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# Serum concentrations of resistin-like molecules β and γ are elevated in high-fat-fed and obese *db*/*db* mice, with increased production in the intestinal tract and bone marrow

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**Abstract** *Aims/hypothesis:* Resistin and the resistin-like molecules (RELMs) comprise a novel class of cysteine-rich proteins. Among the RELMs, RELMβ and RELMγ are produced in non-adipocyte tissues, but the regulation of their expression and their physiological roles are largely unknown. We investigated in mice the tissue distribution and dimer formation of RELMβ and RELMγ and then examined whether their serum concentrations and tissue expression levels are related to insulin resistance. *Methods:* Specific antibodies against RELMβ and RELMγ were generated. Dimer formation was examined using COS cells and the colon. RELMβ and RELMγ tissue localisation and expression levels were analysed by an RNase protection assay, immunoblotting and immunohistochemical study. Serum concentrations in high-fat-fed and *db*/*db* mice were also measured using the specific antibodies. *Results:* The intestinal tract produces RELMβ and RELMγ, and colonic epithelial cells in particular express both RELMβ and RELMγ. In addition, RELMβ and RELMγ were shown to form a homodimer and a heterodimer with each other, in an overexpression system using cultured cells, and in mouse colon and serum. Serum RELMβ and RELMγ levels in high-fat-fed mice were markedly higher than those in mice fed normal chow. Serum RELMβ and RELMγ concentrations were also clearly higher in *db*/*db* mice than in lean littermates. Tissue expression levels revealed that elevated serum concentrations of RELMβ and RELMγ are attributable to increased production in the colon and bone marrow. *Conclusions/interpretation:* RELMβ and RELMγ form homo/heterodimers, which are secreted into the circulation. Serum concentrations of RELMβ and RELMγ may be a novel intestinal-tract-mediating regulator of insulin sensitivity, possibly involved in insulin resistance induced by obesity and a high-fat diet.

**Keywords** Bone marrow · *db*/*db* mice · Heterodimer · High-fat diet · Homodimer · Insulin resistance · Intestinal · Resistin-like molecule β · Resistin-like molecule γ · Serum concentration

**Abbreviations** FIZZ: found in inflammatory zone · GST: glutathione S-transferase · PPARγ: peroxisome proliferator-activated receptor γ · RELM: resistin-like molecules · XCP: ten-cysteine protein

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## Introduction

Type 2 diabetes is characterised by insulin resistance of peripheral tissues such as the liver and muscle and adipose tissue [1–4]. Recent studies have indicated that adipose

tissue is, in addition to being a lipid storage site, an endocrine organ producing hormones, cytokines and other substances [5–7]. Recently, resistin was newly identified as an adipocyte-secreted protein [8]. Serum resistin levels are reportedly elevated in genetically obese mice and are down-regulated by administration of thiazolidinediones [9, 10], peroxisome proliferator-activated receptor γ (PPARγ) agonists, although contradictory data also exist [11]. Resistin has been demonstrated to antagonise insulin action in cultured cells such as 3T3-L1 adipocytes, as well as in rodents [8]. Resistin knock-out mice exhibited lower blood glucose with reduced hepatic glucose production [12]. Thus resistin could be one of the important adipokines causing insulin resistance.

The presence of resistin-like molecules (RELMs) indicates that resistin belongs to a novel family of cysteine-rich secreted proteins (RELM/found in inflammatory zone [FIZZ]/ten-cysteine protein [XCP]). RELMα/FIZZ1 [13] and RELMβ/FIZZ2 [14] are homologous with resistin/FIZZ3 and expressed mainly in white adipose tissue and the colon respectively [14, 15]. The administration of RELMβ to rats resulted in acute impairment of hepatic insulin sensitivity and glucose metabolism [16], which suggested RELMβ is a link between the intestine and hepatic insulin action. Finally, *RELMγ/FIZZ4/XCP1* , a fourth member of the RELM/FIZZ/XCP family, was identified as a gene with decreased expression in rat nasal respiratory epithelium exposed to cigarette smoke [17]. RELMγ was expressed in the bone marrow, spleen, pancreas and colon, and was revealed to play a role as a cytokine in haematopoiesis [18, 19].

The consensus structure of the RELMs is composed of two domains; one half is the N-terminal, including an N-terminal signal sequence and a variable middle portion, and the other half is the C-terminal domain, which has a highly conserved C-terminal signature sequence containing a unique spacing of the cysteine residues. The N-terminal domains of RELMα, RELMβ and RELMγ are 15, 35 and 17% identical to resistin, whereas the C-terminal domains are 47, 54 and 52% identical to resistin. The C-terminus of RELMγ is highly homologous (84%) with RELMβ. Recently, the crystal structures of resistin and RELMβ were revealed to have a unique multimeric structure [20]. Each protomer is comprised of a 'head' and 'tail' segment and circulates as an assembly of hexamers and trimers, which reflect activation of resistin.

We previously identified RELMγ cDNA independently by PCR using degenerated oligonucleotide primers, and investigated its tissue distribution. Since we succeeded in preparing highly specific antibodies against RELMβ and RELMγ, we were able to detect the endogenous proteins and measure serum concentrations as well as tissue contents of RELMβ and RELMγ in the mouse. In this study, we show for the first time that RELMβ and RELMγ form not only a homodimer but also a heterodimer with each other in both tissue and serum. Interestingly, we also found that the serum concentrations of RELMβ and RELMγ are significantly elevated in high-fat-diet-induced and obese diabetic mice. These observations are probably attributable to increased production in the colon and bone marrow. Thus, this report is the first to raise the possibility of a novel intestinal-tract-mediating regulatory mechanism of insulin sensitivity, which may be involved in insulin resistance induced by obesity and a high-fat diet.

## Materials and methods

*cDNA cloning of a novel RELM/FIZZ isoform* Two degenerate oligonucleotide primers were synthesised for PCR. These primers were 5′-ATGAAGA/CCTACAA/CC/TT/GTGTTC/TC-3′ and 5′-TTAG/AGA/CCAG/TTT/CGGC GCAGCG-3′, corresponding to amino acid residues 1–8 and 104–111 of RELMα/β, which are highly conserved among RELM/FIZZ isoforms. PCR was performed using mouse embryonic cDNA. A DNA fragment of approximately 330 bp was then separated by electrophoresis, cloned into TA vector pCRII (Invitrogen, San Diego, CA, USA), and sequenced using a DNA sequencer. We obtained two independent sequences: one, F1, turned out to be completely homologous to mouse RELMα/FIZZ1; the other, F2, encoded a protein to mouse RELMγ/FIZZ4. Then, a mouse embryonic cDNA library produced by a standard method (Stratagene, La Jolla, CA, USA) was screened under standard hybridisation conditions using a $^{32}$P-labelled F2 cDNA fragment as a probe to obtain a full-length cDNA encoding RELMγ/FIZZ4. Positive clones were excised into pBluescript and sequenced.

