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Expression and Regulation of Resistin in Osteoblasts and Osteoclasts Indicate a Role in Bone Metabolism

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Abstract The adipose tissue is the site of expression and secretion of a range of biologically active proteins, called adipokines, for example, leptin, adiponectin, and resistin. Leptin has previously been shown to be expressed in osteoblasts and to promote bone mineralization, whereas adiponectin expression is enhanced during osteoblast differentiation. In the present study we explored the possible role of resistin in bone metabolism. We found that resistin is expressed in murine preosteoclasts and preosteoblasts (RAW 264.7, MC3T3-E1), in primary human bone marrow stem cells and in mature human osteoblasts. The expression of resistin mRNA in RAW 264.7 was increased during differentiation and seemed to be regulated through PKC- and PKA-dependent mechanisms. Recombinant resistin increased the number of differentiated osteoclasts and stimulated NFκB promoter activity, indicating a role in osteoclastogenesis. Resistin also enhanced the proliferation of MC3T3-E1 cells in a PKA and PKC-dependent manner, but only weakly interfered with genes known to be upregulated during differentiation of MC3T3-E1 into osteoblasts. All together, our results indicate that resistin may play a role in bone remodeling. J. Cell. Biochem. 99: 824–834, 2006.

Key words: resistin; adipokines; osteoclastogenesis; NFκB

Resistin, also known as adipocyte secreted factor (ADSF) and found in inflammatory zone 3 (FIZZ3), is a low molecular weight cysteine-rich protein (murine 114 amino acids, human 137 amino acids) secreted from adipocytes [Steppan et al., 2001a; Steppan and Lazar, 2004] Four homologs of the resistin/resistin-like molecules (RELM) family have been identified in mice so far (including RELMα, RELMβ, and RELMγ) [Steppan et al., 2001b].

Resistin acts on skeletal muscle myocytes, hepatocytes, and adipocytes reducing their sensitivity to insulin [Shojima et al., 2002], and, has therefore, been suggested to be a molecular link between obesity and type 2 diabetes [Steppan et al., 2001a]. However, the discovery of multiple sites of resistin expression including human macrophages [Patel et al., 2003], placenta [Yura et al., 2003], pancreas [Minn et al., 2003], and the mouse hypothalamo-pituitary system [Morash et al., 2002] suggest that resistin might play other roles as well.

It is known that body fat is positively correlated with increased bone mineral density and decreased fracture risk [Reid, 2002]. Although mechanical loading may contribute to this relationship, other factors are also involved. Leptin and other adipokines may play a role in the complex relationship existing between adipose tissue and bone metabolism. Leptin [Reseland et al., 2001] and adiponectin [Berner et al., 2004] have previously been

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demonstrated to be expressed and regulated in osteoblasts. Resistin is expressed in several human tissues [Nohira et al., 2004] with the highest level in human macrophages and bone marrow [Patel et al., 2003], and has been proposed to play a role in inflammation [Lehrke et al., 2004; Bokarewa et al., 2005]. Recently, an alternative splicing transcript for the human resistin gene was identified in bone marrow and lung [Nohira et al., 2004]. The RELMβ and γ are also found in high concentration in bone marrow in mice [Schinke et al., 2004; Shojima et al., 2005].

In the current study, we show for the first time that resistin is expressed in mesenchymal bone marrow stem cells, osteoblasts, and osteoclasts. We find that resistin stimulates osteoblast proliferation and cytokine release, as well as osteoclast differentiation.

MATERIALS AND METHODS

Cells Systems and Experimental Design

MC3T3-E1 (murine preosteoblasts, ATCC) cells were maintained in MEM-α (Gibco BRL, Life Technologies Ltd, Scotland) supplemented with 10% fetal calf serum (FCS) (EuroClone, Great Britain). Differentiation of MC3T3-E1 was performed with addition of 25 μg/ml ascorbic acid and 3 mM β-glycerophosphate. Release of cytokines was measured in medium samples collected from MC3T3-E1 cells, and the cytokine concentration was correlated to the amount of total protein.

Mononuclear cells from human bone marrow (iliac crest) were isolated using Lymphoprep (AXIS-SHIELDPoC A/S, Oslo, Norway). The mononuclear cells were pelleted and cultured in MEM-α/10% FCS, 50 U/ml penicillin, and 50 μg/ml streptomycin and 1% Gautama (Gibco BRL). Donor recruitment and acquisition of human liposuction waste material were performed in accordance with a protocol approved by the local ethical committee.

Commercially available primary human osteoblasts from both femur and tibia of different donors (NHOst cell system, Cambrex) were grown in osteoblast growth media (OGM, Cambrex). Osteoblasts cultured to facilitate mineralization were exposed to hydrocortisone hemisuccinate (200 nM) and β-glycerophosphate (10 mM) (Cambrex) in the ambient medium. The phenotype of the osteoblasts was characterized based on the expression levels of alkaline phosphatase (ALP), collagen type 1, bone sialoprotein, osterix, Runx2, osteocalcin, and CD44, and formation of mineralization nodules.

3T3-L1 (murine preadipocytes, ATCC) cells were cultured in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% FCS. 3T3-L1 cells were grown until confluence and differentiated as previously described [Benito et al., 1991]. Briefly, cells were incubated in DMEM added 20% FCS, 1 μM dexamethasone (Sigma-Aldrich, Norway), 0.5 mM 3-isobutyl-1-methylxanthin (IBMX) (Sigma-Aldrich), and 2 μM insulin (Sigma-Aldrich) for 4 days. The following days, cells were incubated in DMEM added 10% FCS and 200 nM insulin.

