Resistin: a newly identified chemokine for human CD4-positive lymphocytes

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Aims
Increased levels of resistin, a peptide secreted by adipocytes and inflammatory cells, circulate in patients with insulin resistance and early type 2 diabetes, a high-risk population for the development of a diffuse and extensive pattern of arteriosclerosis. Recent data suggest that resistin may activate vascular cells such as smooth muscle cells and endothelial cells, but hitherto nothing is known about the role of resistin in CD4-positive lymphocytes. Therefore, the present study examined the effect of resistin on CD4-positive lymphocyte migration, an important process in early atherogenesis.

Methods and results
Resistin stimulated CD4-positive cell chemotaxis in a concentration-dependent manner with a maximal induction of $2.25 \pm 0.54$ at 100 ng/mL ($P < 0.05, n = 7$). This process involves pertussis toxin-sensitive G-proteins as well as activation of Src- and phosphoinositide 3-kinase (PI 3-K). Biochemical analysis showed that resistin induces phosphorylation of Src and PI 3-K activation in human CD4-positive cells. In addition, resistin activates RhoA, Rac-1, and Cdc42 in these cells as shown by affinity precipitation experiments. Finally, resistin-induced phosphorylation of myosin light chain was inhibited by Src short interference RNA transfection, underscoring the importance of the upstream signaling molecule Src in resistin-induced migration.

Conclusion
These data support an active role of resistin in CD4-positive lymphocyte chemotaxis and elucidate molecular mechanisms in resistin-induced cell migration.

Keywords
Resistin • CD4-positive lymphocytes • Migration • Signalling • Atherosclerosis

1. Introduction
Increased levels of resistin, a peptide secreted by adipocytes and inflammatory cells circulate in patients with insulin resistance and early type 2 diabetes, a high-risk population for the development of a diffuse and extensive pattern of arteriosclerosis. Resistin belongs to a novel family of cysteine-rich proteins and was found in inflammatory zone 3 (FIZZ3). Stepan et al. described resistin in 2001 for the first time as a novel peptide synthesized and secreted from murine adipocytes. Subsequent studies suggested that resistin causes increased hepatic glucose production, leading to insulin resistance. Moreover, in rodents, resistin has been considered as a link between obesity and insulin resistance. The role in humans is unclear, because of controversial findings in obesity, insulin resistance, and diabetes and the fact that resistin expression in humans is higher in monocytes/macrophages than in adipocytes. Recent data showed that resistin is secreted from macrophages in atheroma, thus raising the hypothesis of resistin being a contributor to atherogenesis. Moreover, two independent studies recently pointed out direct vasoactive effects of resistin in cultured vascular endothelial cells. Furthermore, Calabro et al. showed that resistin also promotes smooth muscle cell proliferation via activation of Src-kinase, PI-3 kinase, and MAP-kinase. These results suggest a potential causal role of resistin in early atherogenesis in patients with diabetes and insulin resistance.

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The migration of CD4-positive lymphocytes into the vessel wall with subsequent activation of other vascular cells represents an important step in early atherogenesis. CD4-positive lymphocytes are attracted by chemotactic signals such as RANTES and SDF-1 and enter the vessel wall as naïve TH0 cells. In the subendothelium, these cells then encounter antigens like oxidized LDL, thus differentiating into TH1 cells, subsequently releasing pro-inflammatory mediators like TNF-α and interferon-γ (IFNγ). These cytokines then govern the inflammatory response in the vessel wall by activating other cells such as endothelial cells, macrophages, and vascular smooth muscle cells, thus promoting the inflammatory process in atherogenesis. Still, the role of resistin in CD4-positive lymphocytes remains currently unexplored.

Therefore, the present study examined the effect of resistin on CD4-positive lymphocyte migration in vitro and the signalling pathways involved.

2. Methods

2.1 Cells

Human CD4-positive lymphocytes were isolated from freshly drawn blood of healthy volunteers using gradient centrifugation with subsequent magnetic beads (Miltenyi Biotec, Gladbach, Germany) isolation, as previously described. The investigation conforms with the principles outlined in the Declaration of Helsinki and was granted by the university Ethics Review board. After isolation, CD4-positive lymphocytes were cultured in MI media (PAA, Pasching, Austria) with 5% serum for 16 h and then assayed as described in what follows.

