Differential dimerization and association among resistin family proteins with implications for functional specificity

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Abstract

Secreted by white adipose tissue as a hormone, resistin was identified as a possible link between obesity and insulin resistance. High circulating resistin levels were observed to correlate with obesity. Administration of resistin lowered the glucose tolerance threshold and impaired insulin activity; whereas anti-resistin antibodies had the opposite effects. However, contradictory data were subsequently reported in regard to the correlation between resistin expression level and obesity or type 2 diabetes. Two additional proteins that share a highly homologous C-terminus with resistin have been identified in mouse, and one in human, forming a resistin-related protein family. Resistin was shown to dimerize through a disulfide bond formed by the N-terminal-most cysteine (Cys26). Here we demonstrate that while Cys26 is both necessary and sufficient for homodimer formation, all three resistin family members can also interact with one another regardless of the presence of Cys26 through non-covalent interactions. Furthermore, protein crosslinking analysis indicated that resistin and resistin β, but not resistin α, exist as multimers, probably with a dimer as the subunit. The multiple protein complex formation is obviously at a level higher than the Cys26 disulfide bonding. These results suggest the potential importance of considering intermolecular interactions among resistin family members in studying their functions.

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Introduction

A correlation between insulin resistance in type 2 diabetes and obesity is a well-known fact, yet little is understood about the underlying mechanism. Defining such a link will undoubtedly provide new tools for studying and treating diabetes. The hormone resistin is secreted by white adipocytes with expression levels correlated with both obesity and insulin resistance (Steppan *et al.* 2001*a*). It thus appeared to be a strong candidate for the missing link between fat accumulation and type 2 diabetes. New therapies for diabetes could be developed by targeting resistin in obese patient with type 2 diabetes.

However, conflicting data were later found in regard to the correlation between resistin expression and obesity, insulin resistance, or peroxisome proliferator activated receptor-γ (PPARγ) agonists treatment in different rodent models. For instance, instead of the expected increase, decrease of resistin mRNA levels was observed in adipose tissues in obesity (Le Lay *et al.* 2001, Way *et al.* 2001). In other reports, resistin expression appeared to be suppressed or irrelevant to insulin resistance in rats (Juan *et al.* 2001, Levy *et al.* 2002). Further analysis indicated that PPARγ ligands (including thiazolidinedione) reduce resistin expression but not by direct action on PPARγ (Hartman *et al.* 2002, Li & Lazar 2002). Some of these contradictions might be explained by the difference between resistin mRNA levels in fat cells and circulating polypeptides in serum, and between expression levels in individual cells and overall protein levels affected by increased fat cell numbers (Steppan & Lazar 2002).

Resistin was independently cloned as adipose tissue-specific secreted factor (ADSF) and shown to inhibit differentiation of 3T3L1 cells into adipocytes (Kim *et al.* 2001). In that system and in Zucker diabetic fatty rats (Way *et al.* 2001), ADSF/resistin was stimulated by insulin. In another report, however, insulin seemed to suppress the expression of resistin (Haugen *et al.* 2001).

In humans, no correlation between resistin expression and obesity, insulin resistance, and type 2 diabetes was established in one study (Nagaev & Smith 2001). Results from other human studies demonstrated a correlation between central obesity and increased resistin expression (Savage *et al.* 2001, McTernan *et al.* 2002*a*,*b*). However, different observations were made in relation to where human resistin was expressed: in adipose cells or in mononuclear cells.

Resistin was also identified as a homologue of a protein (FIZZ1) found in inflammation zones (Holcomb *et al.* 2000). For convenience and in accordance with the

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standardized nomenclature (Steppan & Lazar 2002), we refer to resistin/FIZZ3/ADSF as resistin, mouse and rat resistin-like molecule α (RELMα)/mouse FIZZ1 as resistin α, and RELMβ/human FIZZ1 as resistin β. Resistin α is overexpressed in allergic inflammation as well as in adipose tissues; resistin β is found in the gastrointestinal tract. These resistin proteins presumably function by binding to common or specific receptors that are yet to be identified. It might be possible that resistins could mediate adipocyte activities in relation to inflammation or link obesity and allergic processes (Shuldiner *et al.* 2001).