RAW 264.7 (murine preosteoclast, ATCC) cells were cultured in DMEM supplemented with 10% FCS. In differentiation studies, the medium was enriched with 50 ng/ml murine M-CSF and 50 ng/ml murine RANKL (both RnD Systems, Oxford, Great Britain).

Osteoclasts were differentiated from human peripheral blood mononuclear cells (PBMC), isolated from buffycoat. Separation of PBMC was performed essentially as described by Bøyum [Bøyum, 1964]. The cells were seeded into 24-well dishes at a concentration of $1.25 \cdot 10^6$ cells/ml in MEM-α including human M-CSF and human RANKL (both 50 ng/ml), and dexamethasone (0.01 μM). The medium was changed at Day 6 and 9. After 12 days, the cells were stained for tartrate resistant acid phosphatase (TRAP) activity using Naphtol AS-BI phosphate and Fast Garnet in the presence of sodium tartrate, as described by the manufacturer (Sigma-Aldrich). The number of multinuclear ($\geq$3 nuclei), TRAP positive cells was counted, and regarded as genuine osteoclasts. In order to investigate direct osteoclast activity, a pit resorption assay was performed. The PBMC cells were seeded on BioCoat Osteologic Discs (BD Biosciences) and cultured as described above. Bone resorption was determined with picture pixel analysis.

The IL-6 dependent mouse hybridoma cell line B9 was cultured in RPMI 1640 (Gibco) supplemented with 10% FCS, 2-mercaptoethanol (50 μM), and IL-6 (1 ng/ml).

Recombinant resistin was purchased from RnD Systems. H-89 (Calbiochem, La Jolla, CA) and GF109203x (Calbiochem) were dissolved in water to a final concentration of 10 mM and in DMSO to a final concentration of 4.8 mM,

respectively. CTP-cAMP and PMA were obtained from Sigma-Aldrich.

NFκB Activity

The Path Detect *cis*-Reporting System pNF-κB-Luc plasmid (Stratagene, Germany) contains five copies of the NF-κB enhancer (TGGGGACTTTCCGC) in front of the luciferase reporter gene. RAW 264.7 cells (5.0×10^4 cells per well) were seeded in 96-well plates the day before the transfection. Cells were transfected using 0.1 μg pNF-κB-luciferase plasmid and 0.3 μl Fugene 6 transfection reagent (Roche Applied Science, Germany) per well. After cultivation for 24 h, cells were treated with test substances for 6 h. Cells were lysed in 15 μl of lysisbuffer/well (Promega, Inc., Madison). Luciferase activity was measured by Turner Luminometer model TD-20/20 (Turner Designs) using the Luciferase reporter Assay System (Promega, Inc.).

Proliferation Assay

Cells/well (4×10^3) (MC3T3-E1) and 3×10^3/well (RAW 264.7) were seeded in 96-well plates, and cultured for 24 h. Then the cells were washed once with 180 μl serum-free medium, before the addition of new medium with or without test substances and inhibitors. After 4 h, 5-bromo-2′-deoxyuridine (BrdU)-labeling solution (Roche Molecular Biochemicals, Mannheim, Germany) was added, and the cells were cultured for additional 18 h before incorporation of BrdU was measured as described by the manufacturer. Briefly, the labeling medium was removed and the cells were fixed and genomic DNA denaturated by adding 150 μl FixDenat per well for 30 min at room temperature. FixDenat-solution was removed and 100 μl of peroxidise-conjugated anti-BrdU antibody solution was added per well, followed by incubation at room temperature for 90 min. The cells were then washed three times with 200 μl washing solution before 100 μl of substrate, Luminol/4-idophenol was added. After 3 min, chemiluminescence was measured (RLU = relative luminiscence units) in a microplate luminometer (Fluoroskan Ascent FL, Labsystems).

ALP Activity

MC3T3-E1 cells were seeded in 6-well plates and cultured in MEM-α supplemented with 10% FCS. After reaching confluence, medium with and without recombinant mouse resistin (5 nM), (Phoenix Pharmaceuticals, Inc., Belmont, CA) was added. The medium was changed three times a week. ALP activity in the culture medium was analyzed after 1, 3, 7, and 14 days. Briefly, aliquotes of 25 μl of culture media was assayed in duplicate for ALP activity by measuring the cleavage of p-Nitrophenyl Phosphate (pNPP) (100 μl) (Sigma, St. Louis, MO). The reaction was stopped after 30 min with the addition of 50 μl of 3 M sodium hydroxide, and absorbance was measured at 405 nm. In parallel to the samples, a standard curve with calf intestinal ALP (CIAP) (Promega, Inc.) was used; 1 μl from the stock CIAP was mixed with 5 ml of ALP buffer (1:5,000 dilution), and subsequently diluted 1:5. Protein concentrations were determined using a BCA protein assay reagent (Pierce Chemical, Rockford, IL) according to the manufacturer's instructions. ALP activity in nmol of pNPP/min/mg of protein was expressed in % of control at the different time points.

