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Resistin Induces Expression of Pro-Inflammatory Cytokines and Chemokines in Human Articular Chondrocytes via Transcription and mRNA Stabilization

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Abstract

Objective—To elucidate the effect of resistin on human articular chondrocytes, and generate a picture of their regulation at the transcriptional and post-transcriptional levels.

Methods—Human articular chondrocytes were cultured with resistin. Changes in gene expression were analyzed at various doses and times. Cells were also treated with the transcriptional inhibitor actinomycin D after resistin treatment, or the nuclear factor kappa B (NF- κ B) inhibitor IKK-NBD before resistin treatment. Gene expression was tested by quantitative real-time polymerase chain reaction. Computational analysis for transcription factor binding motifs was performed on the promoter regions of differentially expressed genes. TC28 chondrocytes were transfected with CCL3 and CCL4 promoter constructs, pNF- κ B reporter, and NF- κ B and CCAAT/enhancer-binding protein β (C/EBP β) expression vectors with or without resistin.

Results—Resistin-treated human articular chondrocytes increased the expression of cytokines and chemokines. The mRNAs for MMP-1, MMP-13 and ADAMTS-4 also increased while COL2A1 and aggrecan were down-regulated. Cytokine and chemokine genes could be categorized into three groups according to the pattern of mRNA expression in a 24 h time course. One pattern suggested rapid regulation by mRNA stability. The second and third patterns were consistent with transcriptional regulation. Computational analysis suggested the transcription factors NF- κ B and C/EBP β were involved in the resistin-induced up-regulation. This prediction was confirmed by the co-transfection of NF- κ B and C/EBP β , and IKK-NBD inhibition.

Conclusion—Resistin has diverse effects on gene expression in human chondrocytes affecting chemokines, cytokines and matrix genes. mRNA stabilization and transcriptional up-regulation are involved in resistin-induced gene expression in human chondrocytes.

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Conflict of interest

The authors declare that they have no conflict of interest.

Obesity is associated with alterations in adipose tissue, including the recruitment of macrophages and T-cells. Adipose tissue is no longer considered to be an inert tissue functioning solely for energy storage. Various secreted products of adipose tissue, called adipokines, have recently been characterized, including adiponectin, leptin, resistin and visfatin (1-3). Adipokines are associated with a chronic inflammatory-response syndrome characterized by abnormal cytokine production, increased acute-phase reactant synthesis and activation of inflammation (1, 3, 4). Recent studies have shown that adipokines represent a potent risk factor for the development and progression of rheumatoid and osteoarthritic joint diseases (5-7).

Resistin is a 12.5 kDa cysteine-rich polypeptide that belongs to a family of resistin-like molecules (RELMs) or “found in inflammatory zone” (FIZZ) molecules (1, 2). Resistin is expressed not only by human adipocytes, but also in high levels by macrophages (1). Many aspects of the biological effects and the regulation of resistin remain controversial, but studies have provided evidence for a role of resistin in inflammatory processes (1, 3, 8). For rheumatoid and osteoarthritic joint diseases, increased levels of resistin are observed in the synovial fluid and tissue of patients with rheumatoid arthritis (RA) or osteoarthritis (OA) (5, 9, 10), and plasma resistin levels are significantly correlated with erythrocyte sedimentation rate and C reactive protein (9). Furthermore, resistin can up-regulate interleukin (IL)-1, IL-6 and tumor necrosis factor α (TNF- α) in blood samples and the synovial fluid of patients with RA, and intra-articular injection of resistin induces arthritis in healthy mouse joints (11).

Cytokines and chemokines are mediators of inflammation and are known to be important in inflammatory diseases, including RA and OA (12-14). Cytokines are a category of signaling molecules that are involved in cellular communication. Chemokines are a specific class of cytokine that mediate chemoattraction (chemotaxis). Chemokines all have a similar protein structure, being 8-10 kDa, with the two major subclasses having conserved cysteine residues either adjacent to each other (CC) or separated by one amino acid (CXC) (15). Using genome-wide expression analysis of human articular chondrocytes, we showed that a large site of chemokines was up-regulated by the pre-inflammatory cytokine, IL-1 β (12).

