Biophysical Analyses of Human Resistin: Oligomer Formation Suggests Novel Biological Function[†]

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ABSTRACT: Resistin, a small secreted peptide initially identified as a link between obesity and diabetes in mice, was shown to be involved in mediating inflammation in humans. We had shown earlier that recombinant human resistin has a tendency to form aggregates by formation of inter/intramolecular disulfide linkages and that it undergoes a concentration-dependent conformational change in secondary structure from α-helical to *β*-sheet form. Here we report that this change in secondary structural conformation is due to the increase in the oligomeric form of human resistin as a function of protein concentration. Gel filtration analysis under different conditions further demonstrated that recombinant human resistin exists as a mixture of oligomer and trimer but is converted to a mixture of monomer and oligomer in the presence of 100 mM NaCl. We show that while the trimeric form of human resistin is stable to urea-induced denaturation, it is highly susceptible to NaCl and NaF, indicating the importance of ionic interactions in stabilization of trimer. In addition, urea was able to destabilize the oligomers indicating the involvement of hydrophobic interactions in oligomerization. Ionic as well as hydrophobic interactions stabilize the monomeric human resistin. Our data suggest that human resistin exists predominantly as oligomer and trimer *in vitro*. The oligomeric form of human resistin shows more potent effect on stimulation of proinflammatory cytokines. Therefore, it is very tempting to propose that the structural conformation of resistin may be involved in maintaining the very fine balance in regulation of macrophage function for successful response to a variety of pathological conditions.

Resistin, a small peptide hormone believed to confer insulin resistance, is regarded as a missing link between obesity and diabetes (*1*−*3*). Expressed by mouse adipocytes, resistin gene is regulated by a group of antidiabetic drugs called thiazolidinediones (*1, 4*). Given the importance of this hormone, there has been considerable interest in the human homologue of resistin. We and others have shown major differences between the human and rodent proteins (*5*−*9*). Human resistin is mostly expressed in macrophages (*10*) and its serum levels do not correlate as clearly with obesity, insulin resistance, or diabetes as with inflammation (*11*−*16*). Similarly, genetic association studies between allelic variants of the resistin gene and metabolic abnormalities have so far been inconclusive (*17*−*20*).

In addition to the different physiological roles of resistin in rodent and human species, significant differences in the structural organization, both at the gene and at the protein level, exist. Mouse resistin gene is present on chromosome 8 while its human counterpart is on chromosome 19. Mouse and human resistin share 64.4% sequence homology at the mRNA level and 59% identity at the amino acid level (*9*).

Recent X-ray crystallographic studies of mouse resistin have revealed a complex hexameric and trimeric structure. Mouse resistin was shown to circulate in two distinct assembly states, the predominant HMM[1] (high-molecular-mass) hexamer and the substantially more bioactive LMM (low-molecular-mass) trimer complex, which is unable to form intertrimer disulfide bonds (*21*). Our earlier observation that human resistin has a tendency to form oligomers (*22*) was further substantiated by Gerber et al. (*23*), who showed the presence of resistin oligomers with a molecular mass >660 kDa and a trimer of 45 kDa in human sera. Additionally, we also reported that human resistin has the ability to form oligomers involving covalent as well as noncovalent linkages (*24*).

Given the reported concentration-dependent increase in *β*-sheet structure (*22*), it would be interesting to address the oligomeric status of human resistin as a function of protein concentration. If covalent linkages are critical for maintaining

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[1] Abbreviations: HMM, high molecular mass; LMM, low molecular mass; HMW, high molecular weight; CD, circular dichroism; TNF-α, tumor necrosis factor-α; IL-1*β*, interleukin-1*β*; NO, nitric oxide; ROS, reactive oxygen species.

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the structure of human resistin, what then is the role of noncovalent interactions in stabilizing human resistin structure? The present study attempts to address some of these questions by probing the biophysical properties of human resistin under various conditions which might help in revealing the importance of noncovalent interactions in relation to the function of different conformational states.

MATERIALS AND METHODS

Materials. Chemicals used for protein purification were procured from Qualigens. Talon was purchased from BD Biosciences, guanidinium chloride (GdmCl) was obtained from Schwarz/Mann, and real-time PCR kits were obtained from Bio-Rad. RAW 264.7 cells were obtained from the National Centre for Cell Science, Pune, India. RPMI 1640 media, FCS, and antibiotics were procured from Invitrogen (Carlsbad, CA). All other chemicals were from Sigma Aldrich Co.

Purification of Recombinant Human Resistin. Mature human resistin gene devoid of the secretory signal sequence was overexpressed in *Escherichia coli* M15 cells as a histidine-tagged protein and was purified as described earlier (*22*). The absorbance of the purified protein at 280 nm was measured to determine protein concentration. The molar extinction coefficient was calculated according to the formula (*24*) ε_{280} (M^{-1} cm^{-1}) = (no. of Trp) (5500) + (no. of Tyr) (1490) + (no. of cystine) (125). The protein concentration was estimated based on the molar extinction value.

Gel Filtration Chromatography. In order to determine the oligomerization property of the recombinant human resistin, size exclusion chromatography was performed using the AKTA Purifier FPLC system (Amersham, GE) equipped with a Superdex-200 HR 10/30 column. Different concentrations (4.0, 2.4, 1.8, and 0.6 mg/mL) of resistin protein in 100 mM NaCl and 50 mM Tris-HCl (pH 8.0) were loaded on the column, and elution of the protein at a flow rate of 0.5 mL/min was monitored at 280 nm. The effect of urea, NaCl, NaF, and GdmCl on the structure of resistin was determined by incubating resistin with varying concentrations of these additives overnight followed by gel filtration. Separation of the protein species was carried out with the same concentration of either urea, NaCl, or NaF in 50 mM Tris-HCl (pH 8.0). Resistin incubated with GdmCl at a given concentration was eluted with the same concentration of GdmCl in 50 mM Tris-HCl buffer containing 100 mM NaCl (pH 8.0). All of the samples were centrifuged at 10000 rpm for 10 min before loading onto the column. The molecular masses of the respective peaks were calculated based on the elution volume of standard protein molecular size markers (Pharmacia Amersham).

Circular Dichroism (CD) Measurements. The far-UV CD spectra of human resistin incubated with varying concentrations of urea, NaCl, NaF, or GdmCl in 50 mM Tris-HCl (pH 8.0) were recorded in steps of 1 nm with 0.5 s averaging per point and a 2 nm bandwidth in a spectropolarimeter (Jasco J-815). All of the samples were equilibrated overnight. The spectra were signal averaged for four accumulations and baselines corrected by subtracting the spectra of the respective blank. The spectropolarimeter was coupled to a Peltier system for recording the spectra at different temperatures. $[\theta]_{222}$, the mean residue ellipticity at 222 nm, was estimated using the relation $[\theta]_{222} = \mathrm{Mo}[\theta]/10lc$. Mo is the mean residue weight of the monomer, θ is ellipticity (mdeg), l is path length (cm), and c is the total protein concentration (mg/mL).

Fluorescence Measurements. The intrinsic tryptophan fluorescence spectra of resistin at various concentrations of NaCl, NaF, and GdmCl were measured in a spectrofluorometer (Perkin-Elmer LS 3B). For these measurements the purified recombinant resistin was incubated overnight with varying concentrations of NaCl, NaF, and GdmCl. The intrinsic fluorescence spectra were recorded with the excitation wavelength set at 280 nm, and the protein emission was scanned in the wavelength range 300−400 nm at 200 nm/min scan speed. The bandwidths for excitation and emission wavelengths were 10 and 5 nm, respectively. All of the measurements of emission spectra were carried out at room temperature (~25 °C). However, the spectrofluorometer was connected to a Peltier system when the emission spectra were recorded at varying temperatures.