OPG Secretion

The concentration of OPG in MC3T3-E1 culture media was determined by ELISA as described by the manufactor (RnD Systems) using anti-mouse OPG-antibody and biotinylated anti-mouse OPG. Detection was performed by labeling with streptavidine-horseradish peroxidise (RnD Systems) and adding 1, 2 phenylenediamine dihydrochloride (OPD) (Dako, Glostrup, Denmark) as substrate. The enzymatic reaction was stopped after 20 min by adding 100 μL of 0.5 mol/L H_2SO_4 to each well. The optical density (OD) was measured as absorbance at 490 nm. Recombinant murine OPG was used as standard. Minimum detectable concentration of mouse OPG was 10 pg/ml. According to the manufacturer, no significant cross-reactivity or interference was observed. Intra-assay and inter-assay variabilities were less than 15% and 9%, respectively. The amount of OPG was related to the amount of total protein in each sample.

RANKL Secretion

RANKL concentration in culture media were determined by an immunoassay kit for quantitative determination of free sRANKL from mouse and rat (Biomedica, Vienna, Austria)

according to the manufacture's protocol. The amount of RANKL was related to the amount of total protein in each sample.

IL-6 Secretion

Concentration of IL-6 in the culture media from MC3T3-E1 was determined by a bioassay utilizing the IL-6-dependent mouse hybridoma cell-line B9. Recombinant human IL-6 (Biosource) was used as a standard. Standard or sample (50 μl) was added as triplicate to a 96-well plate. After that mouse hybridoma B9 cells were seeded (5,000 cells/well) and incubated for 3 days. The growth of cells was assessed by a (3-(4.5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS)/phenazine methosulfate (PMS) reduction assay essentially as previously described [Buttke et al., 1993]. Minimum detectable concentration of IL-6 was 5 pg/ml, and intra-assay and inter-assay variability were both less than 15% for all measured samples. The amount of IL-6 was related to the amount of total protein in each sample.

Western Blot Analysis

Whole cell lysate was prepared from $5-7 \times 10^6$ cells, which were washed twice in PBS, scraped and harvested directly in 500 μl SDS-sample buffer (62.5 mM Tris-HCl, pH 6.8; 8.7% glycerol; 2% w/v SDS; 5% v/v 2-β mercaptoethanol; 0.09% w/v bromophenol blue). Viscosity was reduced by drawing the suspension through a 21-gauge needle, cell debris were removed by centrifugation (15,000*g*, 10 min), and the supernatant was stored at −80°C. Each extract (15 μl) was boiled and separated on a SDS 10% polyacrylamide gel (running buffer: 25 mM Tris-HCl, pH 8.3; 190 mM glycine, 0.1% w/v SDS) prior to electrotransfer onto Hybond-P membranes (Amersham, UK) The transfer was performed in 25 mM Tris-HCl, 190 mM glycine, and 20% methanol, pH 8.3, for 1 h at 175 mA. The membranes were treated with 5% nonfat dry milk (Nestlé, Oslo, Norway) in TBS (50 mM Tris-HCl, pH 7.5 and 150 mM NaCl) for 1 h at room temperature and incubated with primary antibodies diluted 1:1,000 in TBS with 1% BSA and 0.05% Tween 20 overnight at 4°C. The blots were then incubated with peroxidase-conjugated secondary antibodies in TBS with 1% BSA and 0.05% Tween 20 for 1.5 h at room temperature. After washing (4 × 15 min in TBS with 0.05% Tween 20), binding of secondary antibodies (1:1,000) was visualized by the ECL-detection system (Amersham).

Resistin Secretion

Concentrations of resistin in culture media were measured by a competitive radioimmunoassay (Linco Research, St. Charles, MO). The culture medium was concentrated two times compared to the manufacturer's instruction. Intra-assay variation was 1.3%.

Total Protein Measurements

Amount of total protein in media were determined using Sigma Microprotein PR assay kit with Protein Standard Solution Calibrator (Sigma Diagnostics, Dorset, UK). Analyses were performed using Cobas Mira chemistry analyzer (Roche Diagnostics, Germany), according to the manufacturer's protocol, 30 μl of each sample was mixed with 200 μl substrate. Intra-assay and inter-assay variabilities were less than 2.4% and 3.2%, respectively. Detection range for the assay was 10–2,000 mg/L.

mRNA Isolation

Cells were lysed in lysis/binding buffer (100 mM Tris-HCl, pH 8.0, 500 mM LiCl, 10 mM EDTA, pH 8.0, 0.5 mM dithiothreitol [DTT], and 1% sodium dodecyl sulfate [SDS]). mRNA was isolated using magnetic beads [oligo $(dT)_{25}$] as described by the manufacturer (Dynal AS, Oslo, Norway). Beads containing mRNA were resuspended in 10 mM Tris-HCl, pH 8.0, and stored at −70°C until use. One microliter of the mRNA-containing solution was applied directly to obtain a first-strand complementary DNA (cDNA) using the iScript cDNA Synthesis Kit which contains both oligo(dT) and random hexamer primers (Bio-Rad, Hercules, CA).