Resistin-related functional studies have focused mostly on resistin as a single factor. What, if any, other factors might associate with resistin as cofactors? There are currently three resistin family members found in mouse, and two in human (resistin and resistin β). The C-terminal amino acid sequences of all resistin-like proteins are highly conserved across species, highlighted by a strict pattern of 11 cysteine residues. However, the N-terminal sequences diverge significantly and the overall identity between human and mouse resistin is only 53%, even raising the doubt as to whether they are true homologues. Furthermore, an extensive search of the completed human genome yielded no human resistin α or other resistin-like genes (Steppan & Lazar 2002). It is possible that rodents and humans have significant differences in the function of resistin family proteins. It is also possible that, instead of individual factors, the association and networking among resistin-like hormones dictate their roles in balancing signals in circulation.

By transient expression of individual genes, Banerjee & Lazar (2001) demonstrated that secreted resistin or resistin β formed disulfide-linked homodimers when detected in culture medium, whereas resistin α was secreted as a monomer. It was further shown that the first cysteine (Cys26) at the N-terminus, which resistin α lacks, is necessary for dimerization. In the current study, we intended to investigate other potential interactions among resistin proteins by coexpression and coprecipitation. We found that, in addition to homodimerization, resistin family proteins associated with each other, forming heterodimers or multimers. Although resistin α does not form a homodimer, it too bound to resistin or resistin β. Therefore, Cys26 is not required for these heterologous associations. Furthermore, by crosslinking and HPLC experiments, we discovered that multimers, instead of dimers as previously reported, are the existing state of resistin and resistin β.

Materials and Methods

Construction of resistin expression plasmids

Mouse resistin, resistin α, and resistin β complete coding regions were PCR amplified from an embryo cDNA library (Clontech, Palo Alto, CA, USA) and cloned into pcDNA 3·1 with either V5-His tag or FLAG tag at the C-terminus of the fusion proteins. Site-directed mutagenesis for creating resistin mutant M1 (Cys26 changed to Gly26) was achieved with PCR. Similarly, resistin Leu25-Cys26 was changed to Cys25-Gly26 to create mutant M2. Both mutants were cloned into pcDNA 3·1 with V5-His tag at the C-terminus. All plasmids were sequenced to confirm the identities.

Cell culture and transient transfection

COS7 cells were grown in DMEM medium supplemented with 10% fetal calf serum. Resistin-, resistin α- and resistin β-expressing plasmids were transfected into COS7 cells with LipofectAmine (Invitrogen) as described by the manufacturer. Three days after transfection, the cell culture media were collected for affinity precipitation and immunoblot analysis.

Affinity precipitation and Western blot analysis

The cell culture media were first centrifuged at 15 000 ***g*** at 4 °C for 30 min. The supernatants were incubated with either Ni-beads or anti-FLAG M2 beads (Sigma) at 4 °C overnight. The beads were spun down and washed three times with cold wash buffer (20 mM Tris–HCl, 150 mM NaCl, pH 7·4 and 0·05% Tween 20). The precipitated proteins were resolved on a 4–20% gradient SDS-PAGE gel (Invitrogen), and then transferred onto a PVDF membrane (Invitrogen). The membrane was blocked with 2% non-fat dry milk, blotted with either 1:1000 anti-FLAG M2 monoclonal antibody (Sigma) or anti-V5 monoclonal antibody (Invitrogen), followed by blotting with 1:5000 horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (Sigma). The membrane was then developed with an ECL kit (Amersham).

Protein crosslinking

Cell culture media were treated with 5 mM crosslinker ethylene glycol bis(succinimidyl-succinimate) (EGS) (Pierce, Rockford, IL, USA) at room temperature for 20 min. Tris–HCl (pH 8·0) was then added to a final concentration of 100 mM to block unreacted crosslinker molecules and the reactions were allowed to proceed at room temperature for an additional 20 min. The crosslinker-treated media were then subjected to immunoprecipitation and the proteins analyzed by Western blot as described above.

HPLC

Plasmid expressing resistin-FLAG was transfected into COS7 cells as described above. The culture medium was used for purifying the recombinant protein by running through an anti-FLAG M2 affinity column. The column was washed with PBS and eluted with 0·1 M glycine–HCl, pH 2·8. The purified protein was analyzed by

SDS-PAGE and the purity was confirmed to be greater than 90% (data not shown).