Stimulation of Cells. RAW 264.7 cells were maintained in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 units of penicillin/mL, 100 μg of streptomycin/mL, and 10% FCS. Cells were seeded at a density of 2×10^6 cells/mL and allowed to adhere for 2 h at 37 °C in a CO_2 incubator.

To obtain the specific form of resistin, the recombinant human resistin was incubated overnight in the presence of either 5 M urea and 50 mM Tris-HCl, pH 8.0, or 100 mM NaCl and 50 mM Tris-HCl, pH 8.0, at room temperature. Cells were then stimulated with either 10 or 20 μg/mL recombinant human resistin. The buffer controls for urea and NaCl were prepared in the same way without the resistin protein. Cells were also stimulated with 5 μg/mL LPS as a positive control. Total RNA was isolated after 4 h, and the expression of proinflammatory cytokines was determined by real-time PCR.

Isolation of Total RNA from Cells. Total RNA was isolated using Trizol (Sigma) as per the manufacturer's instructions. Briefly, cells were lysed by the addition of Trizol. The aqueous phase was separated by adding 0.2 mL of chloroform/mL of Trizol reagent followed by centrifugation at 12000*g* for 10 min. The aqueous phase was transferred to a fresh tube and RNA precipitated by the addition of 0.5 mL of 2-propanol. The RNA pellet was air-dried after washing with 75% ethanol and resuspended in nuclease-free water. The concentration of RNA was estimated using a NanoDrop ND-1000 spectrophotometer (Rockland).

Real-Time PCR. Real-time PCR was performed with a Bio-Rad iCycler (Bio-Rad) real-time PCR machine using SYBR green as fluorophore. Total RNA was isolated from the treated samples and reverse transcribed using superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). Reactions were set up in duplicates along with a standard serially diluted up to 729-fold in 3-fold increments. For each reaction, cDNA was taken equivalent to 5 ng of total RNA, and 40 cycles of amplification were carried out. The relative amount of each cytokine (MCP1/CCL2, RANTES/CCL5, and TNF-α) was determined from the respective standard curves and normalized to the respective glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels in each sample. Expression data were analyzed using the background subtracted curve-fit algorithm in the iCycler software.

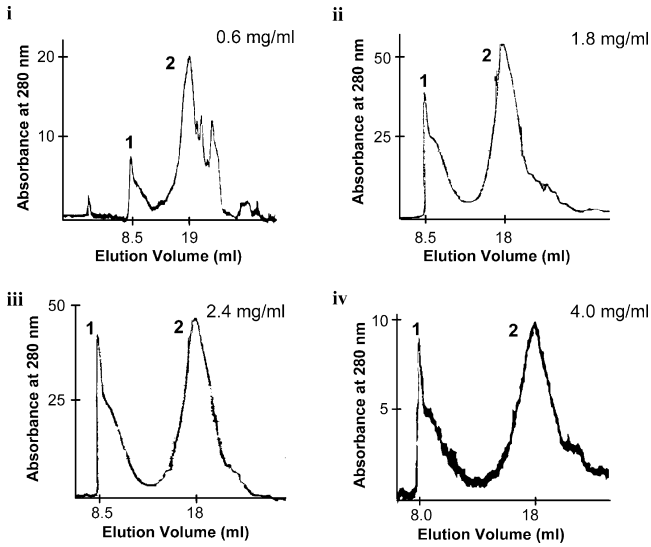

FIGURE 1: Human resistin shows concentration-dependent oligomerization. Different concentrations of human resistin protein in 50 mM Tris-HCl and 100 mM NaCl, pH 8.0, were subjected to gel filtration on a Superdex 200 column. Various concentrations of resistin analyzed are (A) 0.6 mg/mL, (B) 1.8 mg/mL, (C) 2.4 mg/mL, and (D) 4 mg/mL. Peak 1 corresponds to an oligomer (>669 kDa), and peak 2 corresponds to the monomer (~8−12 kDa).

RESULTS AND DISCUSSION

Recombinant resistin was purified under denaturing conditions and refolded by removal of urea using dialysis. The near-UV circular dichroism spectra of the refolded human resistin were recorded to determine whether it possessed tertiary structure. Positive peaks in the 280−290 nm region, contributed by the two tryptophan residues and a number of troughs corresponding to the disulfide bond absorbance (*26*), were observed pointing to the presence of the tertiary structure within the human resistin purified from *E. coli* (data not shown).

We earlier showed that the 10 free cysteine residues in freshly purified, dilute protein are reduced to three free −SH groups as a function of concentration and time. Furthermore, secondary structure analyses revealed a concentration-dependent reversible conformational shift from a predominantly α-helical structure to β-sheet form with implications in disease manifestation (*22*). Thus, at higher protein concentration resistin may form a different molecular organization with distinct structure as well as physiological properties. Therefore, identifying the forces involved in these conformational switches could pave the way toward understanding the implication of resistin structure in its function.

Human Resistin Undergoes a Concentration-Dependent Oligomerization. Given the fact that human resistin undergoes a concentration-dependent change from α-helical to a predominantly β-sheeted secondary structure (*22*), an attempt was made to understand the change in the oligomeric state as a function of protein concentration by performing gel filtration at different protein concentrations in 100 mM NaCl and 10 mM Tris-HCl (pH 8.0). Figure 1 shows the gel filtration of human resistin performed at different protein concentrations (0.6, 1.8, 2.4, and 4 mg/mL) to uncover any changes in the oligomerization of human resistin as a function of protein concentration. Interestingly, we observed a concentration-dependent variation in the oligomerization pattern of human resistin. Human resistin existed as a high molecular weight (HMW) oligomer with molecular mass greater than 669 kDa (Figure 1, peak 1) as well as a monomer (Figure 1, peak 2). At a very low protein concentration (0.6 mg/mL), the majority of the protein existed as a monomer, and the proportion of the oligomer was very low (Figure 1, i). But, as the protein concentration increased, there was an increase in the relative proportion of the oligomer (Figure 1, ii−iv). The proportion of the oligomer and the monomer was almost equal at a protein concentration of 2.4 mg/mL (Figure 1, iii) and higher (Figure 1, iv), indicating an attainment of equilibrium at these protein concentrations. These observations revealed some interesting aspects of resistin aggregation. Human resistin forms higher order oligomer at higher protein concentration which corresponds to the change in conformation to a predominantly β-sheeted structure. This change in conformation and a concomitant increase in protein aggregation have also been reported in other proteins such as prion proteins and crystallins (*27*). Interestingly, the size of the oligomer does not further increase in the case of human resistin as is seen in most of the proteins that undergo aggregation. It has been demonstrated in the case of mouse resistin that the trimeric form is functionally more active than the hexameric form (*21*). The formation of higher oligomers of human resistin, in the form of a complex quaternary structure, at higher protein concentrations might have important functional implications in inflammatory conditions where resistin expression is greatly increased.

Human Resistin Trimer Stability Is Independent of Hydrophobic Interactions. To completely understand the forces stabilizing the protein structure, the effect of different denaturants was studied using far-UV CD, fluorescence spectroscopy, and gel filtration to determine the changes at the secondary, tertiary, and quaternary structural levels, respectively.