Real-Time PCR Quantification With SYBR Green

Reactions were performed and monitored using iCycler iQ (Bio-Rad). The 2X iQ SYBR Green Supermix was based on iTaq DNA polymerase (Bio-Rad). cDNA samples were analyzed both for the genes of interest and reference genes (β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The amplification program consisted of a preincubation step for denaturation of the template cDNA (3 min, 95°C), followed by 50 cycles consisting of a

denaturation step (15 s, 95°C), an annealing step (30 s, 60°C), and an extension step (30 s, 72°C). The Ct value, defined as the number of cycles required to produce a detectable product above background fluorescence, was measured for each sample, and arbitrary units were calculated using a standard curve, which was run for each individual PCR analysis. The standard curves consisted of serial dilutions of cDNA of a sample or a control containing the highest amount of the specific gene analyzed. A negative control without the cDNA template was run with every PCR assay, and contamination by genomic DNA was ruled out by performing PCR analysis where RT-enzyme had been omitted in the RT reactions. β-actin and GAPDH RT-PCR was run in parallel as control to monitor RNA integrity and to be used for normalization. Specificity of each primer pair was confirmed by melting curve analysis and agarose gel electrophoresis. Table I shows the primer sequences designed by using the analysis software Primer3 (University of Massachussetts Medical School) and the expected sizes of the PCR products.

Real-Time PCR Quantification With Taq-Man Probes

After treatment, cells were washed with PBS and total RNA isolated using Qiagen RNeasy kit (Qiagen, Great Britan). cDNA synthesis was performed with 500 ng total RNA in a 20 μl reaction containing 1 × reaction buffer, 5 mM $MgCl_2$, 500 μM each dNTP, 2.5 μM $Oligod(T)_{15}$ primer, 0.4 U/μl Rnase inhibitor, and 1.25 U/μl Euroscript Reverse Transcriptase according to the manufacturer's instruction (Reverse transcription core kit, Eurogentec, Belgium). cDNA synthesis was performed for 10 min at 25°C followed by 1.5 h at 48°C and 5 min at 95°C. After synthesis, the cDNA was diluted 1: 2 with water. Real-time PCR was performed with 1 × Quantitect Probe PCR Master Mix (Qiagen), 400 nM forward primer, 400 nM reverse primer, 200 nM TaqMan Probe (Eurogentec), and cDNA equivalent to 62.5 ng total RNA in a total reaction volume of 25 μl. Real-time PCR was performed in Stratagene's M × 3,000P real-time PCR system; 10 min at 95°C, 45 thermal cycles of 30 s at 95°C, 30 s at 56°C, and 15 s at 76°C.

TABLE I. Primers Used in Real-Time PCR Quantification With SYBR Green or TaqMan Probes

Gene	Primer sequence	Species	Amplicon size (bp)
Alkaline phosphatase	S 5′-AACCCAGACACAAGCATTCC-3′ A 5′-GAGAGCGAAGGGTCAGTCAG-3′	Mouse	151
Bone sialoprotein	S 5′-GAAAATGGAGACGGCGATAG-3′ A 5′-ACCCGAGAGTGTGGAAAGTG-3′	Mouse	141
Runx2	S 5′-GCCTTCAAGGTGGTAGCCC-3′ A 5′-CGTTACCCGCCATGACAGTA-3′	Human/Mouse	67
CD44	S 5′-CTTCCATCTTGACCCGTTGT-3′ A 5′-ACAGTGCTCCTGTCCCTGAT-3′	Mouse	175
Collagen-I	S 5′-AGAGCATGACCGATGGATTC-3′ A 5′-CCTTCTTGAGGTTGCCAGTC-3′	Mouse	177
IL-6	S 5′-CCGGGAGCAGTGTGAGCTTA-3′ A 5′-TAGATGCGTTTGTAGGCGGTC-3′	Mouse	171
Osteocalcin	S 5′-CCGGGAGCAGTGTGAGCTTA-3′ A 5′-TAGATGCGTTTGTAGGCGGTC-3′	Mouse	80
Osteoprotegerin	S 5′-AGACCATGAGGTTCCTGCAC-3′ A 5′-AAACAGCCCAGTGACCATTC-3′	Mouse	131
Osterix	S 5′-ACTGGCTAGGTGGTGGTCAG-3′ A 5′-GGTAGGGAGCTGGGTTAAGG-3′	Mouse	135
RANKL	S 5′-GGCCACAGCGCTTCTCAG-3′ A 5′-TGACTTTATGGGAACCCGAT-3′	Mouse	141
GAPDH	S 5′-ACCCAGAAGACTGTGGATGG-3′ A 5′-CACATTGGGGGTAGGAACAC-3′	Mouse	171
GAPDH	S 5′-TGCACCACCAACTGCTTAGC-3′ A 5′-GGCATGGACTGTGGTCATGAG-3′	Human	254
Resistin	S 5′-CCGAGGCTTCGCCGTCAC-3′ A 5′-CTCAGGGCTGCACACGACAG-3′	Human	137
Resistin TaqMan probe	S 5′-GTACCCACGGGATGAAGAACC-3′ A 5′-GCAGAGCCACAGGAGCAG-3′ 5′-AGGGCACAGCAGTCTTGAGCTGCT-3′.	Mouse	253
β-actin TaqMan probe	S 5′-CTGGCTCCTAGCACCATGA-3′ A 5′-AGCCACCAATCCACACAGA-3′ 5′-CAAGATCATTGCTCCTCCTGAGCG-3′	Human/Mouse	73

Resistin expression was confirmed by sequencing. PCR products were cloned into the pCRII-TOPO vector using the TOPO TA Cloning Kit (Invitrogen) for sequencing, according to the standard protocol. Vectors containing PCR products were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Oslo, Norway) according to the manufacture's protocol.