*RNase protection assay of various tissues* Mice were killed by cervical dislocation, and various tissues (cerebrum, cerebellum, brainstem, heart, lung, liver, oesophagus, stomach, jejunum, ileum, colon, kidney, testis, spleen, pancreas, abdominal fat, epididymal fat, muscle, aorta, femoral bone marrow) were removed. Total RNA was isolated with Isogen (Nippon Gene, Japan). [α-$^{32}$P]UTP-labelled RNA probes were prepared using nucleotides 1–200 of mouse RELMβ, and 83–315 of mouse RELMγ as templates. An RNase protection assay was performed using an RPA III kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions.

*Preparation of the antibodies* An antibody against the whole mouse resistin molecule was prepared by immunising rabbits with the recombinant mouse resistin protein, obtained as described previously [21]. Sequences corresponding to nucleotides 11–173 of RELMβ/FIZZ2 and 47–227 of RELMγ/FIZZ4 were amplified by PCR and cloned into the pGEX-3T expression vector (Pharmacia, Piscataway, NJ, USA). Glutathione *S*-transferase (GST) fusion proteins (GST-RELMβ and GST-RELMγ) were prepared according to the manufacturer's instructions (Pharmacia). The antisera were raised by immunising rabbits with GST-RELMβ and GST-RELMγ. From these antisera, an antibody against GST protein was removed by filtering through Affigel-10 covalently coupled to GST proteins. Then, specific antibodies against RELMβ and RELMγ were affinity purified with Affigel-10 covalently

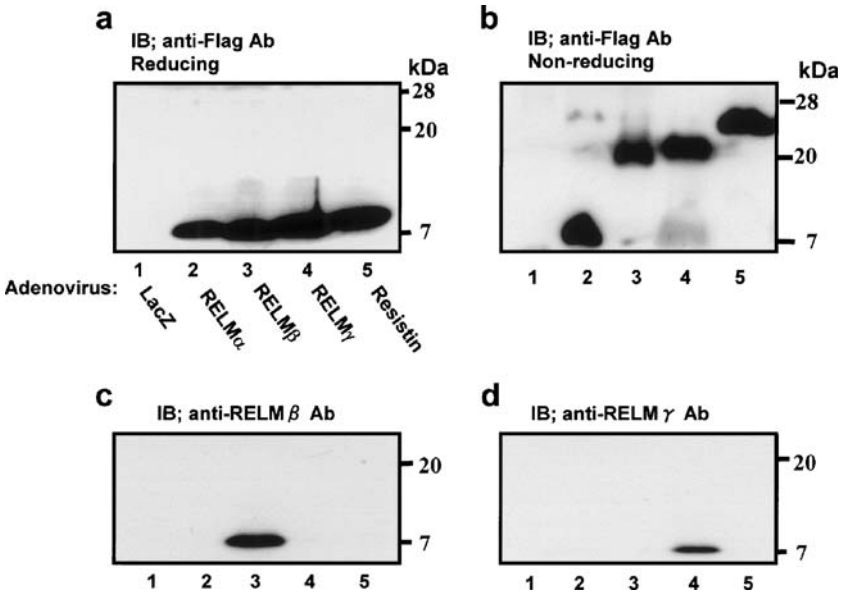


**Fig. 1** Immunoblotting of RELMs secreted by COS7 cells. The four cDNAs coding RELMα, RELMβ, RELMγ and resistin, with the Flag tag at their C-termini, were expressed into COS7 cells and the medium from each cell type was subjected to SDS-PAGE under reducing (**a**, **c** and **d**) and non-reducing (**b**) conditions and immunoblotted (IB) with anti-Flag (**a**, **b**), anti-RELMβ (**c**) and anti-RELMγ (**d**) antibody (Ab)

coupled to GST-RELMβ and GST-RELMγ respectively. Antibodies against Flag tag were purchased from Upstate Biotech, Inc. (Lake Placid, NY, USA).

*Immunohistochemistry* Intestinal tissues removed from the mice were fixed in 10% phosphate-buffered formalin (pH 7.4) and embedded in paraffin. After sectioning, the tissues were dewaxed in ethanol, rehydrated in 10 mmol/l citric acid buffer, and microwaved for 13 min. The tissue sections were blocked with serial incubation in 30% $H_2O_2$, avidin D-blocking reagent, biotin-blocking reagent and protein-blocking reagent. The sections were then incubated with an affinity-purified polyclonal antibody for murine RELMβ and RELMγ or control serum at a 1:100 dilution overnight at 4°C. After washing with PBS, the slides were incubated with a biotinylated goat anti-rabbit secondary antibody followed by detection of horseradish peroxidase.

*Animal studies* Nine-week-old male mice (C57BL/6J) were purchased from Jackson Laboratories (Bar Harbor, ME, USA). They were divided into two groups. One group ($n$=9) was maintained on standard rodent chow, the other ($n$=9) was fed a high-fat diet (60% fat, 25% carbohydrate and 15% protein). Genetically obese *db*/*db* mice ($n$=6) and lean littermates ($n$=6) were purchased from Jackson Laboratories. They were maintained on standard rodent chow. Tissues from the mice were homogenised in ice-cold lysis buffer. Insoluble materials were removed and the cell lysates were incubated for 2 h at 4°C with the indicated antibody. Blood samples were centrifuged at 3,000 rev/min for 20 min and sera containing equal amounts of protein were used for immunoprecipitation. Immunoblotting against these immunoprecipitates was performed as previously described [21]. Animal care and procedures of the experiments were approved by the Animal Care Committee of the University of Tokyo.

*Statistical analysis* Data are expressed as means±SE. Comparisons were made using unpaired $t$-tests. Serum RELM levels were compared with body weight, serum glucose and insulin levels by Pearson's correlation. Values of $p<0.05$ were considered significant.