In order to determine the role of hydrophobic interactions on the oligomerization as well as the overall structure of human resistin, the protein was incubated with increasing concentrations of urea overnight at room temperature, and the samples were analyzed by gel filtration chromatography (Figure 2A). It could be seen that resistin in 50 mM Tris-HCl buffer, pH 8.0 ([urea] = 0 M), exists as a mixture of oligomer (>669 kDa) and trimer (~30 kDa) (Figure 2A, peaks 1 and 2, respectively). Dynamic light scattering (DLS) measurement of the resistin in 50 mM Tris-HCl indicated that oligomer is composed of two predominant species with hydrodynamic radii (R_h) of 21.98 nm (SD = 6.86 nm) and 125.93 nm (SD = 16.27 nm). These two species were polydispersed in nature with polydispersity values of 0.293 and 0.355, respectively. As the urea concentration increased from 0 to 7 M, there was a gradual conversion of the oligomer to a species which eluted earlier than the oligomer (Figure 2A, ii, and Supporting Information Figure 1A, peak 3). This species could represent the unfolded oligomer of human resistin with a higher hydrodynamic volume. The relative concentrations of the trimeric species remained more or less unchanged (Figure 2A, i−iii, and Supporting Information Figure 1A, a−j, peak 2), pointing to the stability of the trimer to urea-induced denaturation. In order to get a clear picture of these observations, percentage peak heights were plotted as a function of molar concentration of urea

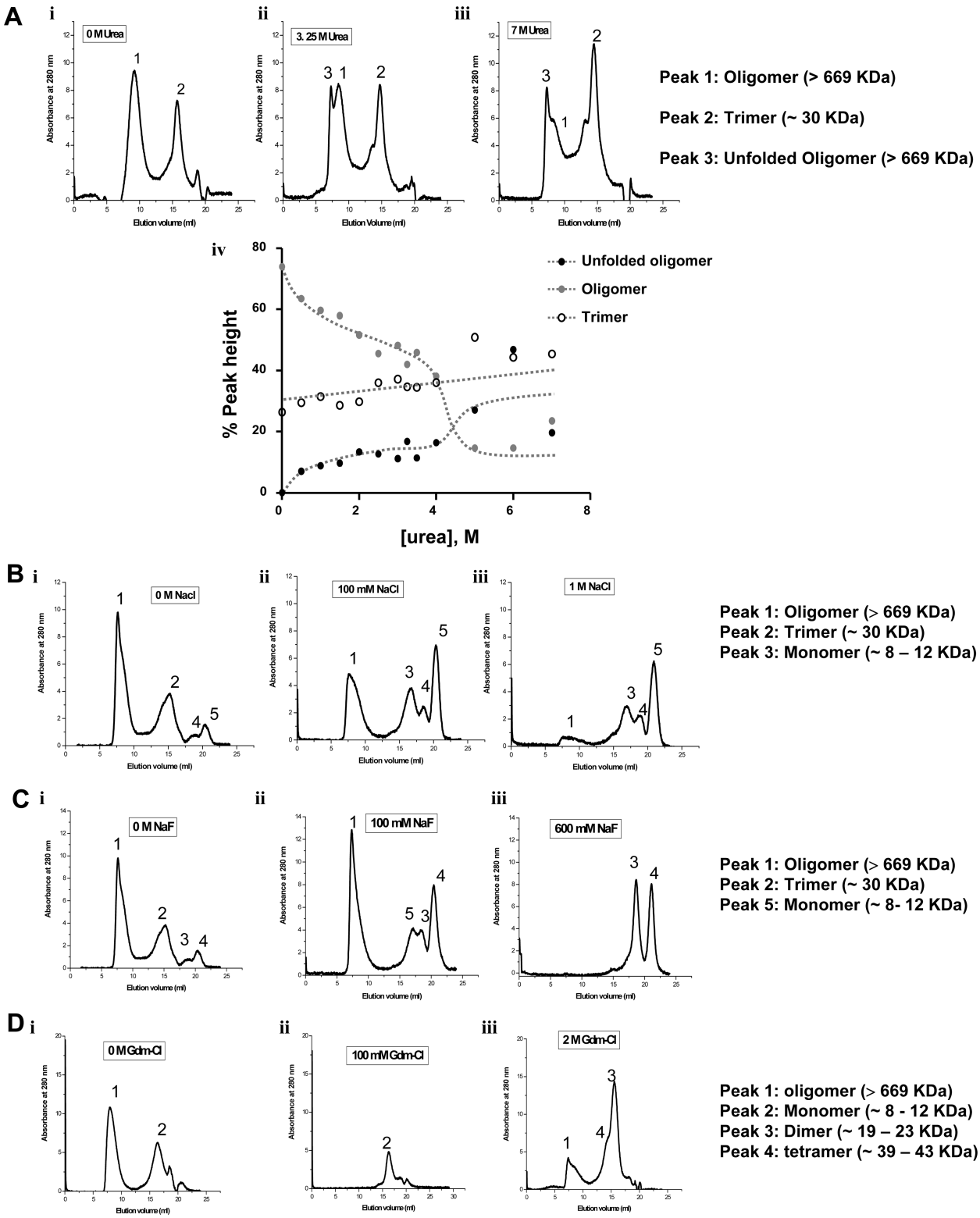

FIGURE 2: Human resistin requires ionic forces for trimerization and ionic and hydrophobic forces for oligomerization. Gel filtration analysis of human resistin at different concentrations of urea, NaCl, NaF, and GdmCl. Human resistin protein was equilibrated with different concentrations of urea, NaCl, NaF, and GdmCl, and the quaternary structure of the protein was analyzed by gel filtration using a Superdex 200 column. The various concentrations used are indicated in the respective chromatograms. Molecular mass of the elution peaks was calculated from the calibration curve obtained by the gel filtration analyses of standards. The estimated molecular masses corresponding to the various peaks are given in the inset. (A) The elution profile of human resistin in the presence of 0 (i), 3.25 (ii), and 7 M (iii) urea is depicted. A plot of % peak heights against [urea] (iv) shows a gradual decrease in the proportion of oligomer (shaded circle) with a concomitant increase in the denatured form (solid circle). The trimer (open circle) remains more or less constant with increasing [urea]. (B) The elution pattern in the presence of 0 (i), 0.1 (ii), and 1 M (iii) NaCl shows the appearance of monomer with addition of 0.1−1 M NaCl while (C) gel filtration analysis of human resistin at 0 (i), 0.1 (ii), and 0.6 M (iii) NaF reveals a complete loss of monomers and oligomers at 600 mM NaF. (D) Gel filtration pattern of resistin in the presence of 0 (i) 0.1 (ii), and 2 M (iii) GdmCl.

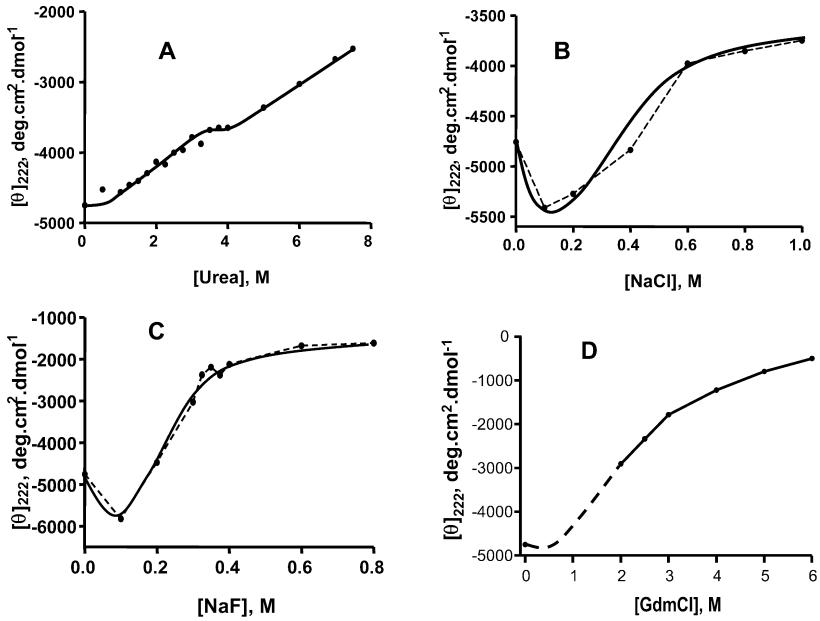

FIGURE 3: GdmCl is able to completely denature human resistin whereas NaCl and urea partially denature human resistin. The far-UV CD spectra of human resistin protein incubated with increasing concentrations of urea (A), NaCl (B), NaF (C), and GdmCl (D) in 50 mM Tris-HCl, pH 8.0, were recorded from 200 to 250 nm. $[\theta]_{222}$ was plotted against concentration of salt denaturants.