2.2 In vitro cell migration assay

CD4-positive lymphocyte chemotaxis was assayed under serum-free conditions in a 48-well microchemotaxis chamber (Neuroprobe, Gaithersburg, Germany). Wells in the upper and lower chamber were separated by a polyvinylpyrrolidone-free polycarbonate membrane (pore size 5 µm; Costar). CD4-positive lymphocytes at a density of 5 x 10^5/mL were incubated for 3 h with chemokines or resistin (Phoenix Pharmaceuticals, Inc., Burlingame, USA) before migration cells on the bottom face of the filter were stained and counted under the light microscope. Cells were counted in five random high-power fields per well. In some experiments, CD4-positive lymphocytes were incubated with resistin (100 ng/mL) pertussis toxin (0.5 µg/mL), 50 ng/mL Clostridium difficile toxin B (Sigma, St Louis, MO, USA), 25 µg/mL Clostridium botulinum exoenzyme B (Calbiochem, La Jolla, CA), the Rho-kinase inhibitor Y-27632 (1.0 µM, Sigma), or the Src-kinase inhibitor PP2 (1.0 µM, Sigma) for 30 min before assays were performed. Checkerboard analysis was performed to differentiate chemotactic from chemo-kinetic activity.

2.3 PI-3 kinase assay

Isolated CD4-positive lymphocytes were stimulated for times indicated before lysis in a buffer containing 20 mMol/L HEPES, pH 7.5, 10 mMOL EGTA, 1% NP-40, 2.5 mMol/L MgCl2, 2 mMOL sodium orthovanadate, 40 mMol/L β-glycerophosphate, 1 mMol/L DTT, 40 µg/mL PMSF, 10 µg/mL leupeptin, 2 µg/mL pepstatin A, and 2 µg/mL aprotinin.

PI-3 kinase was immunoprecipitated with anti-PI3 kinase-antibody (Santa Cruz Biotechnology) in the presence of protein A/G agarose (Santa Cruz Biotechnology). The immunocomplex was washed three times. After washing with lysis buffer and lipid kinase buffer (20 mMol/L TRIS pH 7.4, 4 mMol/L MgCl2, 100 mMol/L NaCl), immunocomplexes were resuspended in 2 µL of 1 × lipid kinase buffer and added to 32 µL of 3 × lipid kinase buffer (60 mMol/L TRIS pH 7.4, 12 mMol/L MgCl2, 300 mMol/L NaCl), 12 µL phosphatidylinositol (10 mg/mL Sigma), and 10 µL [γ-32P] ATP (10 µCi). After incubation for 30 min at 30°C, the reaction was stopped by addition of 150 µL of 1 mol/L HCl. The organic phase was separated by addition of 450 µL methanol/chloroform with the volume ratio of 1:1. The lipids in the organic phase were separated on TLC silica gel plate (Whatman). TLC plates were developed in methanol/chloroform/H2O/ammonia with the volume ratio of 4:76:0:11:3:2. Radioactive PIP products were visualized by autoradiography.