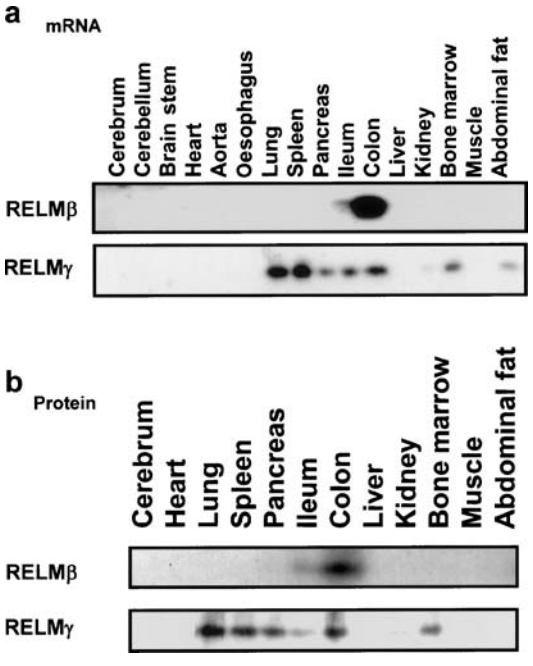


**Fig. 2** RELMβ and RELMγ expression in various tissues. **a** 10 μg of RNA from various mouse tissues were prepared and hybridised with [α-$^{32}$P]UTP-labelled RNA probes for RELMβ or RELMγ. The RNase protection assay was performed using an RPA III kit (Ambion) according to the manufacturer's instructions. **b** Cell lysates from various mouse tissues were prepared and then immunoprecipitated and immunoblotted with anti-RELMβ antibody. Cell lysates from tissues were prepared and then immunoprecipitated and immunoblotted with anti-RELMγ antibody

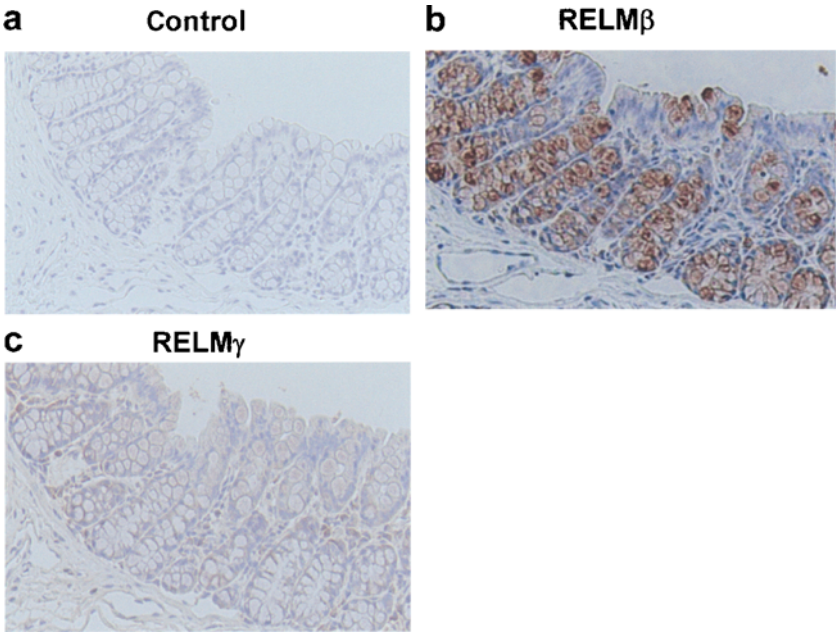


**Fig. 3** Immunohistochemical study of RELMβ and RELMγ. Immunohistochemical study of the colon with control antibody (**a**) confirmed the specificities of specific antibodies (×200). The avidin–biotin–peroxidase complex method with antibodies against RELMβ (**b**) and RELMγ (**c**) was used to detect cells expressing these RELMs. RELMβ and RELMγ staining is *brown*, that of the control *blue*

## Results

*Cloning of RELMγ cDNA* To obtain a cDNA fragment corresponding to a novel isoform of RELM/FIZZ, PCR was performed using degenerate oligonucleotides as primers and mouse embryonic cDNA as a substrate. PCR products, with a length of approximately 300–350 bp, were separated, subcloned and sequenced. Isolated PCR products were shown to consist of two different cDNAs. One corresponded to RELMα cDNA [14], the other to RELMγ. The full-length cDNAs encoding resistin, RELMα and RELMβ were prepared by PCRs based on the reported sequences. The full-length cDNA encoding RELMγ was obtained by screening a cDNA library.

*Overexpression of RELMs in COS7 cells and preparation of specific antibodies against RELMβ and RELMγ* Four cDNAs encoding RELMα, RELMβ, RELMγ and resistin, with the Flag tag at their C-termini, were ligated into adenovirus expression vectors. COS7 cells were infected with these adenoviruses to achieve similar protein expression levels, as assessed by immunoblotting using anti-Flag antibody. The media from cells transfected with these adenoviruses were subjected to SDS-PAGE and immunoblotted with anti-Flag antibody (Fig. 1a). The expression of these proteins was observed as very similar band densities of 7–12 kDa. Thus, it was clear that RELMγ was secreted into the media, like other members of the RELM family, when expressed in COS7 cells.

Next, we investigated the electrophoretic mobilities of these RELMs under non-reducing conditions. As shown in Fig. 1b, RELMβ, RELMγ and resistin migrated with apparent molecular masses twice those of the respective monomers, whereas RELMα behaved as a monomer. Taking previous [22, 23] and current results into account, it was confirmed that resistin, RELMβ and RELMγ form a

