Adenylyl Cyclase-Associated Protein 1 Is a Receptor for Human Resistin and Mediates Inflammatory Actions of Human Monocytes

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SUMMARY

Human resistin is a cytokine that induces low-grade inflammation by stimulating monocytes. Resistin-mediated chronic inflammation can lead to obesity, atherosclerosis, and other cardiometabolic diseases. Nevertheless, the receptor for human resistin has not been clarified. Here, we identified adenylyl cyclase-associated protein 1 (CAP1) as a functional receptor for human resistin and clarified its intracellular signaling pathway to modulate inflammatory action of monocytes. We found that human resistin directly binds to CAP1 in monocytes and upregulates cyclic AMP (cAMP) concentration, protein kinase A (PKA) activity, and NF-κB-related transcription of inflammatory cytokines. Overexpression of CAP1 in monocytes enhanced the resistin-induced increased activity of the cAMP-dependent signaling. Moreover, CAP1-overexpressed monocytes aggravated adipose tissue inflammation in transgenic mice that express human resistin from their monocytes. In contrast, suppression of CAP1 expression abrogated the resistin-mediated inflammatory activity both in vitro and in vivo. Therefore, CAP1 is the bona fide receptor for resistin leading to inflammation in humans.

INTRODUCTION

Resistin was first identified as a mediator of insulin resistance in obese mice (Steppan et al., 2001). It is secreted from mature adipocytes in rodents, and a few studies have implicated murine resistin in the pathogenesis of obesity-mediated insulin resistance and type 2 diabetes (Li et al., 2009; Nakata et al., 2007; Steppan et al., 2001). However, human resistin is quite different from murine resistin. Human resistin is primarily expressed in and secreted from monocytes (Patel et al., 2003). Moreover, the function of resistin in insulin resistance and obesity remains inconclusive in humans (McTernan et al., 2002; Savage et al., 2001; Utzschneider et al., 2005). Interestingly, human resistin seems to be involved in the recruitment of other immune cells and in the secretion of proinflammatory factors (Bokarewa et al., 2005; Silswal et al., 2005), and increasing evidence links resistin with inflammation and atherogenesis (Burnett et al., 2005; Jung et al., 2006; Reilly et al., 2005). As we reported previously (Cho et al., 2011), resistin directly aggravates atherosclerosis by stimulating monocytes to induce vascular inflammation. In terms of the increasing role for inflammation in metabolic disease, the receptor and its intracellular signaling of resistin in human monocytes can be the therapeutic target of chronic inflammation leading to cardiometabolic disease.

Although the inflammatory functions of human resistin appear to be regulated by activation of the NF-κB transcription factor (Silswal et al., 2005), understanding the biological function of human resistin has been slowed by lack of information about its corresponding receptor and signaling mechanisms. Recent reports have suggested an isoform of decorin (Daquinag et al., 2011), mouse receptor tyrosine kinase-like orphan receptor 1 (ROR1) (Sánchez-Solana et al., 2012), and Toll-like receptor 4 (TLR4) (Tarkowski et al., 2010) as potential receptors for resistin. However, both decorin and ROR1 are only putative receptors for murine resistin, and none of these have been shown to mediate the inflammatory effects of resistin in humans. In addition, there was no evidence of the direct interaction...
between TLR4 and human resistin with a lack of biochemical binding assay.

Here, we report the identification of adenylyl cyclase-associated protein 1 (CAP1) as a protein that directly binds to human resistin and elicits inflammatory effects in cultured human monocytes and in white adipose tissue (WAT) in humanized resistin mice in vivo. The gain- and loss-of-function studies implicate CAP1 as a bona fide functional receptor for human resistin.

RESULTS

Identification of a Human Resistin Binding Protein

Human resistin was conjugated with the Fc region of mouse immunoglobulin (denoted as mFc-hResistin) to detect potential receptor proteins using immunoaffinity methods (Figure 1A). The amino terminus of resistin was selected for conjugation with mFc (Figure 1B) because resistin molecules exist as multimeric complexes of coiled-coil trimers that form tail-to-tail hexamers via disulfide bonding near their amino termini (Patel et al., 2004). We hypothesized that the carboxy-terminal globular region constitutes the receptor binding site of resistin, similar to what has been observed for the adiponectin globular domain (Yamauchi et al., 2003).

The mFc-hResistin fusion was expressed and purified to homogeneity after transfecting mFc-hResistin DNA into human embryonic kidney 293F (HEK293F) cells. The western blot analysis, which was performed in nonreducing condition during purification, indicated that the purified mFc-hResistin forms a multimeric assembly, not a monomer (Figure 1C).