2.4 Immunoblotting

CD4-positive lymphocytes were left untreated or incubated at 37°C with 100 ng/mL resistin (Sigma) or with 10 ng/mL SDF-1α (Upstate, Lake Placid, NY, USA) for times indicated. Cells were lysed in lysis buffer [50 mMol/L Hepes pH 7.4, 150 mMol/L NaCl, 1% (w/v) NP40, 1% (w/v) glycerol, 1 mMol/L MgCl2, 1 mMol/L MnCl2, 10 mMol/L NaF, 1 mM Na3VO4, 10 µg/µL aprotinin, 10 µg/µL leupeptin, 0.1 mMol/L PMSF]. Aliquots of cell lysates were boiled in Laemmli buffer before running on SDS–PAGE. Immunoblotting was performed by running samples on SDS–PAGE with subsequent electrotransfer onto nitrocellulose membranes (Amersham Pharmacia Biotech, Amersham, England), blocking with 5% skim milk in TBS buffer with 0.1% Tween 20 for 1 h, and incubating with primary antibody (anti-RhoA was from Santa Cruz Biotechnology (Santa Cruz, CA, USA), anti-Rac1 and anti-Cdc42 were from Upstate (Lake Placid, NY, USA), anti-phospho-LIMK, anti-phospho-PAK, anti-phospho-MLC2, anti-phospho-Src and anti-GAPDH, were from Cell Signaling (Beverly, MA, USA), anti-α-tubulin was from Sigma) and 1:2000 dilution of the secondary antibody (anti-goat, anti-rabbit, or anti-mouse horseradish peroxidase (DAKO, Glostrup, Denmark). Development was done by using enhanced chemiluminescence reagents (Pearson, Rockford, IL, USA) according to the manufacturer’s specifications.

2.5 GTPase activity assays

For the detection of GTP-bound Rac1, Cdc42, and RhoA, isolated CD4-positive lymphocytes were treated at 37°C with 100 ng/mL resistin (Sigma) or with 10 ng/mL SDF-1α (Upstate, Lake Placid, NY, USA) for 15 800 sec. The active, GTP-bound form of Rac1 and Cdc42 was determined by using GST-fusion proteins immobilized to Glutathione Sepharose 4B beads for 45 min at +4°C. The active, GTP-bound form of Rac1 and Cdc42 was determined by using GST-fusion proteins immobilized to Glutathione Sepharose 4B beads for 45 min at +4°C. The active, GTP-bound form of Rac1 and Cdc42 was determined by using GST-fusion proteins immobilized to Glutathione Sepharose 4B beads for 45 min at +4°C. The active, GTP-bound form of Rac1 and Cdc42 was determined by using GST-fusion proteins immobilized to Glutathione Sepharose 4B beads for 45 min at +4°C. The active, GTP-bound form of Rac1 and Cdc42 was determined by using GST-fusion proteins immobilized to Glutathione Sepharose 4B beads for 45 min at +4°C. The active, GTP-bound form of Rac1 and Cdc42 was determined by using GST-fusion proteins immobilized to Glutathione Sepharose 4B beads for 45 min at +4°C. The active, GTP-bound form of Rac1 and Cdc42 was determined by using GST-fusion proteins immobilized to Glutathione Sepharose 4B beads for 45 min at +4°C.
separated on 12% SDS–PAGE, transferred to membrane and detected by using appropriate antibodies.

### 2.6 Short interference RNA transfection

Short interference RNA (siRNA) targeting Src and control siRNA was obtained from Upstate. Freshly isolated CD4-positive lymphocytes were transfected using Amaxa nucleofector kit (Amaxa, Cologne, Germany) according to the manufacturer’s protocol. After 36 h, cells were treated with resistin, and immunoblotting for p-MLC as a read-out for migrating cells was performed. To assess the role of resistin in mock- or SRC siRNA-transfected T-cells, we used myosin light chain (MLC) phosphorylation as a read-out for cell migration since both mock- and siRNA-transfected cells do no longer exhibit a full chemotactic response in our chemotaxis assay (data not shown), most likely due to the fact that the transfection procedure itself interferes with other parts of the migration machinery.

### 2.7 Statistical analysis

Results of the experimental studies are reported as mean ± SD. Differences were analysed by one-way ANOVA followed by the appropriate post hoc test. $P < 0.05$ was regarded as significant.