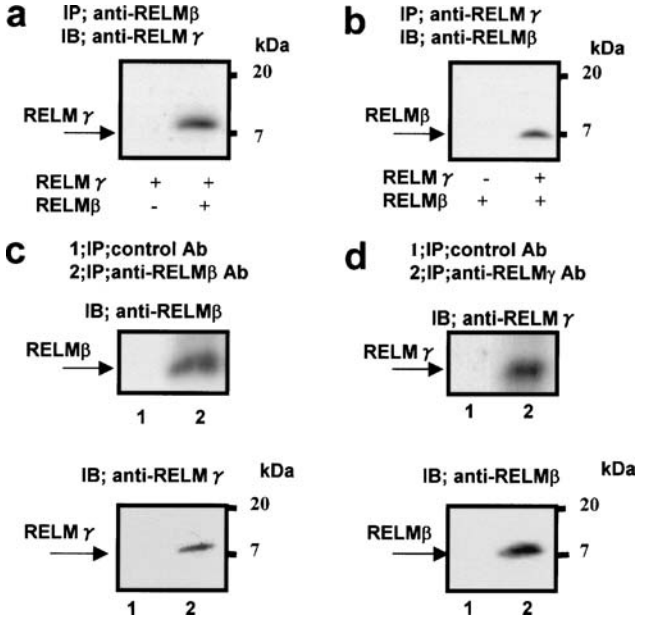


**Fig. 4** Heterodimer formations of RELMβ/RELMγ (**a**, **b**) in COS7 cells and endogenous RELMβ/RELMγ in colon (**c**, **d**). **a** Secreted RELMγ, co-expressed with (*right lane*) or without (*left lane*) RELMβ, was immunoprecipitated (*IP*) with anti-RELMβ antibody (*Ab*) and then immunoblotted with anti-RELMγ Ab. **b** RELMβ, co-expressed with (*right lane*) or without (*left lane*) RELMγ was immunoprecipitated with anti-RELMγ Ab and immunoblotted with anti-RELMβ Ab. **c** Colon cell lysates were immunoprecipitated with control IgG (*lane 1*) and anti-RELMβ Ab (*lane 2*) and then immunoblotted with anti-RELMβ Ab (*upper panel*) and anti-RELMγ Ab (*lower panel*). **d** The colon cell lysates were immunoprecipitated with control IgG (*lane 1*) and anti-RELMγ Ab (*lane 2*) and immunoblotted with anti-RELMγ Ab (*upper panel*) or anti-RELMβ Ab (*lower panel*)

**Table 1** Serum RELMβ and RELMγ in high-fat-fed mice

| | 4 weeks | | 8 weeks | | 12 weeks | |
|---|---|---|---|---|---|---|
| | Control | High-fat | Control | High-fat | Control | High-fat |
| RELMβ | 1±0.05 | 1.08±0.06 | 1±0.05 | 1.74±0.08[a] | 1±0.06 | 2.16±0.09[a] |
| RELMγ | 1±0.05 | 1.05±0.05 | 1±0.06 | 2.00±0.10[a] | 1±0.05 | 2.70±0.12[a] |
| RELMβ/γ | 1±0.05 | 0.97±0.06 | 1±0.05 | 1.65±0.09[a] | 1±0.05 | 2.54±0.11[a] |
| Body weight (g) | 18.3±1.18 | 19.3±1.29 | 26.6±1.32 | 38.8±2.09[a] | 30.1±2.07 | 44.4±1.95[a] |
| Glucose (mmol/l) | 5.03±0.10 | 5.11±0.20 | 5.24±0.16 | 6.77±0.30 | 5.13±0.08 | 7.93±0.27[a] |
| Insulin (pmol/l) | 22.4±1.67 | 22.7±2.64 | 26.0±5.00 | 48.7±2.7[a] | 33.1±1.67 | 62.9±2.45[a] |

Male 12-week-old C57BL/6J mice were fed normal chow ($n$=9) or a high-fat diet ($n$=9) from 4 to 12 weeks of age. Sera were immunoprecipitated and then immunoblotted with RELMβ and RELMγ antibody. The data are shown as ratios to the control diet values and expressed as means±SE

[a] $p<0.01$ relative to control-diet-fed mice

disulphide-linked dimer, while RELMα exists mainly as a monomer.

Immunoblotting with anti-RELMβ and anti-RELMγ antibodies (Fig. 1c, d) revealed that these antibodies do not recognise other members of the RELM/FIZZ family, suggesting anti-RELMβ and anti-RELMγ antibodies to be highly specific for the corresponding isoforms.

*Tissue distribution of RELMβ and RELMγ* We next evaluated RELM mRNA expression with an RNase protection assay (Fig. 2a) and protein expression using Western blotting (Fig. 2b) in various mouse tissues. RELMβ mRNA and protein were abundant in the colon, and to a lesser extent in the ileum. On the other hand, RELMγ mRNA was abundant in the colon, ileum, bone marrow, spleen, pancreas and fat, consistent with previous reports [18–20] (Fig. 2a, lower panel). RELMγ protein expression is also detectable in the colon, ileum, bone marrow, spleen and pancreas (Fig. 2b, lower panel). Thus, we found that the colon and ileum express both RELMβ and RELMγ, and the localisations of RELMβ and RELMγ were investigated by immunohistochemical staining of the colon (Fig. 3). It was demonstrated that epithelial cells throughout the crypt and surface of the colon express both RELMβ and RELMγ, while no significant staining was observed with the control antibody. The highest level of RELMβ expression was observed in goblet cells of the colon, consistent with previous reports [15, 24], and RELMγ protein