A Hitachi Model 655A-11 liquid chromatograph (Hitachi, Ltd, Tokyo, Japan) equipped with a Hitachi controller (Model L-5000LC) and a 300 × 7·80 mm size exclusion column (BioSep-SEC-S2000; Phenomenex, Belmont, CA, USA) were used for the molecular mass analysis. A protein mixture of thyroglobin (670 kDa), IgG (150 kDa), ovalbumin (44 kDa) and myoglobin (17 kDa) was used as molecular mass marker. The column was run at 0·5 ml/min with 100 mM phosphate buffer at pH 6·35.

Results

Resistin, resistin α and resistin β coprecipitate

We first expressed resistin proteins with different tags in COS7 cells, then established conditions for either affinity or immuno-coprecipitation. V5 and His double-tagged resistin was secreted from transiently transfected COS7 cells and could be pulled down by Ni-beads, as detected with anti-V5 antibodies (Fig. 1, lane1). Under non-reducing conditions, resistin-V5-His migrated as an apparent dimer (compare lane 1 between upper and lower panel). Similar to previously reported results with a Cys26 to Ala26 mutation (Banerjee & Lazar 2001), change of Cys26 to Gly26 (resistinM1-V5-His) rendered resistin unable to form dimers (Fig 1, lane 2). When a cysteine was added back by replacing Leu25 with Cys25 in resistinM1-V5-His, dimer was again formed (resistinM2-V5-His; Fig 1, lane 3). From these results we conclude that resistin formed a dimer under our expression and precipitation conditions. Furthermore, the N-terminal-most cysteine is both necessary and sufficient for the dimer formation and its exact location is not critical. Under similar conditions, secreted and precipitated resistin β also formed dimer while resistin α did not (Banerjee & Lazar 2001) (data not shown).

Using this experimental system, we examined interactions between resistin proteins by coexpressing and coprecipitating V5 and His double-tagged proteins with FLAG-tagged proteins. When precipitated with Ni-beads and detected with anti-V5 antibody (Fig 2A, lane 1), resistin-V5-His and resistin-FLAG migrated as two forms of dimers on a non-reducing gel, presumably one was a resistin-V5-His homodimer and the other a dimer of resistin-V5-His and resistin-FLAG. Resistin-FLAG homodimer was also expected but would not be detected by the anti-V5 antibody. As predicted, the same results were obtained by anti-FLAG immuno-coprecipitation and anti-V5 Western blotting (Fig. 2B, lane 1).

We next coexpressed resistin-FLAG with resistin α-V5-His or resistin β-V5-His. Significantly, both resistin α-V5-His and resistin β-V5-His coprecipitated with resistin-FLAG by Ni-beads or anti-FLAG precipitation (Fig. 2A and B). Under non-reducing conditions, the coprecipitated resistin α-V5-His still migrated as a monomer, indicating that its association with resistin-FLAG did not involve dimer formation. On the other hand, when coexpressed with resistin-FLAG, resistin β-V5-His migrated as a dimer with itself and with resistin-FLAG, with the latter slightly bigger than the former (Fig. 2A and B, lane 3, the double bands are difficult to discern because of the high expression level of resistin β-V5-His). Resistin β-V5-His dimer also coprecipitated with resistin α-FLAG (Fig. 2B, lane 4), although at a lower level compared with that with resistin-FLAG. These observations strongly indicate that while resistin and resistin β can form homodimers and heterodimers, all three resistin-like proteins can also interact with each other.

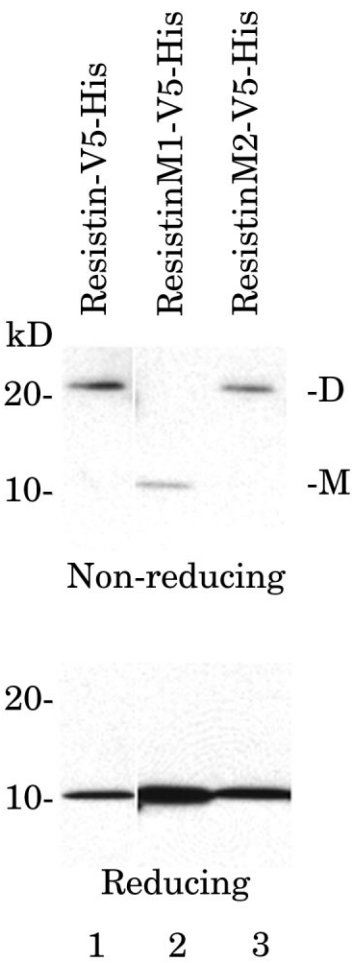

Figure 1 Expression and affinity precipitation of resistin proteins. V5-His-tagged resistin and its mutants M1 and M2 were transiently expressed in COS7 cells. Media from these cells were mixed with Ni-beads and the recombinant fusion proteins isolated. The purified proteins were electrophoresed on non-reducing (upper panel) or reducing (lower panel) gradient gels, followed by Western blotting with anti-V5 antibody. Migration of molecular size markers is shown on the left and monomers and dimers of the expressed proteins are indicated on the right. All blots represent repeated experiments.