### 3. Results

#### 3.1 Resistin induces CD4-positive lymphocyte migration in vitro

Evaluation of resistin’s effect on CD4-positive lymphocyte migration used isolated human CD4-positive cells in an in vitro chemotaxis assay. Stimulation of T-cells with resistin induced cell migration in a concentration-dependent manner with a maximal 2.25 ± 0.54 fold increase at 100 ng/mL ($P < 0.05$ compared with unstimulated cells, $n = 7$; Figure 1A). Pre-incubation with anti-resistin antibodies abolished the migratory effect of resistin (Figure 1B). Heat-inactivated resistin (100 ng/mL) did not exhibit a chemotactic effect, making endotoxin contamination an unlikely responsible mechanism. The extent of resistin-induced T-cell migration resembled the effect of the established T-cell chemokine SDF-1, which increased T-cell migration by 2.8 ± 0.7 fold ($P < 0.05$, $n = 7$; Figure 1A). Testing of resistin’s chemotactic activity (vs. chemokinesis) used a checkerboard analysis with serial dilutions of resistin above and below the filter. As shown in Figure 1C, CD4-positive cell migration depended on the presence of a resistin gradient between the upper and the lower face of the filter, suggesting that resistin induces directed migration (chemotaxis) of T-cells rather than chemokinesis ($n = 7$).

#### 3.2 Resistin-induced CD4-positive lymphocyte migration involves pertussis toxin-sensitive G-proteins as well as activation of Src-kinase

To investigate further intracellular signalling pathways involved in resistin-induced lymphocyte chemotaxis, inhibition migration experiments were performed. Treatment of human CD4-positive lymphocytes with pertussis toxin (0.5 μg/mL) inhibited resistin-induced T-cell migration (Figure 2A), suggesting that pertussis toxin-sensitive G-protein-coupled receptors are involved. Since some G-protein coupled receptors activate Src, and given the involvement of Src in T-cell motility, we next examined the role of Src activation in resistin-induced T-cell migration, employing an inhibitor of Src-kinase (PP2). Thirty minutes pre-treatment of cells with PP2 significantly diminished resistin-induced CD4-positive T-cell migration (Figure 2B), suggesting the involvement of Src-kinase in these signalling pathways. Given the inhibitory effect of PP2 on resistin-induced CD4-positive lymphocyte migration, we next investigated whether resistin activates Src-kinase in these cells. As shown in Figure 2C and D, resistin significantly induced Src phosphorylation in CD4-positive cells within 1–3 min.

**Figure 1** Resistin induces CD4-positive cell migration. (A) Isolated human CD4-positive lymphocytes were stimulated with resistin for 3 h at concentrations indicated and cell migration was assessed using a modified Boyden chamber. Heat-inactivated resistin (HI), as well as SDF-1 served as controls. Data are expressed as fold induction of unstimulated cells. Bars represent mean ± SD ($n = 7$; $*P < 0.05$ vs. unstimulated cells). (B) Pre-incubation with anti-resistin antibodies (ABs) inhibits resistin-induced migration; unspecific antibodies (uABs) with and without resistin served as controls. Data are expressed as fold induction of unstimulated cells. Bars represent mean ± SD ($n = 5$; $*P < 0.05$). (C) Resistin induces T-cell chemotaxis rather than chemokinesis. Checkerboard analysis revealed that CD4-positive lymphocyte migration depended on the presence of a resistin gradient across the filter, suggesting that resistin induces T-cell chemotaxis rather than chemokinesis. Data are expressed as fold induction compared with unstimulated cells. Bars represent mean of seven independent experiments.
3.3 Resistin activates PI-3 kinase in CD4-positive lymphocytes

We next examined the role of PI-3 kinase activation in resistin-induced T-cell migration. Inhibition of PI-3 kinase by LY294002 (500 nmol/L), significantly reduced resistin-mediated T-cell migration (Figure 3A), but did not affect cell viability as examined by trypan blue staining (data not shown). To further explore the effect of resistin on PI-3 kinase, we performed PI-3 kinase activity assays. Stimulation of cells with resistin (100 ng/mL) induced PI-3 kinase activity with a maximum after 3 min (Figure 3B and C).