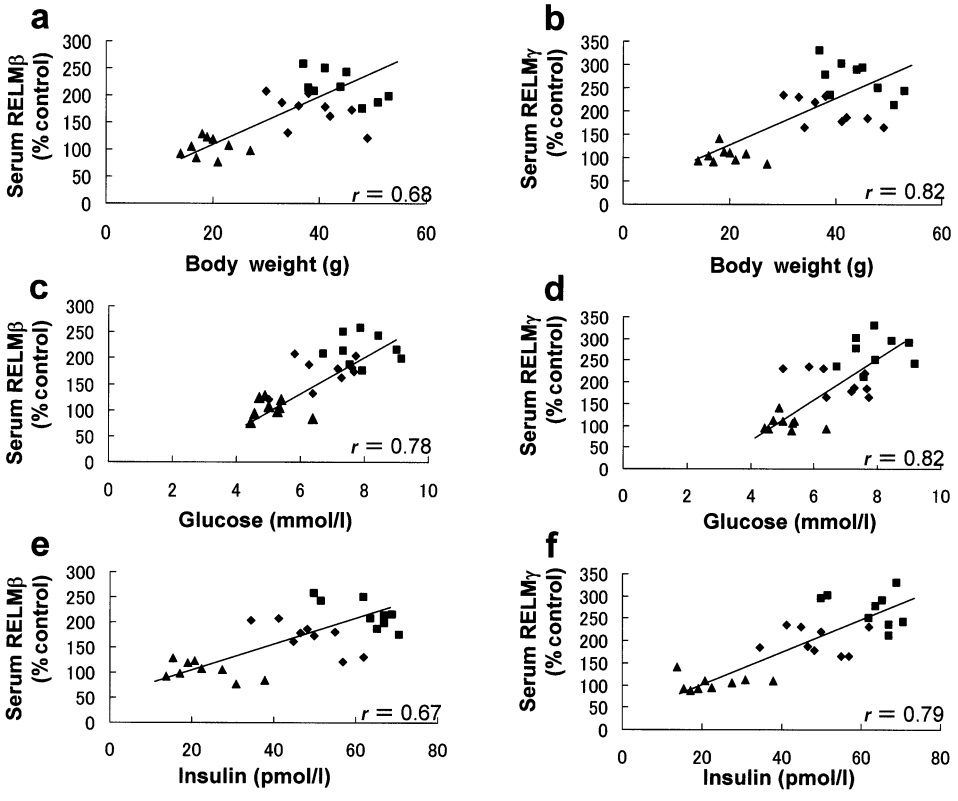


**Fig. 5** Relationships between serum RELMβ and RELMγ concentration and body weight, glucose and insulin in high-fat-fed mice. Male 12-week-old C57BL/6J mice were fed normal chow ($n$=9) or a high-fat diet ($n$=9) from 4 to 12 weeks of age. RELMβ, RELMγ and various parameters were measured at 4, 8 and 12 weeks of age. The data are plotted as the percentage of the means of RELMβ and RELMγ correlated positively with body weight (**a**, $p<0.0001$; **b**, $p<0.0005$), glucose (**c**, $p<0.0005$; **d**, $p<0.0005$) and insulin (**e**, $p<0.001$; **f**, $p<0.0005$)

**Table 2** Expression and regulation of mRNA and protein of RELMβ and RELMγ in high-fat-fed mice

| | | Colon | | Bone marrow | | Spleen | | Lung | | Pancreas | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Control | High-fat | Control | High-fat | Control | High-fat | Control | High-fat | Control | High-fat |
| RELMβ | mRNA | 1±0.04 | 1.97±0.04[a] | – | – | – | – | – | – | – | – |
| | Protein | 1±0.03 | 1.96±0.05[a] | – | – | – | – | – | – | – | – |
| RELMγ | mRNA | 1±0.04 | 1.84±0.03[a] | 1±0.04 | 1.89±0.05[a] | 1±0.04 | 1.03±0.05 | 1±0.04 | 1.05±0.05 | 1±0.04 | 0.97±0.05 |
| | Protein | 1±0.04 | 2.04±0.03[a] | 1±0.04 | 1.78±0.05[a] | 1±0.03 | 1.02±0.04 | 1±0.04 | 1.03±0.04 | 1±0.04 | 0.98±0.05 |

Male 12-week-old C57BL/6J mice were fed normal chow or a high-fat diet from 4 to 12 weeks of age. RNase protection assay and immunoblotting show RELMβ and RELMγ expression in the colon, bone marrow, spleen, lung and pancreas. The data are shown as ratios to the control diet values and expressed as means±SE

[a] $p<0.01$ relative to control-diet-fed mice

was also shown to be localised in goblet cells. In the bone marrow, about 30% of haematopoietic cells were stained with the anti-RELMγ antibody and these cells were myelocytes and metamyelocytes or neutrophils (data not shown), consistent with previous reports [19, 20].

*Heterodimer formation between RELMβ and RELMγ in COS7 cells and tissues* Since colonic cells express both RELMβ and RELMγ, we subsequently investigated whether these RELMs form a heterodimer. The RELMβ and RELMγ antibodies were highly specific and did not immunoprecipitate the respective isoforms of RELM (data not shown). As shown in Fig. 4a, b, when RELMβ and RELMγ were co-expressed, RELMγ or RELMβ was detected in the RELMβ or RELMγ immunoprecipitates respectively. These results suggest that RELMβ and RELMγ associate with each other and form a heterodimer.

Subsequently, we investigated whether or not endogenous RELMβ and RELMγ form a heterodimer using the proximal colon. RELMβ was detected in RELMγ immunoprecipitates but not in those of control antibodies (Fig. 4c). RELMγ was also detected in RELMβ immunoprecipitates but not in those of control antibodies (Fig. 4d). These results indicate that the heterodimerisation between RELMβ and RELMγ is physiological.

*Increased expression of RELMβ and RELMγ in high-fat-fed mice* Male 12-week-old C57BL/6J mice were fed normal chow or a high-fat diet from 4 to 12 weeks of age. A high-fat diet resulted in body weight, serum glucose and insulin increasing time-dependently (30.1±6.21 vs 44.4±5.84 g, 5.13±0.24 vs 7.93±0.81 mmol/l, and 33.1±5.02 vs 62.9±7.35 pmol/l respectively) after 12 weeks of feeding (Table 1). Serum RELMβ and RELMγ levels were increased by 116 and 170% respectively at the end of the 12-week feeding period. We detected the RELMβ/RELMγ heterodimer in serum by immunoblotting of the anti-RELMβ immunoprecipitate with anti-RELMγ antibody, and this heterodimer was also increased.

Serum RELMβ and RELMγ and other parameters were measured at 4, 8 and 12 weeks after initiation of the high-fat diet. Close examination of these three sets of time-dependent data revealed serum RELMβ and RELMγ to correlate positively with body weight (Fig. 5a, $r=0.68$, $p<0.0001$; Fig. 5b, $r=0.82$, $p<0.0005$), serum glucose concentration (Fig. 5c, $r=0.78$, $p<0.0005$; Fig. 5d, $r=0.82$, $p<0.0005$) and serum insulin (Fig. 5e, $r=0.67$, $p<0.001$; Fig. 5f, $r=0.79$, $p<0.0005$).

Subsequently, we investigated the expression levels of RELMβ and RELMγ in tissues at the end of the 12-week feeding period. RELMβ mRNA and protein levels in the distal colon of the high-fat-fed mice were elevated by 97 and 96% respectively (Table 2). Similarly, RELMγ mRNA and protein levels in the distal colon were elevated by 84 and 104% respectively. RELMγ mRNA and protein levels in the bone marrow were also elevated by 89 and 78% respectively. However, no significant alterations were observed in the spleen, lung or pancreas.