Through western blots using the multimeric mFc-hResistin as a ligand (or a primary antibody) to human monocytic leukemia (THP-1) cell lysate, we were able to identify a protein with a molecular mass of about 55 kDa (Figure 1D). This protein nearly disappeared in the presence of an abundant non-Fc-conjugated recombinant human resistin protein (rhResistin) (Figure 1E). Also, when we used just recombinant Fc as a primary antibody instead of Fc-hResistin, we could not detect any protein (Figure 1F). These findings suggested that THP-1 cells expressed a 55 kDa sized protein capable of specifically binding with resistin, similar to what has been observed for the adiponectin globular domain (Yamauchi et al., 2003).

To identify the 55 kDa molecule, which we believed to be a receptor for human resistin, we incubated the purified mFc-hResistin with THP-1 cell lysates and then repurified mFc-hResistin by immunoprecipitation using mFc-specific beads. As expected, SDS-PAGE chromatography detected a specific band corresponding to a protein of approximately 55 kDa (Figure 1G). Then, we excised the gel band and performed MALDI-TOF (Figure 1H). This analysis identified the binding protein as CAP1 (NCBI accession number Q01518). Figure S1 (available online) depicted the full amino acid sequence of CAP1 in Homo sapiens (Yamauchi et al., 2003).

To address whether resistin binds directly to CAP1, whole-cell extracts of THP-1 cells were immunoprecipitated with anti-hCAP1, immunoblotted with anti-hResistin, and vice versa. We observed coinmunoprecipitation of human resistin and CAP1 in THP-1 (Figure 3A). Also, we performed a far western analysis (Wu et al., 2007) to confirm the interaction between resistin multimer and CAP1 in vitro. Briefly, purified mFc-hResistin was transferred to a nitrocellulose membrane, as in standard western blotting, but in nonreducing condition. The membrane then was blocked and incubated with recombinant human CAP1 (rhCAP1). This approach identified CAP1 on spots in the membrane corresponding to the multimeric assembly of mFc-hResistin (Figure 3B), demonstrating that the resistin multimer and CAP1 interact directly. Further, we performed a bimolecular fluorescence complementation (BiFC) assay, which is based on complementation between two nonfluorescent fragments of the fluorescent protein when they are brought together by interaction between proteins fused to each fragment (Hu et al., 2002). Using this nonimmunoglobulin binding approach, we could visualize the interaction between human resistin and CAP1 in living cells (Figure 3C).

In addition, we performed a classical fluorescence-activated cell sorting (FACS)-based binding assay. First, the purified multimeric human resistin was fluorescently labeled, and then we analyzed the binding of the labeled resistin to THP-1 cells, either overexpressed or lacking CAP1. Resistin-binding cells were
increased in the CAP1-upregulated cell group more than in the control group or CAP1-downregulated cell group (Figure 3D). Moreover, a competitive FACS assay further validated the direct interaction, demonstrating that the binding can be effectively competed with nonlabeled resistin (Figure 3E). As another receptor-binding competition assay, rhCAP1 was dispensed into ELISA plates and incubated with mFc-hResistin, in which binding had been quantitated by fluorescence before and after incubation with excessive unconjugated rhResistin. As shown in Figure 3F, mFc-hResistin competed with rhCAP1 in a dose-responsive manner, and the competitive binding curve showed that recombinant resistin competed with...
mFc-hResistin for CAP1 binding (half maximal effective concentration \( [E_{50}] = 0.784 \mu M \); calculated \( E_{50} \) is likely related to the \( K_d \). To calculate \( E_{50} \), we assumed a one-site receptor-ligand binding model and applied the Cheng-Prusoff equation (Cheng and Prusoff, 1973) using GraphPad Prism.

Finally, we underwent a direct binding assay using rhResistin and rhCAP1 (Figure 3G). The binding curves showed increased response unit (RU) between hResistin and hCAP1 in a dose-dependent manner. The calculated dissociation equilibrium constant (\( K_d \)) from the dissociation curves was 0.078 \( \mu M \). These

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**Figure 2. CAP1 Characterization**

(A) The expression of CAP1 mRNA in various human cell lines and multiple organ tissues of rabbits and humanized resistin mice (transgenic mice with macrophage-specific expression of human resistin) compared to general C57BL/6 mice. RT-PCR of resistin in various tissues indicated robust expression of CAP1 and resistin in peripheral blood mononuclear cells (PBMCs). The error bars represent SEM.