Most studies with resistin have focused on cells in the inflammatory cascade. It has recently been shown that resistin is elevated following traumatic joint injury and causes the loss of proteoglycan, PGE₂ production and release of inflammatory cytokines from articular cartilage (16). In this study, we investigated the expression levels of cytokines and chemokines in human articular chondrocytes in response to resistin, and generated an overall picture of their regulation at the levels of transcription and post-transcription.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), Ham's F-12 medium, were from Mediatech Inc. (Herndon, VA). Pronase, Collagenase P, and FuGENE® 6 Transfection Reagent were from Roche (Indianapolis, IN). Recombinant human resistin was from R&D Systems (Minneapolis, MN). RNeasy Mini kit, QIAshredder, DNase I, were from Qiagen (Valencia, CA). Fetal bovine serum (FBS), SuperScript® II Reverse Transcriptase was from Invitrogen (Carlsbad, CA). SYBR Green PCR Master Mix was from Applied Biosystems (Foster City, CA). Penicillin/streptomycin solution, ascorbic acid and Actinomycin D (ActD) were from Sigma (St. Louis, MO). pGL3-basic vector, Reporter Lysis Buffer, Luciferase Assay Reagent were from Promega (Madison, WI). Cell-permeable NEMO binding domain (NBD) synthetic peptides (IKK-NBD peptide and IKK-NBD control peptide), were from BIOMOL (Plymouth Meeting, PA).

Cell culture

Cartilage was obtained with approval of the Washington University Human Studies Review Board and permission of the patient. Normal chondrocytes were obtained from normal articular knee cartilage from a tissue donor (N=1) with traumatic injury. Chondrocytes were also obtained with permission from the preserved area of OA cartilage from donors undergoing total knee replacement surgery (IRB No. 05-0279). For the latter, chondrocytes from macroscopically normal looking cartilage were used: patients were of both sexes and more than 60 years old. Cartilage from 2-4 donors was combined prior to cell isolation (patient pool, N=19). Chondrocytes were isolated and plated for 24 h following previously published procedures (12). Medium without serum was added and cells were allowed to rest for 24 h before adding resistin at the concentrations and times indicated. Resistin was reconstituted in sterile water. The T/C-28a2 human chondrocyte cell line was also used (provided by Dr. Mary B. Goldring, Cornell University), and cultured like human articular chondrocytes.

Total RNA isolation

Total RNA was isolated from chondrocytes with RNeasy Mini Kit with DNase I treatment, following the protocol recommended by the manufacturer. Total RNA (1 µg) was reverse-transcribed with a SuperScript® II Reverse Transcriptase to synthesize cDNA. The cDNA was then used for real-time quantitative PCR (qPCR).

Real-time quantitative PCR analysis

qPCR was performed in a total volume of 20 µl reaction mixture containing 10 µl of SYBR Green PCR Master Mix, 2.5 µl of cDNA, and 200 nM of primers using a 7300 Real-Time PCR System (Applied Biosystems). Primers used for qPCR were optimized for each gene, and the dissociation curve was determined by the Real-Time PCR System (Supplemental Table 1). The parameters of primer design included a primer size of 18 to 21 bp, a product size of 80 to 150 bp, a primer annealing temperature of 59° to 61°, and a primer GC content of 45% to 55%. Results were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The cycle threshold (Ct) values for GAPDH and the genes of interest were measured for each sample, and the relative transcript levels were calculated as $\chi = 2^{-\Delta\Delta C_t}$, in which $\Delta\Delta C_t = \Delta C_t - \Delta C_t^{\text{control}}$ and $\Delta C_t = C_{t\text{treatment}} - C_{t\text{GAPDH}}$; $\Delta C_t^{\text{control}} = C_{t\text{control}} - C_{t\text{GAPDH}}$.

Stability of mRNA

Estimates of changes in mRNA stability were analyzed in two ways. First, the pattern of gene expression was measured over a 24 h period as described by Hao and Baltimore (17). Second, for genes that remained high at 24 h, human articular chondrocytes were treated with 100 ng/ml of resistin for 24 h. The decay of mRNA was evaluated in the presence or absence of resistin, using the transcriptional inhibitor ActD (10 µg/ml). Cells were harvested immediately (time zero) or after 1, 4, 7, and 24 h of ActD treatment. mRNA was measured by qPCR as described above and results were normalized to GAPDH before the calculation of half-lives (i.e., the time when 50% of mRNA remained if the initial value is 100%). The half-life of RNA was calculated from the equation $t_{1/2} = \ln(2)/k$, where $t_{1/2}$ is half-life and k is the degradation rate constant = -2.303(slope) (18). The slope of decay curves was obtained by linear regression analysis of mRNA remaining as a function of time. RNA ratios at respective time points were normalized by the ratio at beginning of the evaluation (i.e. time 0) in each experiment to facilitate direct comparison.