Using computer software from Stratagene (http://www.labtools.stratagene.com), primers and probes were designed to recognize murine resistin and β-actin mRNA sequences.

Fold induction of gene expression level was estimated by the ΔΔCt-method as described by Livak et al. [Livak and Schmittgen, 2001].

$$\text{Fold change} = 2^{-\Delta\Delta Ct} \text{ and}$$
$$\Delta\Delta Ct = (Ct_{GOI} - Ct_{Actin})_{\text{time x}} - (Ct_{GOI} - Ct_{Actin})_{\text{time 0}}$$

GOI is the gene of interest, time x is any time point, and time 0 represents the 1 × expression of the target gene.

Data Presentation

Results are shown as the mean ± SD, normality tests and equal variation test were analyzed before significant differences were calculated using Student's *t*-test. Reporter gene experiments were repeated three times with four wells per condition per experiment. Proliferation experiments were repeated three times, with four wells per experiment. Real-time PCR was repeated three times with two wells per condition per experiment.

RESULTS

Resistin Is Expressed and Regulated in Bone Cells

Resistin mRNA expression was identified in murine preosteoclasts RAW 264.7 (Fig. 1A), murine preosteoblasts MC3T3-E1 (Fig. 1B), human bone marrow mesenchymal stem cells, and human osteoblasts (Fig. 1B). In RAW 264.7, the mRNA expression increased more than sixfold during the first week of differentiation followed by a reduction to basal level after 2 weeks. In MC3T3-E1 cells, however, the mRNA expression remained low and unchanged for 6 weeks of differentiation (data not shown). For undifferentiated cells, the relative amount of resistin mRNA in RAW 264.7 was 360 times lower than in adipocytes (3T3-L1 cells) and about 3,000 times higher than in MC3T3-E1 cells.

The protein was also demonstrated with Western blot in both osteoblast and osteoclast murine cell lines (Fig. 1C). The concentration of resistin in culture media from osteoblasts

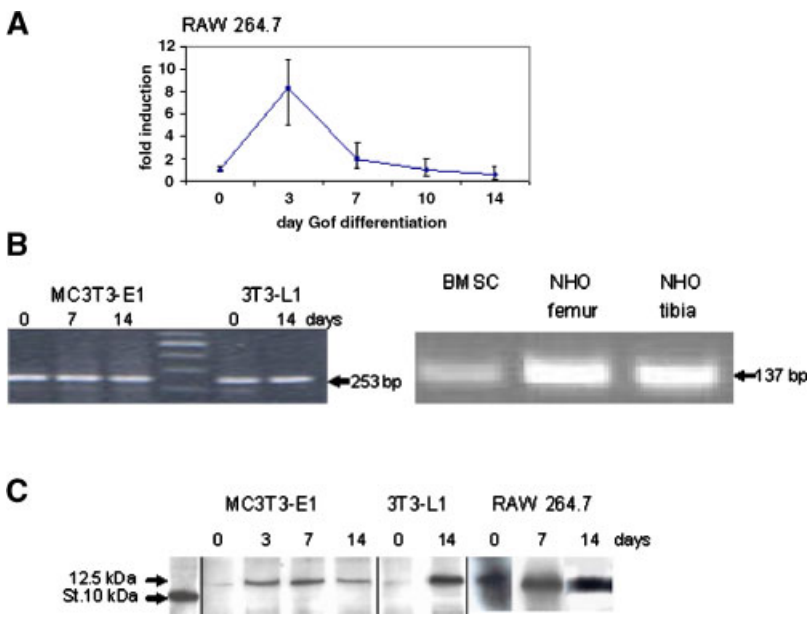

Fig. 1. Resistin mRNA expression in (**A**) murine RAW 264.7; (**B**) murine MC3T3-E1, murine 3T3-L1, human bone marrow mesenchymal stem cells (BMSC), and primary human osteoblasts (NHO), assessed by real-time PCR (A) or visualized in ethidium bromide-stained 1.2% agarose gel (B). **C**: Western blots showing resistin protein expression during differentiation in MC3T3-E1, 3T3-L1, and RAW 264.7.

(MC3T3-E1 cells) was low (0.48 ± 0.18 ng/ml) compared to the content in media from mature adipocytes (3T3-L1 cells; 33.6 ± 2.9 ng/ml). Although we observed higher expression of resistin mRNA in osteoclasts, we were not able to quantify the amount of resistin in media from RAW 264.7 cells by the method used, partly due to the lower amount of total protein in the culture media.

Both PMA (200 ng/ml) and CPT-cAMP (100 μM) stimulated resistin mRNA expression in undifferentiated RAW 264.7. PMA enhanced the resistin level threefold after 6 h and sixfold after 12 h incubation compared to untreated cells (Fig. 2), indicating that resistin expression is regulated by diacylglycerol-sensitive PKCs. The cAMP-analog CPT-cAMP enhanced the resistin mRNA expression more than 300-fold after 12 h (Fig. 2), suggesting that PKA-signaling pathways are involved in regulating resistin expression in preosteoclasts. Neither PMA nor CTP-cAMP increased resistin mRNA expression in MC3T3-E1 cells.