(B) Representative western blotting detecting human CAP1 in the membrane fraction of human monocytic leukemia (THP-1) cells as well as cytosol. Importantly, the exogenously applied resistin elicited a change in CAP1 localization to membrane surface.

(C) Also, the surface flow cytometric analyses (FACS) demonstrated that the number of membrane CAP1-positive cells was increased significantly after the stimulation with Fc-hResistin (\( n = 3, \* p < 0.001 \)). The error bars represent SEM.

(D) Coimmunoprecipitation of human resistin and CAP1 in the membrane fraction as well as in the cytosolic fraction of THP-1 cells.

(E) Colocalization of human resistin and CAP1 as determined by immunofluorescence double staining in THP-1 cells. Scale, 10 \( \mu m \). HUVEC, human umbilical vein endothelial cells; HepG2, human hepatocellular liver carcinoma cells; HDF, human dermal fibroblasts; WAT, white adipose tissue; hCAP1, human CAP1; hResistin, human resistin; Fc-hResistin, mFc-conjugated human resistin fusion protein.
Figure 3. Human Resistin Binds Directly to CAP1

(A) Representative coimmunoprecipitation of human resistin and CAP1.

(B) Representative far western blot of multimeric mFc-hResistin and recombinant human CAP1 (rhCAP1).

(C) Representative bimolecular fluorescence complementation (BiFC) assay visualizing the human resistin-CAP1 interaction in living cells. The highest fluorescence intensity was exhibited when both CAP1 and resistin fused to each fragment (pVC155, pVN173) were expressed (left panel). However, when human CAP1 was expressed alone, detectable fluorescence was not exhibited (middle panel). When SH3- and actin-binding domain deletion mutant was cloned into pVC155 instead of wild-type CAP1 and coexpressed with pVN173-hResistin fusion protein, detectable fluorescence was not exhibited (right panel).

(D) Representative classic background flow cytometry analysis (FACS)-based binding assay. PE-hResistin-binding cells were increased in the CAP1 upregulated cell group more than those in control or CAP1 downregulated cell groups.

(E) Competitive FACS binding assay. FITC-Fc-hResistin was displaced by the nonlabeled rhResistin in a dose-dependent manner (n = 3; *p < 0.001, p < 0.01). The error bars represent SEM.

(F) Competitive ELISA binding assay. Recombinant human resistin (rhResistin) bound to rhCAP1 and was competitively inhibited by mFc-hResistin (n = 3; *p < 0.001). The error bars represent SEM.

(G) Direct binding assay between human resistin and CAP1.
findings provide strong evidence of the direct binding and interaction between hCAP1 and hResistin.

**Signaling Pathway of Resistin via CAP1**

Because CAP1 is known to play a role in adenyl cyclase activation, the finding that resistin binds to CAP1 suggests that resistin might activate adenyl cyclase. As expected, the ELISA showed that resistin significantly increased cAMP levels in THP-1 cells (Figure 4A). Consistent with this, resistin increased the activities of both PKA and NF-κB in THP-1 cells (Figure 4B) and subsequently upregulated the mRNA and protein levels of inflammatory cytokines such as interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), and IL-1β (Figure 4C).

The cellular responses to resistin were greater when this molecule was introduced via adenoviral gene transfection than when cells were directly exposed to recombinant resistin protein (Figure 4B), perhaps reflecting the many cysteine residues in resistin leading to formation of the disulfide-dependent multimeric structure (Aruna et al., 2003; Patel et al., 2004; Raghu et al., 2004) that is active and easily formed by adenoviral transfection but formed in difficulty by treatment with the recombinant protein form. Nonreducing SDS gel analysis of cell lysates transfected with adenovirus-delivered resistin identified the resistin protein in fractions corresponding to molecular masses of approximately 50 kDa and 20 kDa (Figure S2). These likely represented the resistin trimer and dimer forms (Aruna et al., 2008; Gerber et al., 2005).

Since resistin induces cytokine expression via the activation of NF-κB (Silswal et al., 2005), we hypothesized that this activation may be downstream of adenyl cyclase activation. Though unusual, this order would be consistent with reports of crosstalk between cAMP/PKA signaling and proinflammatory NF-κB pathways in macrophages (Peters-Golden, 2009; Wall et al., 2009). Indeed, treatment of THP-1 cells with PKA inhibitors blocked resistin-induced NF-κB activation and cytokine expression (Figure 4C).