(Figure 2A, iv). It could be seen that, with an increase in urea concentration, the oligomer undergoes denaturation while the trimer remains more or less constant. The stability of the trimer in the presence of 8 M urea suggests that hydrophobic interactions may have very little role, if any, in maintaining the structural integrity, and since the oligomer undergoes partial denaturation, it can be concluded that hydrophobic interactions may be required for oligomerization of human resistin.

Although the secondary structure of human resistin in the presence of 8 M urea was not altered (*22*), a decrease in the oligomer was seen as a function of urea in gel filtration. We therefore studied the effect of urea on far-UV CD spectra of human resistin to gain an insight into the secondary structural stability of the protein. The far-UV CD spectrum of human resistin was recorded in the presence of varying concentrations of urea (Figure 3A). Interestingly, a considerable change in the mean residue ellipticity at 222 nm could be seen, indicating that resistin undergoes some spatial rearrangement due to unfolding, resulting in loss of helical content in the presence of urea. It should be noted that the urea-induced denaturation of protein is reversible. An interesting aspect observed here is that human resistin undergoes a two-stage denaturation: the first stage of denaturation resembles a single-step model and proceeds up to 4 M urea beyond which there is a sharp decrease in the helicity. This correlates well with the decrease in the oligomer observed in gel filtration in the presence of urea (Supporting Information Figure 1A, h−j). It is interesting to note that the mean residue ellipticity at 222 nm, $[\theta]_{222}$, in the presence of even 7.5 M urea is much more than that characteristic of a random coil, indicating that human resistin does not undergo complete denaturation even in the presence of 7.5 M urea. The observed $[\theta]_{222}$ in the presence of 7.5 M urea could be contributed by the trimer whose fraction is much less compared to the oligomer (as determined through gel filtration, Figure 2A, iv). The overall secondary structure of resistin remained unaltered except for the loss of helicity as a function of urea concentration pointing to a two-state model of unfolding of human resistin.

Ionic Interactions Play a Major Role in the Stabilization of Oligomeric as Well as Trimeric Forms of Human Resistin. The role of ionic interactions in maintaining human resistin structure was studied using varying concentrations of NaCl and NaF. Human resistin existed mainly as a trimer and an oligomer in 50 mM Tris-HCl, pH 8.0, in the absence of salt (Figure 2B, i). Purification of the trimer from the oligomer could not be achieved since both forms reorganized to form a mixture of oligomer and trimer after purification of individual peaks (data not shown). This indicated that recombinant human resistin has an inherent ability to oligomerize depending on the protein concentration (as determined in Figure 1). Therefore, the importance of ionic interaction was studied in the mixture of oligomer and trimer.

Gel filtration chromatography of human resistin was performed in 50 mM Tris-HCl, pH 8.0, in the presence of different concentrations of NaCl in order to assess the importance of ionic interactions in stabilizing the oligomeric and trimeric forms. Human resistin existed as a combination of oligomeric (>669 kDa) and trimeric (∼30 kDa) (Figure 2B, i, peaks 1 and 2, respectively) species in 50 mM Tris-HCl, pH 8.0. In addition, two peaks that were retarded in the gel filtration column were also observed which may represent the peaks of unexplained origins as they are below the column resolution limit (Figure 2B, peaks 4 and 5). It is evident from Figure 2B, ii, that with the addition of 100 mM NaCl there is disappearance of the trimeric peak and appearance of a peak (∼8−12 kDa) corresponding to monomeric resistin of 11.3 kDa (Figure 2B, ii and iii, peak 3). Addition of NaCl up to 1 M led to a gradual decrease in the proportion of the oligomer but without any change in the monomeric form (Supporting Information Figure 1B). Protein precipitation also increased as a function of salt concentration.

The far-UV CD spectra of recombinant human resistin were recorded in the presence of different concentrations of

NaCl. The secondary structure of human resistin was found to increase up to 0.1 M NaCl, above which it decreased in a concentration-dependent manner in the presence of NaCl (Figure 3B). A plot of $[\theta]_{222}$ of resistin versus [NaCl] showed that the helical content of human resistin was slightly increased in the presence of low concentrations of NaCl (up to 0.1 M). Further increase in NaCl to 1 M resulted in decrease in the ellipticity, indicating loss of helical content of the protein which corresponded to protein denaturation and hence its precipitation.

Surprisingly, although the $[\theta]_{222}$ of human resistin in the presence of 1 M NaCl was greatly reduced as compared to that of the native protein, complete denaturation was not observed. This, as well as the data from gel filtration of human resistin, in the presence of NaCl (Figure 2B) showed that the monomeric form is very stable and resistant to salt-induced denaturation. Further, precipitation of the oligomeric form could be a result of protein denaturation or due to formation of higher order aggregates.

It is also important to note that the helical content of human resistin in the presence of 0.1 M NaCl is greater than that in the absence of NaCl (Figure 3B). Further, the trimeric form disappears, and a peak corresponding to a monomer appears in the presence of 0.1 M NaCl (Figure 2B, i and ii). These observations suggest that the monomeric form of human resistin has a greater helical content than that of the trimer. In other words, monomer undergoes a change in conformation and associates *via* ionic interactions to form a trimer.

Fluorescence emission spectra of human resistin recorded in the presence of increasing concentrations of NaCl showed a trend similar to that observed in the far-UV CD spectra. A decrease in the fluorescence quantum yield was observed with increase in [NaCl] (Figure 4A). Concomitantly, protein precipitation in the samples was also observed. There was a slight initial decrease in fluorescence intensity from 0 to 0.2 M NaCl followed by a drastic decrease in the presence of 0.4 M NaCl. NaCl concentrations greater than 0.4 M (up to 1 M) led to a gradual decrease in fluorescence intensity. No shift in the fluorescence emission maxima was observed even in the presence of 1 M NaCl (Figure 4A), indicating that the tryptophan environment is not perturbed.

Since complete unfolding of human resistin could not be accomplished even in the presence of 1 M NaCl, the effect of NaF, a salt with higher charge screening effect, on the denaturation of the protein was examined. Gel filtration analysis of human resistin in the absence of NaCl or NaF showed that it existed predominantly as a mixture of oligomeric and trimeric forms (Figure 2B, i, and 2C, i, peaks 1 and 2, respectively). Addition of 100 mM NaF to resistin resulted in the complete loss of the trimeric species (Figure 2C, ii), and a new peak corresponding to the monomeric protein appeared (Figure 2C, ii, peak 5). Smaller peaks (<10 kDa) below the resolution limit of the column are of unknown origins (Figure 2C, peaks 3 and 4). In addition, increased protein precipitation was observed with the increase in NaF concentration, and this could be a reflection of the decrease in the proportion of the oligomer. There was a complete loss of the oligomer (peak 1) in the presence of NaF concentration greater than 300 mM (Supporting Information Figure 1C). However, the monomeric form persisted until 400 mM NaF (Supporting Information Figure 1C).