Resistin Stimulates Osteoclast Differentiation and NFκB Activity

Resistin (10 nM) induced a twofold increase ($P = 0.008$) in the number of multinuclear, TRAP positive osteoclasts differentiated from human peripheral monocytes after 12 days (Fig. 3A). The effect of resistin on differentiation of murine cells (RAW 264.7) was similar (Fig. 3B). Resistin (10 nM) also seems to enhance the resorption by differentiated human osteoclasts, though not significantly different from untreated cells in two separate experiments ($P = 0.22$, $n = 3$, and $P = 0.23$, $n = 3$, respectively). In contrast, resistin had no effect on RAW 264.7 proliferation (data not shown).

NFκB is the major signaling pathway involved in osteoclastogenesis. It was, therefore, of interest to study whether the stimulatory effect of resistin on osteoclast differentiation was mediated via this pathway. In RAW 264.7 cells transfected with NFκB reporter plasmid, resistin (10 nM) induced a threefold ($P < 0.05$) increase in luciferase activity compared to untreated cells. In comparison, RANKL (100 ng/ml) induced a fivefold ($P < 0.05$) increase (Fig. 4).

Resistin Increases Osteoblast Proliferation

Resistin (0.1–10 nM) enhanced ($P < 0.05$) the proliferation of MC3T3-E1 preosteoblasts

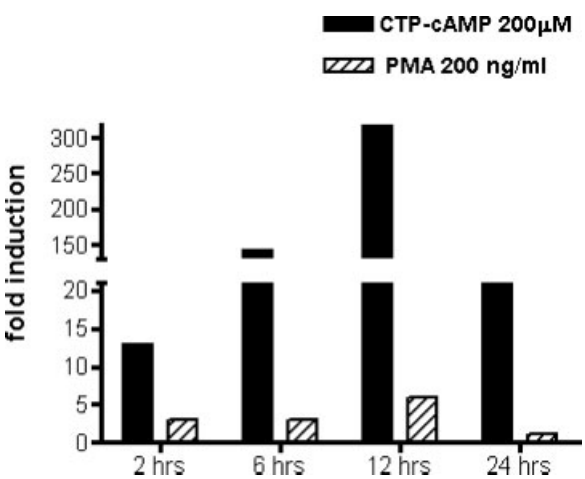

Fig. 2. Resistin mRNA expression in RAW 264.7. Cells were treated with CPT-cAMP (200 μM) or PMA (200 ng/ml) for 2–24 h. Resistin mRNA was assessed by RT-PCR using TaqMan probes, related to β-actin mRNA expression and shown as fold induction compared to untreated cells. The figure shows one representative experiment out of three.

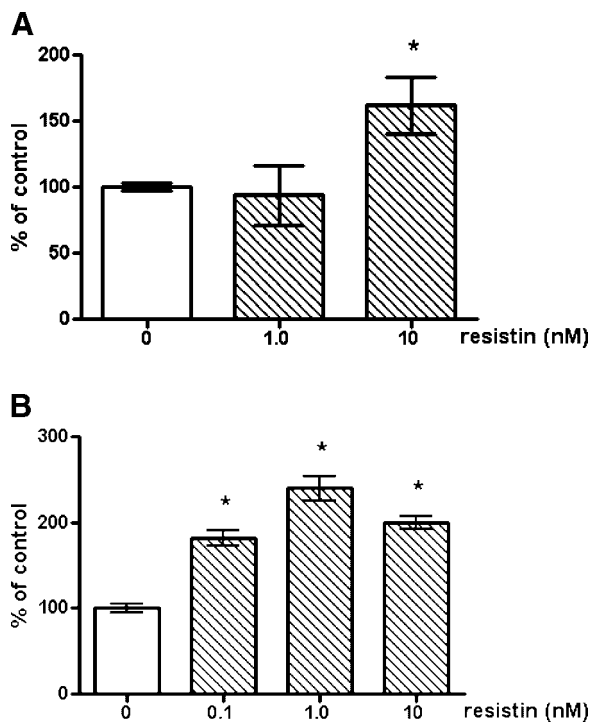

Fig. 3. Effect of resistin on osteoclast differentiation. Human PBMC (**A**) and murine RAW 264.7 cells (**B**) were cultured in medium with M-CSF and RANKL for 12 days with or without resistin (0.1, 1, and 10 nM). Multinuclear (three or more nuclei) TRAP positive cells were counted and regarded as genuine osteoclasts. The data represent mean ± SD of three individual experiments. *indicates statistically significant compared to untreated cells ($P < 0.05$).

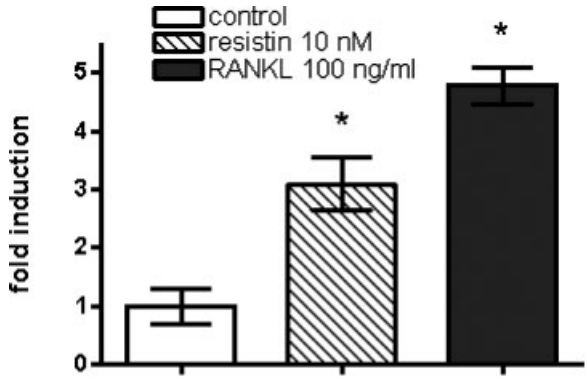

Fig. 4. Resistin induces activation of the NFκB promoter. RAW 264.7 cells were transient transfected with NFκB luciferase reporter plasmid and stimulated with resistin (10 nM) or RANKL (100 ng/ml) for 6 h. The data represent mean ± SD, the figure shows one representative out of four individual experiments. Control = untreated cells.