Computational analysis of cytokine and chemokine genes

Potential regulatory DNA surrounding the cytokine and chemokine genes was analyzed by the Promoter Analysis Pipeline (19, 20). Promoters (defined as 10 kb upstream and 5 kb downstream of the transcription start site) were acquired from six species: human, chicken, chimp, dog, mouse, and rat; and repetitive elements in the promoters were masked. Promoters were aligned and transcription factor binding sites were identified using the TRANSFAC 11.2 database, a curated database of transcription factor profiles (20). Probability scores for each promoter and each transcription factor binding site were calculated and a distribution of probability scores was generated for each transcription factor. *R*-scores were then computed using these distributions (19). This system was used to predict the transcription factors that are most likely to bind to and regulate the set of genes. For each transcription factor binding site motif (identified by the TRANSFAC accession number) and each promoter in the genome, the probability score of the transcription factor binding to the promoter was computed by summing the exponential score of each individual site predicted in the promoter on either strand. This score was set to a minimum value of 1 for a promoter with no sites exceeding the cutoff. The rank of this score was converted to the *R*-score which is related to the fraction of promoters with a higher rank, by $R\text{-score} = \ln N - \ln(\text{rank})$. Promoters ranked in the top half have $R\text{-score} > \ln 2$ (0.693), those in the top 10% have $R\text{-score} > \ln 10$ (2.302), those in the top 1% have $R\text{-score} > \ln 100$ (4.605). The *R*-score for a set of *n* promoters, the average *R*-score, (*R*-score), was calculated by $(R\text{-score}) = (1/n) (R\text{-score})$.

Plasmid constructs

The CCL3 and CCL4 promoter 5'-deletion constructs were generated by PCR using the pGL2-CCL3 (−1972/+75) and pGL3-CCL4 (−1281/+12). The CCL3 and CCL4 promoter constructs, C/EBPβ and IkappaB kinase 2 (IKK2) expression vector, and pNF-κB luciferase reporter were provided by the following: the human pGL2-CCL3 (−1972/+75) was from Dr. G. David Roodman (University of Pittsburgh); human pGL3-CCL4 (−1281/+12) was obtained from Dr. Sheau-Farn Yeh (National Yang-Ming University, Taipei); human IKK2 in the pCDNA3 vector and pNF-κB luciferase reporter were from Dr. Yousef Abu-Amer (Washington University); human C/EBP-full-length in the pCDNA3 vector was from Dr. Erika Crouch (Washington University).

Transient transfection and luciferase assay

DNA transfections of T/C-28a2 cells were performed using FuGENE 6 transfection reagent. 2×10^5 of T/C-28a2 cells were cultured on a 6-well plate overnight. The transfection mixture containing 6 μl of FuGENE 6, 500 ng of various promoter constructs, and 200 ng of pCMV-β-gal was then added, and the cells were cultured for 24 h. For the co-transfection assay using IKK2 and C/EBPβ expression vector, expression vectors or empty vector were added to the 100 μl transfection mixtures as indicated. FBS was added to transfection medium 4 h later to a final concentration of 10%. After 24 h of incubation, cells were replaced with fresh complete medium and incubated for an additional time with or without resistin as indicated. The cells were then harvested with Reporter Lysis Buffer, and the lysate was analyzed for luciferase activity using Promega Luciferase Assay Reagent. The β-galactosidase activities were also measured to normalize variations in transfection efficiency. Each transfection experiment was performed in triplicate and repeated at least twice.

RESULTS

Effect of resistin on human articular chondrocytes

Resistin induced gene expression of multiple cytokines and chemokines in human articular chondrocytes (one normal and three patient pools from preserved area of OA cartilage). The response of proinflammatory cytokines, chemokines and matrix molecules to resistin (100 ng/ml) was not different between normal cartilage and the preserved area of OA cartilage (Figure 1, and supplemental Figure 1). With the exception of CXCL12, resistin stimulated the expression of the other 20 cytokines and chemokines tested. Fifteen genes were up-regulated over 10-fold (Figure 1B). Bone morphogenetic protein 2 (BMP-2), TNF- α , CCL2, and CX3CL1 were up-regulated from 2 to 10-fold (Figure 1A). A selection of other genes related to cartilage growth and degradation were also monitored. mRNAs for matrix metalloproteinase (MMP)-1 and MMP-13 increased while mRNAs for the matrix genes type II collagen (COL2A1) and aggrecan were down-regulated slightly (Figure 1A, and Supplementary Figure 1).