**Table 3** Serum RELMβ and RELMγ in *db*/*db* mice

| | 4 weeks | | 5 weeks | | 6 weeks | |
|---|---|---|---|---|---|---|
| | Control | *db*/*db* | Control | *db*/*db* | Control | *db*/*db* |
| RELMβ | 1±0.05 | 1.34±0.06 | 1±0.06 | 1.57±0.09[a] | 1±0.07 | 1.82±0.11[a] |
| RELMγ | 1±0.06 | 1.68±0.04 | 1±0.04 | 1.70±0.08[a] | 1±0.04 | 2.27±0.09[a] |
| RELMβ/γ | 1±0.04 | 1.48±0.05 | 1±0.05 | 1.58±0.08[a] | 1±0.05 | 1.79±0.06[a] |
| Body weight (g) | 19.8±0.90 | 25.3±0.92 | 21.0±1.00 | 27.5±1.13 | 23.2±0.92 | 30.1±1.24[a] |
| Glucose (mmol/l) | 4.71±0.12 | 5.65±0.20 | 4.94±0.12 | 5.91±0.22 | 4.62±0.07 | 6.13±0.18[a] |
| Insulin (pmol/l) | 21.4±1.64 | 44.4±2.99 | 22.0±1.74 | 48.2±2.63 | 26.3±1.68 | 51.9±2.57[a] |

Male lean littermates ($n=6$) and *db*/*db* mice ($n=6$) were fed a standard diet. Sera were immunoprecipitated and then immunoblotted with RELMβ and RELMγ antibody. The data are shown as ratios to the control lean littermate values and expressed as means±SE

[a] $p<0.01$ relative to lean littermates

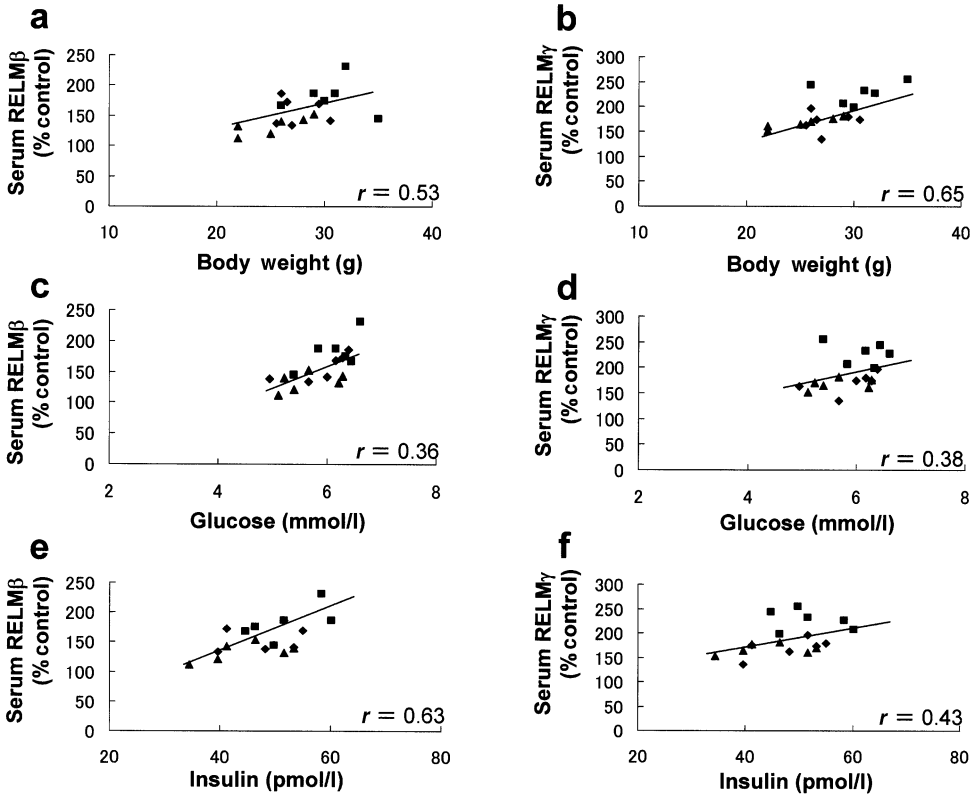


**Fig. 6** Relationships between serum RELMβ and RELMγ concentrations and body weight, glucose and insulin in *db*/*db* mice. Male lean littermates ($n$=6) and *db*/*db* mice ($n$=6) were fed a standard diet. RELMβ, RELMγ and other parameters were measured at 4, 5 and 6 weeks of age. The data are plotted as percentage of the means of control lean littermates. RELMβ and RELMγ correlated positively with body weight (**a**, $p<0.05$; **b**, $p<0.01$). Neither RELMβ nor RELMγ showed any correlation with glucose (**c**, **d**). RELMβ (**e**, $p<0.01$), but not RELMγ (**f**), also positively correlated with insulin

*Increased expression of RELMβ and RELMγ in db/db mice* Serum RELMβ and RELMγ and other parameters in male *db*/*db* mice and their littermates were measured at 4, 5 and 6 weeks of age. Male 6-week-old *db*/*db* mice weighed more (30.1±3.03 vs 23.2±2.26 g), and had higher serum glucose (4.62±0.16 vs 6.13±0.45 mmol/l) and insulin (51.9±6.29 vs 26.3±4.11 pmol/l), than their lean littermates. Serum RELMβ and RELMγ levels of *db*/*db* mice were higher and were increased by 82 and 127% respectively at the age of 6 weeks, as compared with the controls (Table 3). The RELMβ/RELMγ heterodimer in serum was also increased. Serum RELMβ and RELMγ correlated positively with body weight (Fig. 6a, $r$=0.53, $p<0.05$; Fig. 6b, $r$=0.65, $p<0.01$), but not with the serum glucose concentration (Fig. 6c, d). Serum insulin correlated positively with RELMβ (Fig. 6e, $r$=0.63, $p<0.01$) but not with RELMγ (Fig. 6f).

RELMβ mRNA and protein levels in the distal colons of *db*/*db* mice at the age of 6 weeks were elevated by 94 and 87% respectively (Table 4). Similarly, RELMγ mRNA and protein levels in the distal colon were elevated by 77 and 98% respectively (Table 4). RELMγ mRNA and protein levels in the bone marrow were elevated by 68 and 53% respectively. There were no significant changes in RELMγ levels in the spleen, lung or pancreas.