(Fig. 5). To better understand the molecular mechanisms involved in resistin-induced MC3T3-E1 proliferation, we investigated the possible involvement of PKC and PKA signaling pathways. Cells were treated with the PKC inhibitor GF 209103x (3.5 μM) or the PKA inhibitor H-89 (10 μM) before and during the resistin stimulation. The observed increase in proliferation by resistin was reversed when cells were pre-treated with The PKA and PKC inhibitors (Fig. 5). The PKC inhibitor alone had no effect on proliferation, whereas the PKA inhibitor alone reduced the proliferation by 15–20% compared to untreated cells. The results indicate that the effect of resistin on proliferation is partly mediated through both PKC and PKA-dependent mechanisms in osteoblasts.

Furthermore, we examined the effect on genes known to be regulated during osteoblast differentiation. MC3T3-E1 cells were treated with resistin (5 nM) for 1, 3, 7, and 14 days. Resistin weakly increased the mRNA expression of collagen type I (Day 7 and 14), whereas the mRNA expression of Runx2, osterix, CD44, bone sialoprotein, osteocalcin, and ALP was not different from untreated cells at any time point tested (data not shown). Resistin weakly increased ALP activity after 14 days ($P < 0.05$), but did not influence ALP activity at other periods of time (data not shown).

Resistin Increases IL-6 Release but Only Weakly Influences OPG and RANKL in Osteoblasts

Resistin enhanced IL-6 mRNA expression (data not shown), and IL-6 release from MC3T3-E1 after 48 and 72 h, in a dose-dependent manner (Fig. 6). Treatment with 5 nM resistin for a period of 14 days, weakly decreased RANKL mRNA levels at Day 1 and 3,

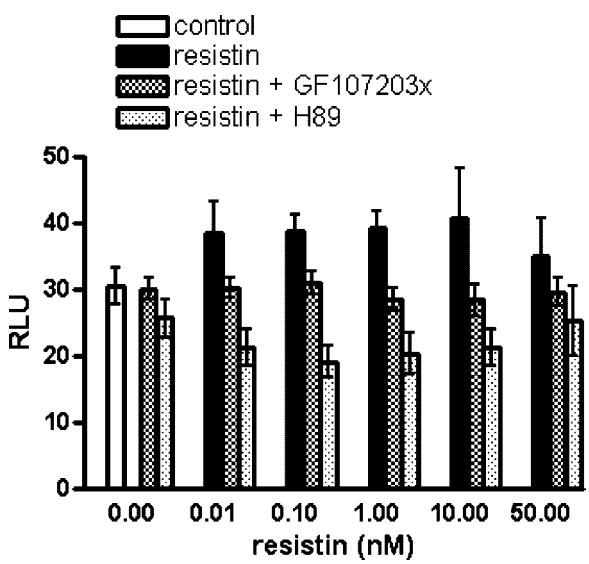

Fig. 5. Resistin (0.1–10 nM) induces proliferation in MC3T3-E1 ($P < 0.05$). Cells were also pre-treated with H-89 (10 μM) or GF109203x (3.5 μM) for 1 h prior to administration of resistin (0.01–50 nM) for 24 h. Proliferation was assessed by BrdU incorporation and expressed as RLU (relative luminescence). The data represent mean ± SD. The figure shows one representative out of four individual experiments.

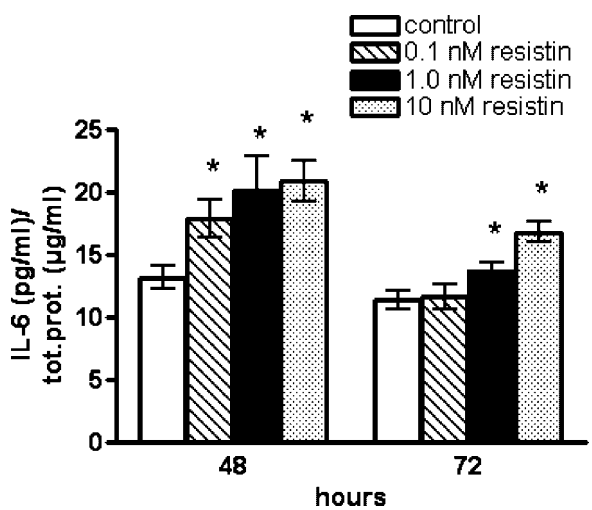

Fig. 6. Resistin enhances IL-6 release from MC3T3-E1. Cells were stimulated in serum-free medium for 12–72 h with different concentrations of resistin. Medium was harvested and assayed for resistin by IL-6 bioassay. The IL-6 concentration was related to the amount of protein and presented as mean ± SD of three parallels. The figure shows one representative experiment out of five. *indicates statistically significant compared to untreated cells ($P < 0.05$).

while RANKL mRNA levels were unchanged after 2 weeks of incubation. No change in OPG or RANKL secretion was observed for a period of 72 h, and there was no change in OPG mRNA levels except for at Day 14 where its expression was decreased by 20% (data not shown).