To determine whether CAP1 is a functional signaling receptor for human resistin, we manipulated CAP1 expression and evaluated its impact on intracellular signals and proinflammatory actions of resistin in monocytes in vitro. CAP1 levels were reduced using specific small interfering RNAs (siRNAs) in THP-1 cells (Figure 4D). CAP1 knockdown inhibited the ability of resistin to increase cAMP concentration (Figure 4E), PKA, NF-κB activity (Figure 4F), and cytokine production (Figure 4G). Conversely, overexpression of CAP1 by adenoviral transfection (Figure 4H) enhanced the intracellular signals and biological responses of THP-1 cells to resistin, including increased PKA, NF-κB activity (Figure 4I), and cytokine expression (Figure 4J).

**Resistin-Binding Domain of CAP1**

CAP1 is comprised of three major structurally and functionally distinct domains (Figure 5A). The highly conserved carboxy-terminal domain of CAP1 binds to monomeric actin and is necessary for normal cellular morphology. The centrally located proline-rich domain interacts with Src homology 3 (SH3) domains of specific proteins, including the yeast actin-associated protein Abp1p (Freeman et al., 1996; Lilja and Drubin, 1997). Finally, the amino-terminal domain of CAP1 interacts with adenyl cyclase in yeast. In higher eukaryotes, however, the function of the CAP1 N terminus is unknown. The amino termini of CAP1 molecules can interact with each other or with the CAP1 carboxyl terminus, suggesting the possibility of either parallel or antiparallel dimers (Hubberstey and Mottillo, 2002). Notably, either dimer topology could allow the polyproline SH3-interacting domain to bind target proteins.

We cloned three deletion mutants of human CAP1 using lentiviral vectors (Figure 5B): an adenyl cyclase binding domain deletion mutant ([ΔAC binding domain [BD] deletion], a mutant in which both the SH3 binding domain and the actin binding domain were deleted ([ΔSH3Δactin BD deletion), and an actin binding domain deletion mutant ([Δactin BD deletion). Following resistin stimulation, PKA/NF-κB signaling (Figure 5C) and inflammatory cytokine production (Figure 5D) were not affected in the Δactin BD deletion mutant overexpressed cells, whereas they were suppressed in the ΔAC BD deletion and in the ΔSH3Δactin BD deletion mutant overexpressed cells. These data indicate that the actin binding domain of CAP1 is not necessary for the resistin ligand binding and/or receptor signaling, whereas the other two domains are crucial. Further, to identify the exact resistin binding sites on CAP1, we performed in vitro binding assays between each CAP1 mutant and rhResistin. After overexpressing His-tagged CAP1 mutants in 293A cells and treating rhResistin, we immunoprecipitated the whole-cell extracts with His antibodies. Then, samples were western blotted using antibodies against both resistin and His. The band at approximately 12 kDa corresponding to rhResistin was not detectable in the ΔSH3Δactin BD deletion mutant, whereas it was detected in the ΔAC BD deletion mutant and in the Δactin BD deletion (Figure 5E). These observations confirm that human resistin binds to CAP1 via the proline-rich SH3 BD and that the AC domain likely plays a key role in receptor signaling.

Neither X-ray crystallography nor nuclear magnetic resonance spectroscopy data are available to deduce the molecular structure of CAP1. Thus, we predicted the structure of the resistin-binding domain using homology modeling (Fernandez-Fuentes et al., 2007; Sternberg et al., 1999) with the known structure of cytidylate/transferase from Thermus thermophilus HB8 (2PX7), which has 20% sequence identity and 32% sequence homology with the region of CAP1 (Figure S3A). Using Discovery Studio 2.5 (Accelrys), we were able to visualize the predicted structure of the resistin-binding domain and the known structures of the resistin trimer (Patel et al., 2004). Then, to predict the structure of the resistin-CAP1 complex, we virtually analyzed the surface geometry of the complex using a protein-protein docking simulation and the score function analysis. Using the Pairwise Shape Complementarity function-based docking algorithm, ZDOCK (Chen et al., 2003; Chen and Weng, 2003), we inferred several 3D binding structures between the resistin trimer and the resistin-binding domain of CAP1. Then, we evaluated the binding free energy of each complex using the Poisson-Boltzmann surface area method (Zoete et al., 2010). Figure 5F depicts the structure of the resistin trimer-CAP1 binding complex with the lowest binding free energy, which could be the conformation observed in nature. Further, we performed molecular dynamics (MD) simulations for predicting the binding energy between resistin and mutated CAP1. We found that the point mutations in CAP1-SH3 BD abolish interaction between resistin and CAP1-SH3 BD. As shown in Figure 5F (magnified circle), hydrophobic
Figure 4. Resistin Signaling Pathway via CAP1

(A) cAMP concentration was increased with the treatment of a physiologic dose (50 ng/ml) of recombinant human resistin in THP-1 cells. \((n = 3; \*p < 0.001, \#p < 0.01, \#\#p < 0.05)\).