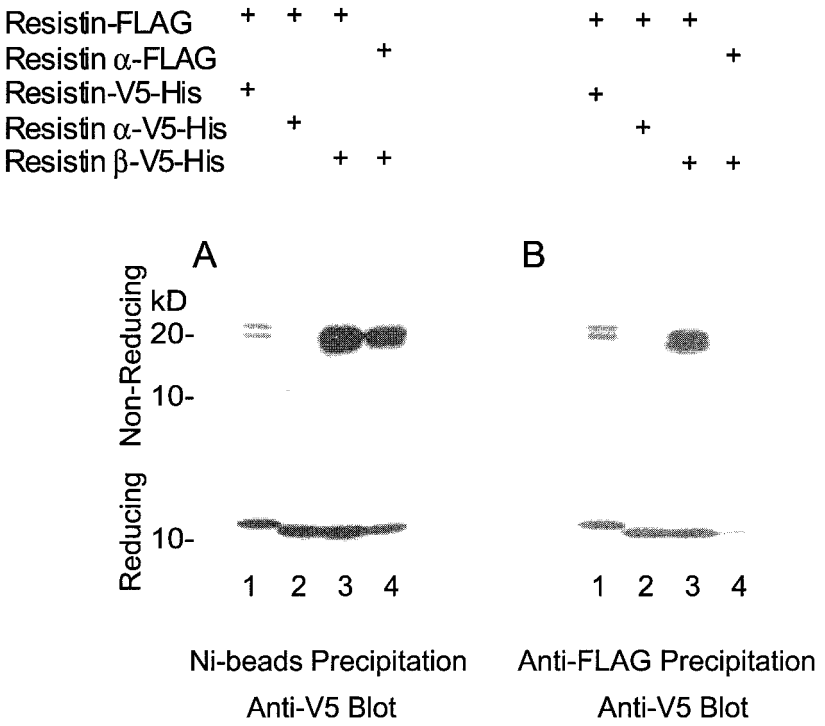

Figure 2 Coprecipitation of resistin family proteins. Combinations of FLAG-tagged and V5-His-tagged resistin, resistin α, and resistin β were transiently expressed in COS7 cells for coprecipitation assays as indicated above each lane. After precipitation with either Ni-beads (A) or with anti-FLAG antibody (B), the isolated proteins were analyzed by anti-V5 Western blotting as in Fig. 1.

Resistin proteins exist as higher-order complexes than dimers

Based on the results that resistin α could interact with resistin and resistin β even without the N-terminal cysteine, we hypothesized that resistin family proteins might interact with one another by interactions in addition to the Cys26 disulfide bonding. This would predict that resistin and resistin β could exist as congregates of more than two monomers. To test that, we performed chemical crosslinking before running resistin-FLAG on reducing or non-reducing gels. Most of the resistin-FLAG dimers were covalently linked so that even on reducing gel the dimer dominated over the monomer (Fig. 3). An even more prominent band than that of the dimer, which we estimated to be a tetramer, appeared on the reducing gel. There were a number of bands that migrated above the presumed tetramer band, demonstrating a very complex pattern of resistin proteins in solution. This observation could also be explained by different degrees of partial crosslinking within one form of multimer that would migrate as the highest band if all of its subunits were linked together. On non-reducing gel, the resistin monomer completely disappeared as shown before (Fig. 1 and Banerjee & Lazar (2001)). More interestingly, the dimer and tetramer also seemed to be integrated into higher