## Discussion

Resistin and the three RELMs comprise a novel class of cysteine-rich proteins. Resistin is expressed exclusively in adipose tissues and reportedly causes insulin resistance [8]. RELMα was originally identified in broncho–alveolar

**Table 4** Expression and regulation of mRNA and protein of RELMβ and RELMγ in *db*/*db* mice

| | | Colon | | Bone marrow | | Spleen | | Lung | | Pancreas | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Control | *db*/*db* | Control | *db*/*db* | Control | *db*/*db* | Control | *db*/*db* | Control | *db*/*db* |
| RELMβ | mRNA | 1±0.04 | 1.97±0.07[a] | – | – | – | – | – | – | – | – |
| | Protein | 1±0.05 | 1.87±0.07[a] | – | – | – | – | – | – | – | – |
| RELMγ | mRNA | 1±0.06 | 1.77±0.07[a] | 1±0.07 | 1.63±0.07[a] | 1±0.04 | 0.98±0.05 | 1±0.05 | 0.96±0.07 | 1±0.06 | 1.10±0.07 |
| | Protein | 1±0.05 | 1.98±0.07[a] | 1±0.05 | 1.53±0.07[a] | 1±0.06 | 1.02±0.07 | 1±0.05 | 1.05±0.07 | 1±0.06 | 1.02±0.07 |

Male lean littermates and *db*/*db* mice were fed a standard diet. RNase protection assay and immunoblotting show RELMβ and RELMγ expression in the colon, bone marrow, spleen, lung and pancreas. The data are shown as ratios to the control lean littermate values and expressed as means±SE

[a] $p<0.01$ relative to control-diet-fed mice

lavage fluid in experimentally induced pulmonary inflammation, but is most abundant in adipose tissues [13]. RELMβ is specifically expressed in the intestinal tract, especially abundantly in the colon [14] and has been suggested to be related to bacterial colonisation [24]. Injecting RELMβ reportedly induced insulin resistance in rats [16]. RELMγ is expressed in the colon, bone marrow, spleen and lung [17–19] and reportedly increases the proliferation rate of promyelocytic cells and modulates their differentiation [18]. RELMγ is also expressed in rat nasal respiratory epithelium, and is altered by cigarette smoke [17]. Although the physiological roles of these isoforms are still unclear, a series of previous reports seems to suggest their interaction with inflammatory processes and/or insulin resistance.

In this study we showed that RELMγ overexpressed in and secreted by COS7 cells forms a homodimer. Homodimerisation of RELMβ and resistin, but not RELMα, was previously reported and conserved cysteines (Cys26 of resistin and Cys25 of RELMβ) were considered to be required for disulphide bond formation [23, 24]. In RELMγ, however, the corresponding cysteine is not conserved. Therefore, Cys11 or Cys45 in RELMγ may be regarded as critical for dimer formation. Considering that Cys11 is conserved while Cys45 is missing from RELMα, which is not capable of forming a homodimer, Cys45 in RELMγ is very likely to be involved in dimerisation. Interestingly, we also found that RELMβ and RELMγ secreted by COS7 cells and also endogenously expressed in colonic tissues partially heterodimerised (Fig. 4).

RELMγ reportedly binds α-defensin, a cysteine rich 3–4-kDa antimicrobial peptide stored in the cytoplasimic granules of neutrophils, some macrophages and intestinal Paneth cells [19]. Thus, it is possible that some protein(s), like α-defensin, which bind to RELMγ, also bind to RELMβ and may form polymers with disulphide bonds. In this case, this heteromeric formation may modulate the antimicrobial effects of defensin. In addition, homodimerisation and heterodimerisation of RELMγ would affect binding to other molecules such as their receptor(s) and their resultant functions. Thus, whether or not RELMγ heterodimers and homodimers have different roles in insulin resistance and/or inflammatory processes is important. Further analysis, considering the crystal structure data of resistin and RELMβ, is necessary to clarify the mechanism of homo/heterodimer formation of resistin family proteins [20].

This is the first study to investigate the serum concentrations and tissue contents of RELMβ and RELMγ in insulin-resistant animals, while resistin expression has been analysed in several models of obesity and diabetes [8, 11, 25–30]. In high-fat-fed mice and *db*/*db* mice, serum levels of RELMβ and RELMγ were apparently increased. Taking into consideration that RELMβ causes insulin resistance by impairing hepatic glucose production [16], it is reasonable to consider the increased serum concentrations of RELMβ and RELMγ to be among the molecular mechanisms underlying the insulin resistance in these diabetic mice. Furthermore, we demonstrated that elevated serum concentrations of RELMβ and RELMγ are attributable to increased production in the colon (both RELMβ and RELMγ) and bone marrow (only RELMγ). Since the heterodimer consisting of serum RELMβ and RELMγ was also increased, a considerable portion of the RELMβ and RELMγ in serum appears to be derived from the colon and ileum.

We observed that the PPARγ agonist rosiglitazone had no effect on expression levels (10 mg $kg^{-1}$ $day^{-1}$ for 2 weeks, orally; data not shown). We can suggest two possible mechanisms of RELMβ and RELMγ upregulation in the insulin-resistant diabetic mouse. One involves the expression of these isoforms being regulated by exposure of intestinal and bone marrow cells to nutrients and/or factors such as glucose, lipid, insulin or certain cytokines or hormones. The other involves signals related to inflammation triggering increased RELMβ and RELMγ expression. Since inflammation has mechanistic significance in obesity and insulin resistance [8, 31–34] and some resistin family proteins have been implicated in the inflammatory response [13, 17], increased expression of RELMβ and RELMγ may be involved in the mechanisms connecting inflammation and insulin resistance, which are associated with obesity and/or diabetes. In this regard, a transcriptional factor may be involved in the inflammation-induced increased expression of RELMβ and RELMγ. Indeed, the promoter region of RELMβ contains a binding sequence of nuclear factor κB and signal transducer and activator of transcription 6 [24] and *RELMγ* is reportedly a target gene of CCAAT/enhancer binding protein ε mediating the role of promyelocytic cell development [19]. Our other interesting finding was that the expression of RELMγ was unchanged in the spleen, lung and pancreas in the insulin-resistant as compared with the control mice. A similar observation was that RELMα expression in adipose tissue responds to food deprivation, except in the lung [16]. Thus, it seems that organs specifically responding to nutritional or inflammatory conditions have a system which allows expression of RELMα and RELMγ to be regulated, although the underlying molecular mechanism is unclear.

