRP receptor together with calcitonin receptor-like receptor (45), are expressed in adipose tissue (46). Another possible target of AM secreted by adipocytes may therefore be adipocytes themselves. Biological effects of AM on adipocytes, however, remain to be determined.

IFN- γ has been shown to affect fat metabolism and adipocyte gene expression (47–50). It has been demonstrated that IFN- γ treatment results in the development of insulin resistance through down-regulation of PPAR- γ (51). IL-1 β stimulates lipolysis and inhibits lipogenesis (52). In the present study, however, IFN- γ or IL-1 β did not affect AM or resistin gene expression in 3T3-L1 adipocytes.

In the present study, we have shown that AM expression was down-regulated and resistin expression was up-regulated during adipocyte differentiation of 3T3-L1 cells. We also demonstrated roles for TNF- α as a potent negative regulator of resistin expression and a potent positive regulator of AM expression in adipocytes. Studies on expression of AM and resistin in adipose tissue of lean and obese subjects or animal models may further clarify their significance in the pathogenesis of insulin resistance and other obesity-related diseases.

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