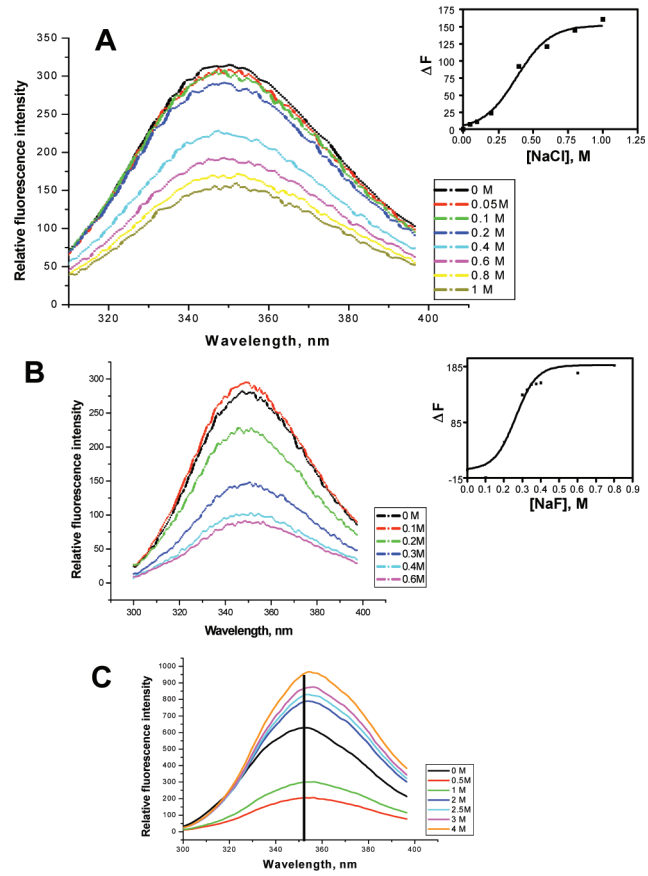

FIGURE 4: Fluorescence emmision spectra of human resistin at different concentrations of NaCl (A), NaF (B), and (C) GdmCl. The fluorescence emission spectra of resistin in the presence of varying concentrations of NaCl, NaF, and GdmCl were recorded from 300 to 400 nm by exciting the protein at 280 nm. Different NaCl, NaF, and GdmCl concentrations are shown with different colors. Insets in panels A and B represent a plot of the change in fluorescence intensity versus [NaCl] and [NaF], respectively.

Interestingly, NaF at 600 mM completely denatured human resistin as evident from the total loss of oligomeric as well as monomeric forms (Figure 2C, iii).

To study the secondary structure of resistin in the presence of NaF, the far-UV CD spectrum of human resistin was recorded in the presence of increasing concentrations of NaF. The results obtained were similar to that of NaCl with the effect being more drastic as expected (Figure 3C). A plot of mean residue ellipticity at 222 nm versus [NaF] revealed that the helical content of the protein is slightly increased at a concentration up to 0.1 M NaF (as was observed in the presence of 0.1 M NaCl, Figure 3B) followed by a one-step denaturation (Figure 3C). The overall mean residue ellipticity of human resistin decreased in the presence of increasing concentrations of NaF. Complete loss of helicity ($[\theta]_{222} > -2000$) was observed at [NaF] of 0.4 M with no significant change in ellipticity at higher concentrations of NaF pointing to complete denaturation of human resistin at 0.4 M NaF.

The intrinsic fluorescence of human resistin was found to decrease in the presence of increasing concentrations of NaF. The profile of fluorescence intensity decrease was similar to that observed with NaCl. A drastic decrease in the fluorescence intensity with concomitant protein precipitation was detected in the presence of 0.2, 0.3, and 0.4 M NaF (Figure 4B). However, no significant change in the fluorescence emission was observed at concentrations more than 0.4 M

NaF, indicating complete exposure of tryptophan residues to polar environment.

Incubation of human resistin with increasing concentrations of NaCl led to partial denaturation of the protein and a decrease in secondary as well as tertiary structure. A similar, but more pronounced, effect was seen in the presence of NaF. Since NaF has a greater charge shielding effect compared to NaCl, the effect of 1 M NaCl on human resistin was similar to that seen in the presence of 0.3 M NaF. Gel filtration analysis of human resistin in the presence of NaCl and NaF suggests that the human resistin structure is mainly stabilized by ionic interactions.

Human Resistin Oligomer and Monomer Are Stabilized by both Hydrophobic and Ionic Interactions. Urea-induced denaturation of resistin showed the importance of hydrophobic interactions in conferring conformational stability to the oligomer (Figure 2A, iv). These results also suggested that hydrophobic interactions do not have much contribution in maintaining the trimeric form. Furthermore, results from gel filtration in the presence of different concentrations of salts indicate that human resistin oligomer as well as trimer is stabilized by ionic interactions (Figure 2B,C). These observations taken together could explain to some extent why urea, which disrupts hydrophobic interactions, was not able to denature the trimeric form of resistin. Further, the monomeric form of human resistin was stable even in the presence of 1 M NaCl (Figure 2B, iii), and denaturation of the monomeric form could be achieved only in the presence of 600 mM NaF (Figure 2C, iii). This indicates that very high ionic strength is required to disrupt the structure of human resistin monomer. In order to assess the importance of hydrophobic and ionic interactions in stabilizing the monomeric structure of human resistin, we studied the effect of GdmCl-induced denaturation.

Human resistin as determined earlier exists as an oligomer and a monomer in the presence of 100 mM NaCl (Figure 2B, ii). To analyze the role of GdmCl on the stabilization of resistin structure, gel filtration chromatography was performed in the presence of different concentrations of GdmCl. Resistin was incubated with varying concentrations of GdmCl. The samples were then analyzed by gel filtration on a Superdex 200 column (Figure 2D, i−iii). A complete loss of the oligomeric species (Figure 2D, ii, peak 1) in the presence of very low concentrations of GdmCl (0.1 M) was evident with manifestation of protein precipitation. The monomeric form, however (Figure 2D, peak 2), was stable up to 1 M (Supporting Information Figure 1D). As the concentration of GdmCl was increased to 2 M, salting-in of the protein precipitate was observed. Gel filtration in the presence of 2 M GdmCl and above revealed the formation of soluble multimers corresponding to several oligomeric forms (Supporting Information Figure 1D).

Since human resistin existed in different multimeric forms at 2 M and higher concentrations of GdmCl, it was important to assess the secondary structure of resistin in the presence of GdmCl. Hence, the far-UV CD spectra of human resistin at various GdmCl concentrations were recorded to assess the secondary structure of human resistin monomer as well as the soluble higher molecular weight multimers. Intriguingly, it was observed that GdmCl-induced denaturation of human resistin is very unique. At 2 M GdmCl, the protein showed a mean residue ellipticity that was lower than that of the native protein, indicating the denaturation of human resistin. As the concentration of GdmCl is increased from 2 to 6 M, there is a gradual decrease in the ellipticity of the protein indicating further denaturation of the protein (Figure 3D). This indicates that the different multimers observed in the presence of 2 M (Figure 2D, iii) and higher concentrations of GdmCl (Supporting Information Figure 1D) are nonspecific soluble aggregates of denatured human resistin species.

Furthermore, the intrinsic fluorescence emission of human resistin was recorded in the presence of increasing concentrations of GdmCl in 50 mM Tris-HCl (pH 8.0). A drastic decrease in the fluorescence intensity from 0 to 1 M GdmCl was observed. Increasing the concentration of GdmCl further led to an increase in fluorescence quantum yield, and this could possibly be attributed to solvent interactions (Figure 4C). Interestingly, a red shift in the fluorescence emission maxima of human resistin was observed in the presence of 2 M and higher concentrations of GdmCl, indicating the shift of tryptophan environment to a more polar region due to structural perturbation in human resistin protein. This observation further strengthens the conclusion that the multimers seen at higher concentrations of GdmCl are nonstructured oligomers.