DISCUSSION

Resistin has previously been shown to be present in high concentrations in bone marrow [Patel et al., 2003]. Here, we demonstrate for the first time the transcription, translation, and secretion of resistin from bone cells. We found resistin to be expressed in both osteoclasts (differentiated from human monocytes and RAW 264.7 cells) and osteoblasts (human primary mesenchymal stem cells, mature human osteoblasts from tibia and femur, and MC3T3-L1 cells). The highest expression among bone cells was found in osteoclasts at an early stage of differentiation. An enhancement of resistin expression during early differentiation of monocytes is in accordance with the observation in differentiated macrophages [Patel et al., 2003]. In MC3T3-E1 cells, however, resistin expression was unchanged during differentiation in contrast to adipocytes where an increase of resistin is found [Steppan et al., 2001a; Li et al., 2003]. The relative amount of resistin expressed and secreted from the preosteoclast cell line RAW 264.7 and the preosteoblast cell line MC3T3-E1 cells was, however, low compared to the adipocyte cell line 3T3-L1.

We found resistin expression to be upregulated by PMA and the cAMP- analog CTP-cAMP in murine RAW 264.7 cells. Analysis of the 5′-flanking sequence of resistin has revealed several potential binding sequences for transcription factors activated through PKC and PKA signaling pathways [Hartman et al., 2002; Lu et al., 2002]. The mouse resistin promoter contains a CCAAT/enhancer-binding protein (C/EBPα) binding site, which has been shown to be necessary and sufficient for resistin expression in 3T3-L1 adipocytes [Hartman et al., 2002]. Moreover, the C/EBPα transcription factor is known to be activated through cAMP and PKA signaling pathways [Wilson et al., 2002], and our results may reflect that these signaling pathways are essential in the transcriptional activation of resistin in RAW 264.7 cells as well. The expression of resistin in MC3T3-E1 was not found to be regulated by the cAMP analog or PMA, probably due to low levels of resistin in MC3T3-E1, or low stability of the mRNA product.

Enhanced osteoclastogenesis leading to bone loss is the main etiological factor in the development of osteoporosis. Osteoclast differentiation is regulated by the coordinated synthesis and actions of cytokines produced by bone marrow stromal cells and osteoblasts. RANKL/OPG ratio plays a central role in this regulation. In the present study, we found that resistin may affect osteoclastogenesis in different manners in vitro. The observed decrease in RANKL/OPG mRNA ratio, although weak, may indicate an indirect inhibitory effect, while the increase in IL-6 secretion may result in an indirect stimulatory effect on osteoclastogenesis. On the other hand, resistin was found to have a direct stimulatory effect on osteoclast differentiation. This effect was observed both in cultures of the murine preosteoclasts and human monocytes. Although we were unable to demonstrate a significant increase in osteoclast activity with resistin, the osteoclasts formed were biologically active and able to resorb bone.

Interestingly, resistin induced activation of the NFκB promoter in RAW 264.7 cells, even in the absence of RANKL. The NFκB signaling pathway plays a central role in osteoclastogenesis, and the transcription factor NFκB participates in regulation of genes coding for cytokines and cytokines receptors [Baeuerle and Henkel, 1994]. Recently Bokarewa and co-workers have reported that resistin activates NFκB and induces expression of IL-6, IL-1β, and TNFα in human peripheral monocytic cells [Bokarewa et al., 2005]. Resistin is also shown to induce IL-12 and TNFα secretion in RAW 264.7 cells [Silswal et al., 2005]. The effect of resistin on osteoclast differentiation could thus be mediated directly through NFκB or via release of other cytokines. In contrast, no effect of resistin on the release of proinflammatory cytokines and prostaglandin from human placenta and adipose tissue has been found, while adiponectin exerted proinflammatory effects by increasing the release of cytokines from the same tissues [Lappas et al., 2005]. This is partly in contrast to previously reported studies, which indicate the opposite effect of both adipokines [McTernan et al., 2003; Masaki et al., 2004; Wolf et al., 2004]. Thus, the conflicting findings may reflect tissue and/or cell-specific differences.

Administration of recombinant resistin enhanced the proliferation of preosteoblasts (MC3T3-E1). This effect seems to be mediated through PKA and PKC signaling pathways as incubation with resistin in combination with the inhibitors of these pathways reduced the proliferation compared to resistin alone. This is in accordance with previous findings showing that PKA and PKC signaling pathways are involved in MC3T3-E1 proliferation [Datta et al., 2005; Ghayor et al., 2005]. We also found a weak, but significant resistin-mediated increase in collagen type 1 mRNA expression and ALP activity. However, no effect was observed on other genes known to be upregulated during osteoblast differentiation. Resistin, thus, participates in recruitment of osteoblasts, but we cannot conclude from this study that it drives the differentiation in an osteoid direction, at least not in MC3T3-E1 cells.

Although in vivo studies are necessary to elucidate the net skeletal effects of resistin, we present data indicating that the high level of resistin found in bone marrow is not generated only by bone marrow adipocytes and monocytes [Patel et al., 2003; Botolin et al., 2005], but also by mesenchymal stem cells, osteoblasts, and osteoclasts. Resistin may affect bone metabolism via several mechanisms, where the two main actions seem to be the enhancement of osteoclast differentiation and the recruitment of osteoblasts. The observed increase in resistin during osteoclast differentiation could stimulate, in turn, the osteoblast recruitment, indicating all together a potential role for resistin in bone metabolism and remodeling.

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