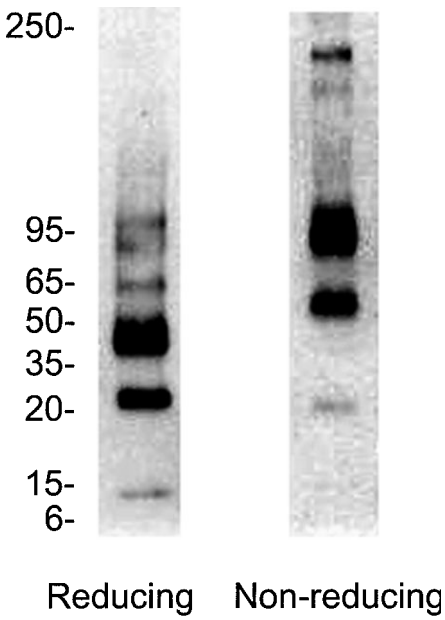

Figure 3 Resistin exists as multimers shown by chemical crosslinking. FLAG-tagged resistin was treated with crosslinker EGS and purified by immunoprecipitation. After electrophoresis through reducing or non-reducing 4–20% gradient gel, the protein complexes were detected with anti-FLAG antibody.

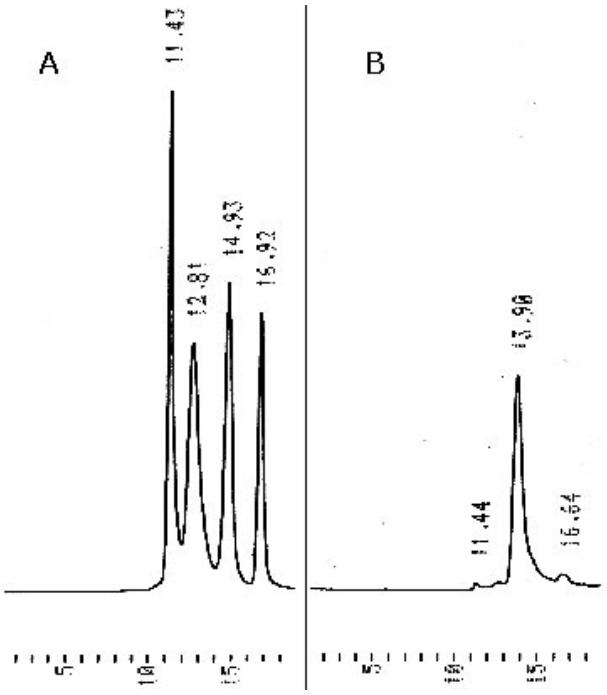

Figure 4 Resistin exists as multimers shown by HPLC. Molecular mass standard proteins (A) or purified resistin-FLAG (B) were analyzed by HPLC. The retention time of each protein is indicated above the corresponding peak. The molecular mass standards were, from left to right, thyroglobin (670 kDa), IgG (150 kDa), ovalbumin (44 kDa) and myoglobin (17 kDa). The retention time and the molecular mass of the last three molecular mass standard proteins fell within a linear range as represented by the equation: y(kDa)=554·92 − 32·552x(min) with R^2=0·906. The relative molecular mass of the dominant resistin-FLAG peak with retention time of 13·90 min was calculated to be 102 kDa. Knowing that the molecular mass of a monomeric resistin-FLAG is about 13 kDa, this species appears to be composed of eight monomers.

complexes. We thus concluded that resistin exist as multimers composed of several protein molecules. When expressed alone, resistin α always appeared to be a monomer regardless of crosslinking treatment whilst resistin β also formed higher complexes when crosslinked (data not shown).

To confirm the above notion, we performed HPLC analysis with resistin-FLAG. After a set of protein mass standards (Fig. 4A), resistin-FLAG protein, purified to above 90% as analyzed by SDS-PAGE (data not shown), was run through a BioSep-SEC-S2000 column under the same conditions (Fig. 4B). A linear relationship between the retention time and the known molecular mass of the standard proteins was deduced and used to calculate the apparent molecular mass of resistin-FLAG. A single dominant peak corresponding to a calculated octamer was seen with the resistin-FLAG sample. There were three minor peaks presumably of multimers with different compositions. The overall distribution of the HPLC peaks agrees well with that of the crosslinked resistin-FLAG bands on non-reducing gel (Fig. 3). Taken together, it is clear that resistin exists as multimers instead of a dimer.