These results clearly show that the multimeric forms of human resistin observed in the presence of 2 M and higher GdmCl are soluble, non-native, denatured species characterized by loss of secondary structure and altered tertiary structure. This species then associates nonspecifically to form high molecular size intermediates observed in gel filtration but possesses no definite secondary structure represented by a decrease in helical content and a red shift in the fluorescence maxima at GdmCl concentrations higher than 2 M. The GdmCl-induced denaturation of human resistin monomer, therefore, suggests that both hydrophobic as well as ionic interactions are involved in the stabilization of the monomer.

Human Resistin Is Stable to Thermal Denaturation. To evaluate the effect of temperature on its secondary structure, far-UV CD spectra of human resistin at increasing temperatures were recorded. With an increase in the temperature to 50 °C a slight increase in the mean residue ellipticity of resistin protein at 222 nm was observed. A further increase in temperature of the protein caused a minor decrease in the helicity. But, it is interesting to note that even at 90 °C the ellipticity at 222 nm is similar to that when the protein is at 4 °C. Loss of secondary structure indicated by decrease in ellipticity was observed only at 105 °C although complete conversion to a random coil was not evident (Figure 5A). This indicated that the secondary structure of human resistin is highly stable to thermal denaturation.

The intrinsic fluorescence of human resistin was recorded at different temperatures to assess the stability of the tertiary structure. Increased quenching of fluorescence intensity was observed as the temperature of the protein was increased from 4 to 100 °C (Figure 5B), indicating greater exposure of the tryptophan residues to the polar solvent. A plot of fractional quenching against temperature (Figure 5B, inset) pointed to a slight perturbation in the environment of tryptophan residues. To be noted here is that although the tertiary structure of human resistin is slightly altered as the temperature increases (Figure 5B, inset), the secondary structure is largely intact with no major loss of helicity (Figure 5A). The

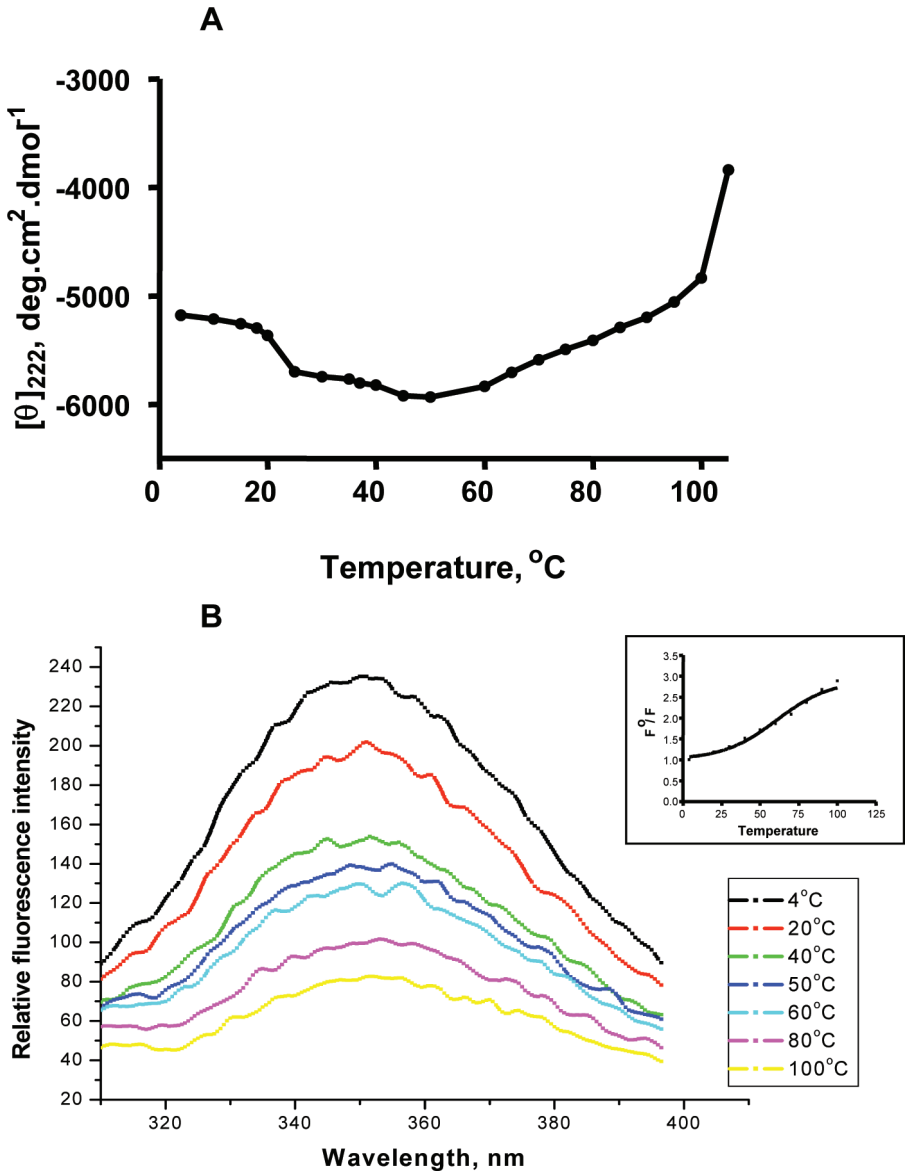

FIGURE 5: Human resistin is stable to thermal denaturation. (A) The far-UV circular dichroism spectra of human resistin at different temperatures. These were recorded by varying the temperature from 4 to 105 °C using Peltier. (B) The temperature dependence of fluorescence emission spectra of human resistin. The intrinsic protein fluorescence of human resistin was recorded at varying temperatures. The inset shows a plot of the fractional fluorescence quenching as a function of temperature.

slight changes in the tertiary structure of human resistin as a function of temperature could be important in functional activity of the protein. This is in agreement with our earlier report that heat-inactivated human resistin could not stimulate the expression of TNF-α in macrophages (*15*).

These results clearly demonstrate that the secondary and tertiary structure of human resistin is largely stable upon thermal denaturation. Only partial loss of secondary structure was evident at a temperature as high as 105 °C. This could be due to strong hydrophobic and ionic interactions involved in maintaining the integrity of human resistin.

The relative proportion of the oligomeric and trimeric species varied with protein concentration, and the change in secondary structure from α-helical to *β*-sheet is associated with an increase in the oligomer proportion. The trimer is stabilized by weak ionic interactions as there is a dissociation of the trimer to the monomer at very low salt concentrations (Figure 2B,C). The trimers associate to form the oligomer, and noncovalent interactions (both ionic and hydrophobic) seem to play a major role in maintaining the structural stability of the oligomer as was observed by the denaturation of the oligomer in the presence of urea, NaCl, NaF, and GdmCl. The ionic interactions in the oligomer are more recalcitrant to NaCl-induced denaturation as compared to the trimer dissociation. Relatively high concentrations of NaF (Figure 2C) are required to denature the monomer. Further, the monomer undergoes conformational changes to associate as a trimer with the monomer having a greater helical content than the trimer.

It is likely that the human resistin monomer is stabilized by intramolecular disulfide linkages possibly formed between the 10 cysteine residues at the C-terminal end. Three human resistin monomers associate through ionic interactions to form the trimer, which further associates through noncovalent and probably intermolecular disulfide linkages to form a higher order oligomer.