(B and C) Both recombinant protein resistin (rhRe; 50 ng/ml) and gene-transfected resistin (adv-hRe) increased the activities of PKA and NF-κB significantly \((n = 3; \*p < 0.001, \#p < 0.01, \#\#p < 0.05)\). (B) and subsequently induced inflammatory cytokines at the mRNA and protein levels (C). The PKA inhibitor, KT5720, abolished resistin-induced NF-κB activation and inflammatory cytokine expression \((n = 3; \*p < 0.001, \#p < 0.01, \#\#p < 0.05)\).

(D–G) CAP1 levels were reduced using specific small interfering RNAs (siRNAs) in THP-1 cells \((n = 3; \*p < 0.001, \#p < 0.01, \#\#p < 0.05)\).

(H–J) We overexpressed human CAP1 in THP-1 cells using adenovirus (H), which enhanced the intracellular signals (I) and biological effects (J) of resistin in THP-1 cells. The expression data were quantified using real-time qPCR, and the mRNA expression of cytokines was validated by ELISA \((n = 3; \*p < 0.001, \#p < 0.01, \#\#p < 0.05)\). The error bars represent SEM. See also Figure S2.
interactions increase the binding affinity between the side chain of Val27 in CAP1-SH3 BD and Trp82A, Trp82C in the resistin trimer. Also, the hydrogen bonds between Ser28 in CAP1-SH3 BD and Ser64A, Ser60C in the resistin trimer increase the binding affinity. So, we targeted Val27 and Ser28 in CAP1-SH3 BD to induce point mutations and substitute glycine for Val27 and Ser28. After 2 ns of MD simulations, we calculated the difference of binding free energy between the wild-type complex and mutant complex. The wild-type CAP1 is predicted to bind more favorably than its mutant, with ΔG ∼9.0 kcal/mol. It suggests that substituting glycine for Val27 and Ser28 in CAP1-SH3 BD may abolish interaction between resistin and CAP1-SH3 BD.
We previously demonstrated that resistin has chemoattractive effects on monocytes (Cho et al., 2011). Using the same assay of vertical collagen gel invasion (Cho et al., 2011; Hur et al., 2007; Yoon et al., 2005), in the present study we show that adenovirus-mediated CAP1 overexpression significantly enhanced the invasion of human THP-1 cells toward resistin (Figure 6A). In contrast, suppression of CAP1 expression by short hairpin RNAs (shRNAs) abrogated the resistin-mediated infiltration of monocytes (n = 3; p < 0.001, p < 0.01) (Figure 6A). We suppressed human decorin, TLR4, and ROR1 expression in human monocytes (C), and they showed little effect on resistin-induced proinflammatory cytokine production (D) (n = 3; p < 0.001, p < 0.01, p < 0.05; ns, not significant), (E) Also, human decorin, TLR4, and ROR1 did not affect the migration of human monocytes toward human resistin (n = 3; p < 0.01; ns, not significant). The error bars represent SEM. See also Figure S4.

**CAP1 and Migration Activity of Monocytes**

We previously demonstrated that resistin has chemoattractive effects on monocytes (Cho et al., 2011). Using the same assay of vertical collagen gel invasion (Cho et al., 2011; Hur et al., 2007; Yoon et al., 2005), in the present study we show that adenovirus-mediated CAP1 overexpression significantly enhanced the invasion of human THP-1 cells toward resistin (Figure 6A). In contrast, suppression of CAP1 expression by short hairpin RNAs (shRNAs) abrogated the resistin-mediated infiltration of monocytes (Figure 6A). These data indicate that the chemotaxis of macrophages or THP-1 cells to resistin is dependent upon CAP1. Further, for evaluating the role of the actin BD on chemotaxis, we performed the transwell migration assay by comparing wild-type CAP1 with the deletion mutants of CAP1. Interestingly, the migration ability of THP-1 cells was not changed significantly in ΔAC BD deletion mutant group, whereas overexpressing the ΔSH3Δactin BD deletion or Δactin BD deletion CAP1 mutant inhibited the resistin-mediated infiltration of monocytes (Figure 6B). Thus, the actin binding domain of CAP1 might have a pivotal role on the resistin-related migration activity of human monocytes.