3.4 Resistin activates RhoA, Rac1, and Cdc42 in CD4-positive cells

Downstream of PI-3 kinase Rho GTPases are important signalling molecules involved in leukocyte migration. To examine the role of Rho GTPases in resistin-induced migration of CD4-positive
lymphocytes, we first performed in vitro chemotaxis assays employing Clostridium difficile toxin B, an inhibitor of all Rho GTPases. As shown in Figure 4A, Clostridium difficile toxin B significantly reduced resistin-induced cell migration, suggesting that Rho GTPases are involved in this process. In addition, treatment of human CD4-positive lymphocytes with Clostridium botulinum C3 transferase (C3) for 30 min before stimulation with 100 ng/mL resistin for migration experiments. Data are expressed as fold induction of unstimulated cells. Bars represent mean ± SD (n = 7 for toxin B; n = 5 for C3 transferase; *p < 0.05). (B) Resistin activates RhoA, Rac1, and Cdc42. CD4-positive lymphocytes were stimulated with resistin (100 ng/mL) for times indicated. Cells were then lysed and lysates were subjected to affinity precipitation assays to determine RhoA, Rac1, and Cdc42 activity in the presence of GST-Rhotekin or GST-PAK. Equal loading of protein was confirmed by immunoblotting of aliquots against total RhoA, Rac1, or Cdc42. Three independent experiments yielded similar results.

To further investigate the role of these small Rho GTPases upon resistin stimulation, we assessed RhoA, Rac1, and Cdc42 activity performing affinity precipitation experiments with GST-Rhotekin or GST-PAK to which only the active, GTP-bound form of RhoA, or Rac1 and Cdc42, respectively, can bind. Stimulation of cells with resistin activated all three GTPases RhoA, Rac1, and Cdc42 in a time-dependent manner with a maximal effect after 1–3 min of stimulation (Figure 4B).

3.5 Resistin induces activation of PAK and LIMK in CD4-positive lymphocytes

Since resistin stimulation of CD4-positive cells also activates Rac1 and Cdc42, we then investigated the effect of resistin on downstream targets of these GTPases. During chemokine-induced cell migration, activation of Rac1 and Cdc42 has been shown to induce threonine phosphorylation of PAK, a process critical for the maintenance of full PAK activity. Activated PAK then phosphorylates and activates LIMK. Stimulation of cells with resistin induced PAK phosphorylation within 3 min and this process was prolonged up to 10 min after stimulation (Figure 5A/B). Treatment of human CD4-positive lymphocytes with resistin also led to a time-dependent threonine phosphorylation of LIMK, a direct downstream target of PAK (Figure 5C).

3.6 Rho-kinase activation as well as MLC phosphorylation is involved in resistin induced CD4-positive lymphocyte migration

Next, we investigated signalling pathways downstream of RhoA. RhoA activation is known to stimulate actin–myosin contractility...
through activation of ROCK and MLC. Treatment of cells with the specific ROCK inhibitor Y-27632 significantly reduced resistin-induced CD4-positive cell migration, indicating that ROCK activity is required for this process (Figure 6A). Moreover, resistin treatment of CD4-positive lymphocytes resulted in phosphorylation of MLC. Isolated CD4-positive lymphocytes were stimulated with resistin for times indicated. Total lysates were analysed by immunoblotting employing antibodies against phospho-MLC. Equal loading of intact protein was confirmed by staining for GAPDH. (C) Densitometric analysis of five independent experiments. Data are expressed as p-MLC normalized to GAPDH. Bars represent mean ± SD. *P < 0.05 compared with unstimulated cells.

3.7 Transfection with Src siRNA inhibits resistin-induced phosphorylation of MLC

Since Src-kinase is the most upstream signalling molecule involved in resistin-induced migration, we performed Src siRNA transfection experiments in human CD4-positive lymphocytes and assessed MLC phosphorylation as a read-out for cell migration after resistin treatment. Resistin stimulation of mock-transfected cells significantly increased phosphorylation of MLC, whereas transfection of cells with Src siRNA abolished this induction (Figure 7), underscoring the importance of Src in resistin-induced CD4-positive lymphocyte migration.
Resistin induces CD4+ cell migration

4. Discussion

The present study demonstrates that resistin, a peptide secreted from adipocytes and inflammatory cells, exhibits chemotactic activity on CD4-positive lymphocytes. This process involves pertussis toxin-sensitive G-proteins as well as activation of Src- and PI-3 kinase. In addition, resistin activates RhoA, Rac-1, and Cdc42 in these cells and induces the phosphorylation of MLC.