The formation of high molecular weight oligomer by human resistin was reported earlier (*23*). But our observation

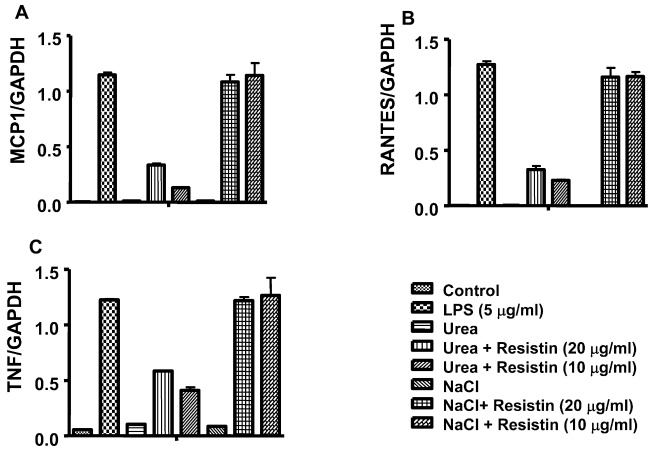

FIGURE 6: Human resistin oligomers are biologically more active. Human resistin was incubated with 5 M urea or 100 mM NaCl at room temperature overnight to obtain predominantly the trimeric or oligomeric forms, respectively. RAW 264.7 cells were stimulated with resistin pretreated with 5 M urea or 100 mM NaCl. The expression of proinflammatory cytokines (A) MCP-1, (B) RANTES, and (C) TNF-α was estimated using quantitative real-time PCR. The relative amount of each cytokine was normalized to the respective GAPDH levels in each sample. LPS (5 μg/mL) was used as a positive control.

of trimer formation in human resistin, similar to mouse resistin (*21*), is novel. The trimers in human resistin associate to form an oligomer with a marked absence of intermediate molecular weight forms, while the mouse resistin protein was shown to form hexamer similar to adiponectin (*28*). These results, while throwing new light on the biophysical attributes of resistin protein, point to likely additional physiological role for this protein. The observation of the concentration-dependent conformation changes from predominantly α-helical of human resistin to β-sheet form is reminiscent of the pathophysiology of neurological disorders such as Alzheimer's and is therefore suggestive of a similar role of resistin in human disorders where the pathophysiology is a function of protein structural abnormality.

Human Resistin Oligomers Are Biologically More Active. Human resistin has an inherent property to oligomerize, thus making it impossible to study the physiological attributes of the individual assembly state. Resistin exists mostly as a trimer in the presence of 5 M urea which is toxic to the cells at such a high concentration whereas 100 mM NaCl is not toxic where resistin exists mostly as oligomer. Therefore, to evaluate the biological significance of these resistin multimers, we first incubated the recombinant human resistin in conditions that would irreversibly generate predominantly either trimer or oligomer, and these were used to stimulate RAW 264.7 cells. Cells were stimulated for 4 h with recombinant human resistin treated with either 5 M urea or 100 mM NaCl, the conditions where human resistin is assumed to exist predominantly as a trimer and a mixture of oligomer and monomer, respectively (Figure 2A, iv, and 2B, ii). Total RNA was isolated, and the expression of proinflammatory markers was determined by quantitative real-time PCR (Figure 6). We observed that both urea- and NaCl-treated resistin caused an increase in the expression of proinflammatory cytokines such as TNF-α, MCP-1 (CCL2), and RANTES (CCL5) differently in these cells. The 4 h treatment of urea alone or NaCl alone did not have a significant effect on the levels of these cytokines when compared to unstimulated control (Figure 6). However, a dose-dependent effect of these multimers can be observed in either NaCl- or urea-treated resistin. Interestingly, in urea-treated resistin, where the trimeric form prevails, the induction of these proinflammatory cytokines was not as pronounced as that observed in NaCl-treated resistin where the oligomers make the bulk of the resistin protein. That resistin function is dependent on the trimeric state of the species has already been shown for mouse resistin (*21*). This was more than evident from physiological clamp studies where a cysteine mutant form of mouse resistin was unable to form intertrimer disulfide bonds and consequently showed difference in bioactivity. Although the crystal structure of human resistin is not known, the cysteine residues are highly conserved. Human resistin is known to be expressed in the macrophages (*10*). Although its role in insulin resistance in humans is not free from controversy, what is certainly known is its action as a proinflammatory molecule. Macrophages are a critical component of immune machinery, both innate and adaptive. In response to an stimulus caused by injury or a pathogen the macrophage releases the so-called immune artillery comprising of proinflammatory cytokines which include TNF-α and IL-1β, as well as cytotoxic and inflammatory molecules like NO (nitric oxide) and ROS (reactive oxygen species). A fine balance between pro- and antiinflammatory molecules is perfectly achieved by a healthy macrophage so as to be able to fight an injury without causing any damage to normal cells. The present observation that resistin possesses a highly stable trimeric structure might point to its importance in maintaining the redox balance with the three free cysteine residues acting as a sink (*29*). It therefore becomes very tempting for us to predict that human resistin, which differs from the mouse homologue in terms of genomic organization, structure, and function (*9*), by virtue of its proinflammatory and antioxidant nature may also have a novel role in eliciting a successful response against an invading pathogen. This view is supported by our observation that human resistin is a proinflammatory molecule that upregulates the expression as well as secretion of TNF-α and IL-12 in both mouse and human macrophages (*15*).

The observation that resistin can exist in different structural forms in the presence of different chaotrophs tempted us to speculate on the existence of a structural switch that may regulate its function differently *in vivo*. Although these structural isomers were observed under extreme conditions *in vitro*, *in vivo* they could be transformed dynamically depending on concentration or the presence of hitherto unknown switching agent(s). We already know that human resistin can undergo a concentration-dependent dynamic structural transition (*22*). However, pinpointing the biological roles of these transitional structures appears to be difficult, partly because of their transitional nature. Our observations, therefore, indicate that unlike mouse resistin where trimers show more potent biological effect, the oligomeric form of human resistin is physiologically more active. Also, unlike in mouse where resistin had an effect on hepatic insulin sensitivity, the human resistin oligomers have more potent activity in inducing proinflammatory response. This possibly explains why human resistin, unlike mouse resistin, has not yet been implicated to have a direct role in insulin resistance. As shown for mouse (*21*), human resistin also circulates in different assembly states in human serum (*23*). Therefore,

while the specific function of purified individual assembly states of human resistin cannot be determined *in vitro*, our results certainly provide evidence of possibly distinct roles of these resistin isomers that are generated *in vivo*. It will be tempting to speculate that human resistin with structural similarities to proprotein convertase subtilisin/kexin type 9 (PCSK9) may belong to the same class of proteins whose functions are regulated by a dynamic structure (*30*).