In addition, to demonstrate that CAP1 is indeed the bona fide receptor of human resistin, we assessed the role of the three previously reported receptors in the inflammatory actions of monocytes induced by human resistin. After suppressing the expression of human decorin, ROR1, and TLR4 using specific shRNAs (Figure 6C), we found that the expression levels of human decorin, ROR1, and TLR4 in human monocytes had little effect on the production of proinflammatory cytokines that was stimulated by human resistin (Figure 6D). Transwell migration assay also demonstrated that human decorin, ROR1, and TLR4 did not affect the migration of human monocytes toward human resistin (Figure 6E). Only CAP1 had a great effect on the production of proinflammatory cytokines and the infiltration property of human monocytes that were induced by human resistin. When compared to CAP1 expression, moreover, both...
decorin and ROR1 were scarcely expressed in human cells, particularly in human PBMCs or THP-1 (Figures S4A and S4B). Even if resistin was applied exogenously or using viral transfection, the expression levels of decorin and ROR1 were not increased (Figure S4C).

**In Vivo Analysis of Resistin-CAP1 Interaction**

Mice humanized by expressing human resistin in monocytes/macrophage lineage were known to develop an exacerbation of inflammation in the WAT when fed with a high-fat diet (Qatanani et al., 2009). Before starting animal experiments using these transgenic mice (humanized resistin mice), we addressed evidence of the interaction between human resistin and murine CAP1 (Figure S5). Murine CAP1 was known to have 97% homology with human CAP1, although its function was rarely known in both species so far (Hubberstey and Mottillo, 2002). Finally, we evaluated whether alteration of CAP1 expression in the monocytes of humanized resistin mice affects resistin-induced WAT inflammation. The time course of the in vivo experiments is illustrated in Figure 7A.

Following a 1-month period of a high-fat diet to induce WAT inflammation, interestingly, CAP1 expression was elevated in WAT of humanized resistin mice along with human resistin and monocyte chemotactic protein-1 (MCP-1) (Figure 7B). Consequently, humanized resistin mice exhibited an accumulation of CAP1-overexpressed macrophages in the WAT greater than that of control mice (Figure 7C). However, when CAP1-suppressed monocytes were administered into humanized resistin mice, the accumulation of macrophages in WAT decreased significantly (Figure 7C). This effect was concordant with a reduction in several inflammatory markers, including TNF-α (Figure 7D). The identity of the infiltrating macrophages in WAT was confirmed by immunofluorescence staining, which indicated that the infused monocytes migrated to inflamed WAT and that monocytes overexpressing CAP1 infiltrated WAT to an extent much greater than did CAP1-suppressed monocytes (Figure 7E). These findings strongly suggest that CAP1 serves as a receptor for resistin in vivo, while also acting as a key physiological regulator of the resistin-induced inflammatory action of monocytes.

**DISCUSSION**

Here we identified CAP1 as a resistin receptor that mediates the proinflammatory effects of human resistin in vitro and in vivo. The various competitive and direct biochemical binding assays that were lacking in previous receptor studies validated the direct interaction between CAP1 and resistin. Moreover, we have identified the region of CAP1 that binds resistin and provided structural evidence of the plausibility of the resistin-CAP1 interaction.

Resistin is part of a unique family of cysteine-rich peptides that includes resistin-like molecules (RELMs) α and β, raising the question of whether CAP1 might bind to the RELMs. RELMα is not conserved in humans (Yang et al., 2003), but in preliminary studies we found that human resistin-like molecule β (RELMβ) interacts with the same domain of CAP1 that binds resistin (Figure S3B), and CAP1 had an effect on the production of proinflammatory cytokines induced by human RELMβ (Figure S3C).

RELMβ is known to contribute to local immune responses in gut and bronchial epithelial cells (He et al., 2003), and a recent report showed that RELMβ is abundantly expressed in activated macrophages and contributes to atherosclerosis development via lipid accumulation and inflammatory facilitation (Kushiyama et al., 2013). That is similar to the role of resistin in aggravating atherosclerosis by stimulating monocytes, which is demonstrated in our previous study (Cho et al., 2011).

In the present study, we further assessed the role of the three previously reported receptors for resistin: isoform of decorin, ROR1, and TLR4. The gain- and loss-of-function study demonstrated that all of the three putative receptors had little effect on the proinflammatory actions of human resistin in human monocytes. Furthermore, decorin and ROR1 were scarcely expressed in human mononuclear cells, and the expression levels of decorin and ROR1 were not increased, regardless of resistin treatment. Also, the decorin identified as a potential receptor was an extracellular cleavage product of decorin, and their model cell line may not perform the necessary proteolytic cleavage. Taken together, our results support that CAP1 is indeed the bona fide resistin receptor in humans.