Originally, resistin was suggested to be a linking molecule between obesity and insulin resistance in rodents. In humans, the role of resistin in insulin resistance remains controversial despite the fact that elevated levels of resistin have been found in patients with insulin resistance. However, recent work has shown the presence of resistin in human atheroma, most likely derived from monocytes/macrophages, as well as stimulatory effects of resistin on other vascular cells like endothelial cells and vascular smooth muscle cells. As such, resistin was found to induce endothelial dysfunction in vitro and increases the expression of ET-1 (Endothelin-1), vascular cell adhesion molecule-1, intracellular adhesion molecule-1, matrixmetalloproteinases, pentraxin-3, and PAI-1, suggesting a pro-inflammatory and pro-thrombogenic action of this mediator. In vascular smooth muscle cells resistin induces cell proliferation through activation of extracellular signal-regulated kinase 1/2 and phophatidylinositol 3-kinase.

The present study now extends our knowledge on the role of resistin in the vasculature by suggesting a chemotactic effect on CD4-positive lymphocytes. Migration of CD4-positive cells into the vessel wall is a critical step in lesion development. These cells, once recruited into the subendothelial space differentiate to TH1-cells, thus releasing pro-inflammatory cytokines which then orchestrate the inflammatory activation of other cells in the vessel wall. Given the presence of resistin in atherosclerotic lesions, resistin could affect atherogenesis by promoting the recruitment of CD4-positive cells into the vessel wall. However, the physiological role of resistin in the context of lymphocyte recruitment remains unclear, but one could imagine that resistin released from macrophages could be an additional mediator, like RANTES and SDF-1, in the redundant system of macrophage-mediated T-cell chemotaxis.

To date, the resistin receptor remains unidentified, but our data suggest that the chemotactic effect of resistin on T cells was mediated by a pertussis toxin-sensitive G-protein-coupled receptor. Downstream, resistin stimulation of CD4-positive lymphocytes activates Src-kinase and leads activation of PI-3 kinase. Previous work has shown that Src-kinase is involved in IP-10 (interferon-gamma-inducible protein-10) mediated chemotactic responses of lymphocytes. A specific inhibitor of Src-kinase, PP22, inhibits resistin-induced migration of CD4-positive lymphocytes, as well as transfection of Src siRNA abolished resistin-induced phosphorylation of PLC in our experiments, suggesting that resistin also signals through this pathway. Various chemokines, like CXCL12a (SDF-1α) and CCL5 (RANTES) stimulate lymphocyte migration through activation of small Rho GTPases. Our data, showing a reduction of resistin-induced chemotaxis by inhibitors of RhoA, Rac1, and Cdc42 by resistin suggest that similar mechanisms are involved in resistin-mediated CD4-positive lymphocyte migration. Downstream of Rac1 and Cdc42 PAKs are important effectors in migrating cells, leading to actin filament stabilization, via phosphorylation of LIMK. Resistin-mediated activation of Rac1 and Cdc42 also results in phosphorylation of PAK and LIMK at the leading edge of the migrating cell. Further downstream, LIMK leads to phosphorylation of cofilin, thus allowing actin filament stabilization. In the uropod, resistin activates Rho-kinase and its effector MLC which is required for chemokine-induced cell motility downstream of RhoA.

Together, these mechanisms mediate cell migration by inducing myosin contraction at the uropod of the cell and actin filament stabilization at the leading edge.

Clinical data showed that hyperresistinemia is associated with hypertension in patient with type 2 diabetes. Moreover, resistin expression is positively correlated with insulin resistance in humans and serum resistin levels are elevated in obesity and type 2 diabetes. Serum levels of resistin in diabetic patients are in the range of 50 ng/mL, suggesting that the resistin concentrations employed in this study (10–100 ng/mL) are in the physiological range.

Taken together, the data presented here—in concert with previous findings—suggest that resistin could play an important role in the development of atherosclerotic lesions. These data support an active role of resistin in CD4-positive lymphocyte migration and elucidate the molecular mechanism of resistin-induced cell migration. Future studies are warranted to identify the respective resistin receptor in vascular cells and to demonstrate the role of resistin in atherogenesis in vivo.

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