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SUPPORTING INFORMATION AVAILABLE

Gel filtration chromatograms of human resistin in the presence of different concentrations of urea (Figure SlA), NaCl (Figure S1B), NaF (Figure S1C), and GdmCl (Figure S1D). This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Steppan, C. M., Bailey, S. T., Bhat, S., Brown, E. J., Banerjee, R. R., Wright, C. M., Patel, H. R., Ahima, R. S., and Lazar, M. A. (2001) The hormone resistin links obesity to diabetes. *Nature 409*, 307–312.
2. Flier, J. S. (2001) Diabetes. The missing link with obesity? *Nature 409*, 292–293.
3. Ehtesham, N. Z. (2001) Molecular link between diabetes and obesity: The resistin story. *Curr. Sci. 80*, 1369–1371.
4. Way, J. M., Gorgun, C. Z., Tong, Q., Uysal, K. T., Brown, K. K., Harrington, W. W., Jr., Willson, T. M., Kliewer, S. A., and Hotamisligil, G. S. (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.
5. Nagaev, I., and Smith, U. (2001) Insulin resistance and type 2 diabetes are not related to resistin expression in human fat cells or skeletal muscle. *Biochem. Biophys. Res. Commun. 285*, 561–564.
6. Janke, J., Engeli, S., Gorzelniak, K., Luft, F. C., and Sharma, A. M. (2002) Resistin gene expression in human adipocytes is not related to insulin resistance. *Obes. Res. 10*, 1–5.
7. McTernan, C. L., McTernan, P. G., Harte, A. L., Levick, P. L., Barnett, A. H., and Kumar, S. (2002) Resistin, central obesity, and type 2 diabetes. *Lancet 359*, 46–47.
8. Azuma, K., Katsukawa, F., Oguchi, S., Murata, M., Yamazaki, H., Shimada, A., and Saruta, T. (2003) Correlation between serum resistin level and adiposity in obese individuals. *Obes. Res. 11*, 997–1001.
9. Ghosh, S., Singh, A. K., Aruna, B., Mukhopadhyay, S., and Ehtesham, N. Z. (2003) The genomic organization of mouse resistin reveals major differences from the human resistin: functional implications. *Gene 305*, 27–34.
10. Patel, L., Buckels, A. C., Kinghorn, I. J., Murdock, P. R., Holbrook, J. D., Plumpton, C., Macphee, C. H., and Smith, S. A. (2003) Resistin is expressed in human macrophages and directly regulated by PPAR gamma activators. *Biochem. Biophys. Res. Commun. 300*, 472–476.
11. Kaser, S., Kaser, A., Sandhofer, A., Ebenbichler, C. F., Tilg, H., and Patsch, J. R. (2003) Resistin messenger-RNA expression is increased by proinflammatory cytokines in vitro. *Biochem. Biophys. Res. Commun. 309*, 286–290.
12. Lehrke, M., Reilly, M. P., Millington, S. C., Iqbal, N., Rader, D. J., and Lazar, M. A. (2004) An inflammatory cascade leading to hyperresistinemia in humans. *PLoS Med. 1*, e45.
13. Bokarewa, M., Nagaev, I., Dahlberg, L., Smith, U., and Tarkowski, A. (2005) Resistin, an adipokine with potent proinflammatory properties. *J. Immunol. 174*, 5789–5795.
14. Reilly, M. P., Lehrke, M., Wolfe, M. L., Rohatgi, A., Lazar, M. A., and Rader, D. J. (2005) Resistin is an inflammatory marker of atherosclerosis in humans. *Circulation 111*, 932–939.
15. Silswal, N., Singh, A. K., Aruna, B., Mukhopadhyay, S., Ghosh, S., and Ehtesham, N. Z. (2005) Human resistin stimulates the pro-inflammatory cytokines TNF-α and IL-12 in macrophages by NF-κB dependent pathway. *Biochem. Biophys. Res. Commun. 334*, 1092–1101.
16. Pang, S. S., and Le, Y. Y. (2006) Role of resistin in inflammation and inflammation-related diseases. *Cell. Mol. Immunol. 3*, 29–34.
17. Osawa, H., Onuma, H., Murakami, A., Ochi, M., Nishimiya, T., Kato, K., Shimizu, I., Fujii, Y., Ohashi, J., and Makino, H. (2002) Systematic search for single nucleotide polymorphisms in the resistin gene: the absence of evidence for the association of three identified single nucleotide polymorphisms with Japanese type 2 diabetes. *Diabetes 51*, 863–866.
18. Smith, S. R., Bai, F., Charbonneau, C., Janderova, L., and Argyropoulos, G. (2003) A promoter genotype and oxidative stress potentially link resistin to human insulin resistance. *Diabetes 52*, 1611–1618.
19. Ochi, M., Osawa, H., Onuma, H., Murakami, A., Nishimiya, T., Shimada, F., Kato, K., Shimizu, I., Shishino, K., Murase, M., Fujii, Y., Ohashi, J., and Makino, H. (2003) The absence of evidence for major effects of the frequent SNP + 299G → A in the resistin gene on susceptibility to insulin resistance syndrome associated with Japanese type 2 diabetes. *Diabetes Res. Clin. Pract. 61*, 191–198.
20. Osawa, H., Yamada, K., Onuma, H., Murakami, A., Ochi, M., Kawata, H., Nishimiya, T., Niiya, T., Shimizu, I., Nishida, W., Hashiramoto, M., Kanatsuka, A., Fujii, Y., Ohashi, J., and Makino, H. (2004) The G/G genotype of a resistin single-nucleotide polymorphism at−420 increases type 2 diabetes mellitus susceptibility by inducing promoter activity through specific binding of Sp1/3. *Am. J. Hum. Genet. 75*, 678–686.
21. Patel, S. D., Rajala, M. W., Rossetti, L., Scherer, P. E., and Shapiro, L. (2004) Disulfide-dependent multimeric assembly of resistin family hormones. *Science 304*, 1154–1158.
22. Aruna, B., Ghosh, S., Singh, A, K., Mande, S. C., Srinivas, V., Chauhan, R., and Ehtesham, N. Z. (2003) Human recombinant resistin protein displays a tendency to aggregate by forming intermolecular disulfide linkages. *Biochemistry 42*, 10554–10559.
23. Gerber, M., Boettner, A., Seidel, B., Lammert, A., Bar, J., Schuster, E., Thiery, J., Kiess, W., and Kratzsch, J. (2005) Serum resistin levels of obese and lean children and adolescents: biochemical analysis and clinical relevance. *J. Clin. Endocrinol. Metab. 90*, 4503–4509.
24. Raghu, P., Ghosh, S., Kanukolanu, S., Haseeb, A., Aruna, B., and Ehtesham, N. Z. (2004) Dimerization of human recombinant resistin involves covalent and noncovalent interactions. *Biochem. Biophys. Res. Commun. 313*, 642–646.
25. Pace, C. N., Vajdos, F., Fee, L., Grimsley, G., and Gray, T. (1995) How to measure and predict the molar absorption coefficient of a protein. *Protein Sci. 4*, 2411–2423.
26. Schmid, F. X. (1990) Spectral methods of characterizing protein conformation and conformational changes, in *Protein Structure: A practical approach* (Creighton, T. E., Ed.) IRL Press, Oxford University Press, New York.
27. Pan, K. M., Baldwin, M., Nguyen, J., Gasset, M., Serban, A., Groth, D., Mehlhorn, I., Huang, Z., Fletterick, R. J., Cohen, F. E., and Prusiner, S. B. (1993) Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion protein. *Proc. Natl. Acad. Sci. U.S.A. 90*, 10962–10966.
28. Pajvani, U. B., Du, X., Combs, T. P., Berg, A. H., Rajala, M. W., Schulthess, T., Engel, J., Brownlee, M., and Scherer, P. E. (2003) Structure-function studies of the adipocyte-secreted hormone Acrp30/adiponectin: Implications for metabolic regulation and bioactivity. *J. Biol. Chem. 278*, 9073–9085.
29. Sah, N. K., Taneja, T. K., Pathak, N., Begum, R., Athar, M., and Hasnain, S. E. (1999) The baculovirus antiapoptotic p35 gene also functions via an oxidant-dependent pathway. *Proc. Natl. Acad. Sci. U.S.A. 96*, 4838–4843.
30. Hampton, E. N., Knuth, M. W., Li, J., Harris, J. L., Lesley, S. A., and Spraggon, G. (2007) The self-inhibited structure of full-length PCSK9 at 1.9 Å reveals structural homology with resistin within the C-terminal domain. *Proc. Natl. Acad. Sci. U.S.A. 104*, 14604–14609.

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