With regard to CAP1 localization to the cell membrane, our detection of CAP1 in the membrane fraction of human monocytes using surface FACS as well as western blotting and immunofluorescence staining is consistent with an earlier report that showed that CAP1 is mainly associated with the plasma membrane in human monocytes (Wakeel et al., 2009). Moreover, we demonstrated that human resistin elicited a change in CAP1 localization to the membrane surface. As CAP1 lacks a transmembrane domain, the cell biological mechanism underlying its membrane location is unclear and will require future investigation.

Resistin and receptor CAP1 upregulated intracellular cAMP concentration, PKA activity, and NF-κB-related transcription of many inflammatory cytokines in human monocytes. As a contributor to the CAP1 receptor signaling that precedes inflammation, the cAMP/PKA-dependent signaling pathway might be the key mechanism of the action of resistin, modulating monocytes to lead chronic inflammation. Considering that resistin was produced by activated monocytes and that CAP1 was detected in monocytes both in cytosol and near the plasma membrane, the present study suggests that CAP1 may serve as an autocrine/paracrine receptor or an endogenous receptor for resistin, particularly during inflammatory conditions, so that it can aggravate the inflammatory actions of monocytes more. Therefore, CAP1 could be a target in the treatment of inflammatory cardiometabolic diseases, such as obesity or atherosclerosis.

**EXPERIMENTAL PROCEDURES**

See also Supplemental Experimental Procedures.

**SDS-PAGE Chromatography**

To identify the molecule that binds to mFc-conjugated hResistin in THP-1, which is believed to be a receptor for human resistin, we performed immunoprecipitation (IP) using mFc-conjugated hResistin protein as a primary antibody with mFc-specific beads. Briefly, THP-1 cells were incubated with Human Fc Receptor Binding Inhibitor (Cat. No. 16-9161; eBioscience) for 1 hr to inhibit the nonspecific receptor-mediated binding, and then THP-1
Figure 7. In Vivo Analysis of the Resistin-CAP1 Interaction

(A) The time course of in vivo experiments.

(B) White adipose tissue of humanized resistin mice (CD68hR) fed with a high-fat diet exhibited levels of hResistin, MCP-1, and CAP-1 higher than those of resistin knockout control mice (R+/C0/C0) (n = 9 for each group; *p < 0.001; ns, not significant). The error bars represent SEM.

(C) Immunohistochemical detection of the monocyte/macrophage-specific antigen CD11b in adipose tissue from humanized resistin and control mice. When CD68hR mice were systemically infused with monocytes overexpressing CAP1, macrophages heavily infiltrated into the white adipose tissue. When CAP1-suppressed monocytes were infused instead, macrophages infiltrated the white adipose tissue less heavily (n = 9; yp < 0.01, zp < 0.05). Magnification: upper panels, 200×; lower panels, 400×. The error bars represent SEM.

(D) Consequently, inflammatory cytokines in white adipose tissue were induced more following systemic administration of CAP1-overexpressing monocytes than after administration of CAP1-suppressed monocytes (n = 9; p < 0.01, p < 0.05). The error bars represent SEM.

(E) Representative immunofluorescence staining images indicate that infused monocytes migrated to inflamed white adipose tissue and that CAP1-overexpressing monocytes infiltrated adipose tissue much more heavily than did CAP1-suppressed cells. Scale, 100 μm. See also Figure S5.
cells were washed with ice-cold PBS, and whole-cell lysates were prepared with a lysis buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 1% Triton X-100, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM NaF, and 1 mM Na3VO4 containing protease inhibitor cocktail (Roche). The cell lysates were incubated with mFc-conjugated hResistin or recombinant mouse IgG1 Fc (Cat. No. 10690-MNAH; Sino Biological) as a negative control at 4°C on a rotator overnight. After incubation, the protein complexes were pulled down using CaptureSelect Multi Species Fc matrix (Bio Affinity Company) and microcentrifuged to pellet the protein-mFc matrix. After washing with PBS, the immuno-precipitated sample was resuspended with 2× reducing electrophoresis buffer and boiled at 95°C–100°C for 5 min to denature the protein and separate it from the protein-mFc matrix. The proteins were separated by electrophoresis on 8% polyacrylamide SDS gel, and the gel was stained with Coomassie Brilliant Blue R-250. We found the specific band around the size of 55 kDa only in the lane of THP-1 whole-lyse with mFc-hResistin fusion protein. To identify this unknown molecule, we excised the band from the gel and performed MALDI-TOF.