Discussion

It was previously shown that resistin and resistin β were homodimers linked via a disulfide bond at Cys26 (Banerjee & Lazar 2001). Resistin α lacks Cys26 and exists as a monomer. Here we further demonstrate that all resistin family proteins can form heterodimers or multimers when coexpressed. The most striking among these observations is that resistin α, while not capable of forming dimers by itself, can still associate with resistin or resistin β. In the resistin-FLAG and resistin α-V5-His coprecipitation experiments, resistin α-V5-His migrated as a monomer under both reducing and non-reducing conditions (Fig. 2). It therefore indicates that the association between resistin α-V5-His and resistin-FLAG was not by a disulfide bond at their C-terminal cysteines. A single resistin β-V5-His dimer band in non-reducing gel was observed after coprecipitation with resistin α-FLAG, suggesting that no disulfide-bonded heterodimer was formed; thus cysteines were not involved in the interaction between these two proteins either. This also serves as evidence that a resistin family protein dimer could further interact with other proteins. On the other hand, the coprecipitation of resistin-FLAG and resistin β-V5-His resulted in two dimer bands under non-reducing conditions. This would indicate that resistin and resistin β could form heterodimers through disulfide bonds. In addition, it is known that when expressed separately then mixed together, resistin and resistin-FLAG do not dimerize with each other (Banerjee & Lazar 2001). The same is true with resistin-V5-His and resistin-FLAG or resistin β-V5-His and resistin β-FLAG (data not shown). Such results point to the fact that the dimerization occurs as resistin family proteins are produced inside the cell, probably during folding, and the dimers are stable in solution. The coprecipitation of resistin β-V5-His dimer by resistin-FLAG would then support the above conclusion that a dimer could still interact with other resistin proteins. From the above results, we conclude that resistin family proteins could associate with each other in both disulfide bond-dependent and -independent manners.

Knowing that disulfide bonding is not the only mode of interaction, we speculated that resistin proteins might form higher-order complexes. We chose to use chemical crosslinking to detect any such complexes. The results showed that in either reducing or non-reducing gel, a group of bands were detected for resistin-FLAG (Fig. 3) or resistin β-FLAG (data not shown). The apparent molecular masses corresponding to these bands suggest that they were multimers formed from dimers. It seems reasonable to speculate that disulfide-bonded dimers were formed first, which in turn associated to form tetramer, hexamer,

octamer and so on. Interestingly, even though resistin α can bind to resistin and resistin β without disulfide bonding, it could not be crosslinked to itself (data not shown). It thus appears that the associations involving resistin α may be of lower affinity compared with the others. The result by HPLC confirms that the existing state of resistin (and likely resistin β as well) is of multimers. It would be interesting to find out which parts of the proteins are responsible for the secondary associations by deletion mutations in the future.

These observations raise the possibility that resistin does not necessarily function by itself. A specific combination of resistin family proteins could potentially provide specific effects. Further experiments by administering different combinations of resistin proteins or their antibodies in animal models should help to elucidate this possibility. Given the divergence of mouse and human resistin-related proteins, it might be important to find out whether, instead of individual proteins, specific combinations of these proteins in different species share more conserved functions.

Resistins are a newly discovered family of potentially very important protein hormones. Their unique expression pattern and change of expression levels in relation to other hormones suggest their roles in adipocyte development, metabolism and inflammation in general, and as a link between obesity and insulin resistance in particular. Nonetheless, the current understanding of resistin functions is very limited and often contradicting. Part of the controversy could be due to the discrepancy between the RT-PCR-detected cellular levels of resistin mRNA and the circulating protein in serum. Some of the related questions might be answered with effective detection reagents such as antibodies that can specifically detect low levels of serum resistin and its related proteins in humans. Unfortunately, our attempts to generate such specific antibodies have so far been unsuccessful and antibodies from a few commercial sources did not provide satisfactory specificity or sensitivity (data not shown).

With certain discrepancies, previously published data suggest that both resistin and resistin α are expressed at significant levels in mouse adipose tissues, where intracellular association is possible. On the other hand, resistin β, found mainly in the intestine, has not been shown to be coexpressed with resistin or resistin α (Holcomb *et al.* 2000, Steppan *et al.* 2001*b*). The levels of circulating resistin proteins remain unclear. Therefore, to what level the associations among resistin family proteins occur and function biophysically is yet to be assessed at the current stage. Here we propose that the close and complicated association among resistin-related hormones in the circulation should be taken into account when studying their functions. When adequate antibodies become available, the obvious next step will be to demonstrate that the complexes exist in the plasma of animals in different pathological or nutritional states and to elucidate their physiological importance.

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