In summary, adipose tissue and the intestinal tract are major tissues which produce RELM family proteins (resistin and RELMα from adipose tissue, and RELMβ and RELMγ from the intestinal tract). The production of RELMβ and RELMγ in the intestinal tract was clearly demonstrated to be increased in diet-induced and genetically obese mice, with elevated serum concentrations suggesting a hormonal link between the intestinal tract and insulin sensitivity. Furthermore, this link may respond to the total calorie and/or nutrient content of the food ingested. Future studies will examine whether the serum concentrations of RELMβ and RELMγ are also increased in obese or insulin-resistant diabetic human subjects. If so, the measurement of serum RELMβ and RELMγ concentrations may be diagnostically useful. We can also suggest the possibility that RELMβ and RELMγ are potential molecular targets for future antidiabetic drugs, and additional studies are needed to investigate these possibilities.

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## References


1. Kahn CR (1994) Banting lecture. Insulin action, diabetogenes, and the cause of type II diabetes. Diabetes 43:1066–1084
2. Birnbaum MJ (2001) Turning down insulin signaling. J Clin Invest 108:655–659
3. Saltiel AR, Kahn CR (2002) Insulin signalling and the regulation of glucose and lipid metabolism. Nature 414:799–806
4. Czech MP, Corvera S (1999) Signaling mechanisms that regulate glucose transport. J Biol Chem 274:1865–1868
5. Mora S, Pessin JE (2002) An adipocentric view of signaling and intracellular trafficking. Diabetes Metab Res Rev 18:345–356
6. Kahn BB, Flier JS (2000) Obesity and insulin resistance. J Clin Invest 106:473–481
7. Yamauchi T, Kamon J, Waki H et al (2001) The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. Nat Med 7:941–946
8. Steppan CM, Bailey ST, Brown ER et al (2001) The hormone resistin links obesity to diabetes. Nature 409:307–312
9. Olefsky JM (2000) Treatment of insulin resistance with peroxisome proliferator-activated receptor $\gamma$ agonists. J Clin Invest 106:467–472
10. Saltiel AR, Olefsky JM (1996) Thiazolidinediones in the treatment of insulin resistance and type II diabetes. Diabetes 45: 1661–1669
11. Way JM, Gorgun CZ, Tong Q et al (2001) Adipose tissue resistin expression is severely suppressed in obesity and stimulated by peroxisome proliferator-activated receptor $\gamma$ agonists. J Biol Chem 276:25651–25653
12. Banerjee RR, Rangwala SM, Shapiro JS et al (2004) Regulation of fasted blood glucose by resistin. Science 303:1195–1198
13. Holocomb IH, Kadakoff RC, Chan B et al (2000) FIZZ1, a novel cysteine-rich secreted protein associated with pulmonary inflammation, defines a new gene family. EMBO J 19:4046–4055
14. Steppan CM, Brown EJ, Wright CM et al (2001) A family of tissue-specific resistin-like molecules. Proc Natl Acad Sci U S A 98:502–506
15. Rajala MW, Lin Y, Ranalletta M et al (2002) Cell type-specific expression and coregulation of murine resistin and resistin-like molecule $\alpha$ in adipose tissue. Mol Endocrinol 16:1920–1930
16. Rajala MW, Obici S, Sherer PE, Rossetti L (2003) Adipose-derived resistin and gut derived resistin-like molecule-$\beta$ selectively impair insulin action on glucose production. J Clin Invest 111:225–230
17. Gerstmayer B, Kusters D, Gebel S et al (2003) Identification of RELM$\gamma$, a novel resistin-like molecule with a distinct expression pattern. Genomics 81:588–595
18. Schinke T, Haberland M, Jamshidi A et al (2004) Cloning and functional characterization of resistin-like molecule $\gamma$. Biochem Biophys Res Commun 314:356–362
19. Chumakov AM, Kubota T, Walter S, Koeffler HP (2004) Identification of murine and human XCP1 genes as C/EBP-$\varepsilon$-dependent members of FIZZ/resistin gene family. Oncogene 23: 3414–3425
20. Patel SD, Rajara MW, Rossetti L et al (2004) Disulfide-dependent multimeric assembly of resistin family hormones. Science 304:1154–1158
21. Ogihara T, Asano T, Ando K et al (2001) Insulin resistance with enhanced insulin signaling in high-salt diet-fed rats. Diabetes 50:573–583
22. Banerjee RR, Lazar MA (2001) Dimerization of resistin and resistin-like molecule is determined by a single cysteine. J Biol Chem 276:25970–25973
23. Chen J, Wang L, Boeg YS, Xia B, Wang J (2002) Differential dimerization and association among resistin family proteins with implications for functional specificity. J Endocrinol 175: 499–504
24. He W, Wang M, Jing H et al (2003) Bacterial colonization leads to the colonic secretion of RELM$\beta$/FIZZ2, a novel goblet cell-specific protein. Gastroenterology 125:1388–1397
25. Le Lay SI, Boucher J, Rey A et al (2001) Decreased resistin expression in mice with different sensitivities to a high-fat diet. Biochem Biophys Res Commun 289:564–567
26. Juan CC, Au LC, Fang VS et al (2001) Suppressed gene expression of adipocyte resistin in an insulin-resistant rat model probably by elevated free fatty acids. Biochem Biophys Res Commun 289:1328–1333
27. Chen L, Nyomba BL (2003) Glucose intolerance and resistin expression in rat offspring exposed to ethanol in utero: modulation by postnatal high-fat diet. Endocrinology 144:500–508
28. Levy JR, Davenport B, Clore JN, Stevens W (2002) Lipid metabolism and resistin gene expression in insulin-resistant Fischer 344 rats. Am J Physiol Endocrinol Metab 282:E626–E633
29. Hirosumi J, Tuncman G, Chang L et al (2002) A central role for JNK in obesity and insulin resistance. Nature 420:333–336
30. Li J, Yu X, Pan W, Unger RH (2002) Gene expression profile of rat adipose tissue at the onset of high-fat-diet obesity. Am J Physiol Endocrinol Metab 282:E1334–E1341
31. Hotamisligil GS, Shargill NS, Spiegelman BM (1993) Adipose expression of tumor necrosis factor alpha: direct role in obesity-linked insulin resistance. Science 259:87–91
32. Miles PD, Romeo OM, Higo K et al (1997) TNF$\alpha$-induced insulin resistance in vivo and its prevention by troglitazone. Diabetes 46:1678–1683
33. Lehrke M, Lazar MA (2004) Inflamed about obesity. Nat Med 10:126–127
34. Yuan M, Konstantopoulos N, Lee J et al (2001) Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkbeta. Science 293:1673–1677