Bimolecular Fluorescence Complementation Assay
Human CAP1 (both wild-type and ΔSH3Δactin BD deletion mutant) and human resistin were cloned into pVC155 and pVN173, respectively. pVC155 and pVN173 were a kind gift from Professor Sunghoe Chang, from Seoul National University College of Medicine. See the Supplemental Experimental Procedures for more details about lentiviral vector cloning of full-length human CAP1 and three different deletion mutants of human CAP1. After verifying sequences, HEK293A cells were transfected with 500 ng of each corresponding BiFC pair using polyethylenimine (PEI). After transfection for 24 hr, cells were fixed with 2% paraformaldehyde, and fluorescence was imaged with a confocal microscope (LSM710; Zeiss).

Direct Binding Assay: Surface Plasmon Resonance
The protocol of surface plasmon resonance spectroscopy (SPR) in this study of real-time direct binding of hResistin and hCAP1 was performed by using a Biacore X100 (GE Healthcare). The Sensor Chip CM5 with preimmobilized hResistin (200 µg/ml) in one flow cell was first saturated with hResistin protein. To analyze the binding kinetics, various concentrations of hCAP1 diluted in HBS-EP buffer (consisting of 0.01 M HEPES [pH7.4], 0.15 M NaCl, 3 mM EDTA, 0.005% Surfactant P20) were injected onto the sensor chip for 120 s at 30 µl/min, and the response unit (RU) was then recorded. After injection of the analyte was stopped, HBS-EP buffer was poured over the chip for 130 s at 95°C–100°C for 5 min to denature the protein and separate it from the protein-mFc matrix. The proteins were separated by electrophoresis on 8% polyacrylamide SDS gel, and the gel was stained with Coomassie Brilliant Blue R-250. We found the specific band around the size of 55 kDa only in the lane of THP-1 whole-lyse with mFc-hResistin fusion protein. To identify this unknown molecule, we excised the band from the gel and performed MALDI-TOF.

Transwell Migration Assay
Monocyte chemotaxis was measured using a 24-well Micro Transwell Permeable Supports (Corning). Wild-type CAP1—and its three different deletion mutants—overexpressed THP-1 cells, or CAP1, Decorin, TLR4, and ROR1 knockdown THP-1 cells using lentivirus were transferred to the upper chamber of the microchemotaxis chamber. See Supplemental Experimental Procedures for more details about RNAi and cloning of shRNA constructs. As a chemoattractant, recombinant human resistin (50 ng/ml) was added to the lower chamber. The lower and upper chambers were separated by a polycarbonate membrane (5 µm pore size). Transmigration was performed for 6–12 hr at 37°C in a humidified atmosphere with 5% CO2. All cells migrating through the polycarbonate membrane to lower chamber were counted and taken by light microscopy.

In Vivo Analysis of Resistin-CAP1 Interaction
All animal experiments were performed after receiving approval from the Institutional Animal Care and Use Committee (iACUC) of Clinical Research Institute in Seoul National University Hospital, Korea.

Age-matched (9- to 10-week-old) male humanized resistin mice expressing human resistin in their monocyte/macrophage lineages and not expressing murine resistin (Retn−/− CD68hR) were used in this study. Retn−/− littersmates were used as controls (R+/−). All animals were fed a high-fat diet (60% fat, D12492; Uni-Faith, Inc.) for 1 month, which induced WAT inflammation. During this period, excess mononuclear cells (2 × 106 cells) that either overexpressed or suppressed CAP1 were infused systemically. To obtain the mononuclear cells expressing human resistin, we harvested bone marrow and/or spleen from humanized resistin mice and then isolated the mononuclear cells using panning procedures and cell sorting. CAP1 expression was modulated by lentiviral transduction, as described above.

Statistics
All experiments were repeated at least three times, and all data were calculated as mean ± SEM. In each experiment, biological duplicates or triplicates were included. Comparisons between groups were performed by Student’s t test, and the results of in vivo experiments were compared using paired t tests. SPSS v.11.0 (SPSS) was used for all analyses, and statistical significance was assigned at p < 0.05. The error bars represent SEM.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2014.01.013.

AUTHOR CONTRIBUTIONS
The first three authors contributed equally to this work. The other authors of this research paper also have directly participated in the planning, execution, or analysis of the study. They have read and approved the final version submitted.

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