Dose dependence of resistin-induced changes in phenotype

As the response to exposure to 100 ng/ml was reproducibly strong, we determined the effect of different concentrations of resistin from 20 ng/ml to 500 ng/ml. It has been reported that the physiological concentrations of resistin range from 22.1 ng/ml to up to 70 ng/ml in synovial fluid, and from 10 ng/ml to over 25 ng/ml in serum in OA and RA patients (9, 10, 11, 16). Human articular chondrocytes were exposed to resistin at 0, 20, 100 and 500 ng/ml. The down-regulation of COL2A1 gene showed a dose-dependent response beginning at 20 ng/ml. Aggrecan, MMP-1, MMP-13, and ADAMTS-4 were induced by 100 ng/ml (Supplementary Figure 1). At the concentration of 100 ng/ml of resistin, many of the cytokine and chemokine mRNAs were dramatically increased (Figure 1C and D). The genes that continued to increase at 500 ng/ml were TNF- α , IL-1 α , IL-1 β , CCL2, CCL3, CCL3L1, CCL4, CCL8, CCL5, CXCL1, CXCL2, CXCL3, and CXCL6. IL-1 β , CCL3, and CCL8 reached levels of 400-600 fold (Figure 1C). In contrast, the induction of BMP-2, IL-6, IL-8, CCL20, CXCL5, and CX3CL1 reached their maximum levels with 100 ng/ml (Figure 1D).

Time course of resistin-induced changes in phenotype

In order to begin to ascertain which genes are coordinately regulated by resistin, RNA was isolated at 0, 1, 4, 8, and 24 h after treatment. The expression of genes tested was changed significantly at 4 h, but three patterns of regulation emerged. The expression of genes in group I (Figure 2A) was highest at 4 h, but then quickly decreased during the remaining time period. Genes in group II (Figure 2B) were also induced quickly after resistin stimulation, but thereafter their high expression was sustained. Genes in group III (Figure 2C) were induced more slowly, and gradually and steadily increased, not reaching peak expression even by the end of the 24 h observation period. Thus, there appear to be a number of pathways leading to the phenotypic changes induced by resistin.

Resistin enhanced cytokine and chemokine mRNA stability in human articular chondrocytes

To begin to determine the mechanism of gene regulation by resistin, the ability of resistin to alter mRNA half life was measured in human articular chondrocytes. For some genes in the group I (TNF- α , IL-6 and CXCL2), previous studies by Baltimore and colleagues have shown that they are primarily regulated by mRNA stability (17). We have shown that BMP-2 gene expression induced by TNF- α is also regulated by mRNA stability (21). Here, we investigated the mRNA stability of cytokines and chemokines in groups II and III by blocking transcription with ActD after 24 h of resistin treatment. The results showed that the

extension of half-lives in group II was more significant than group III, with extension varying from approximately 2 times to 10 times (Table 1). Thus, the involvement of a post-transcriptional mechanism in the induction of these genes by resistin in human chondrocytes is indicated. Baltimore and colleagues (17) have shown that multiple adenine-rich elements (AREs) were present in chemokine genes regulated by mRNA stability. The average number of AREs present in these groups of transcripts is correlated with mRNA stability (Table 1).

Computational analysis to predict regulatory domains

Genes that are transcriptionally co-expressed often contain common regulatory motifs in their DNA flanking domains. To begin to analyze the regulatory mechanism of the cytokines and chemokines by human chondrocytes in response to resistin, the up-regulated cytokines and chemokines were subdivided into two categories: Group A mRNAs were increased over 10-fold when exposed to 100 ng/ml resistin. Group B mRNAs were increased 2 to 10-fold. The promoters of group A genes were analyzed (Table 2). The *R*-score indicates the probability that the transcription factor corresponding to this motif will bind to the promoter of these genes: the higher the *R*-score, the more likely it is to bind. Although the binding must be verified experimentally, *R*-scores over 2 have been demonstrated to have a high likelihood of functional significance (19, 20). Overall, several transcription factor binding motifs known to be involved in the expression of pro-inflammatory cytokine-induced genes were identified: NF- κ B, p65, c-Rel, myocyte enhancer binding factor-3 (MEF-3), Ikaros-1 (Ik-1), and C/EBP β .

NF- κ B and C/EBP β are involved in up-regulation of cytokines and chemokines by human chondrocytes in response to resistin

In order to experimentally verify the transcription factor regulation predicted by computational analysis in human chondrocytes, we looked directly at NF- κ B function by using pNF- κ B luciferase reporter in TC28 chondrocytes. TC28 cells responded to resistin similar to human primary chondrocytes (Supplemental Figure 2). The activity change of pNF- κ B luciferase reporter was up-regulated at 1 h, remained up-regulated at 8 h, but was reduced by 24 h in the presence of resistin (Figure 3A).

Because other transcription factors are potentially important in cytokine and chemokine gene expression, we also investigated the role of C/EBP β . To investigate the function of NF- κ B and C/EBP β in detail, C/EBP β and IKK2 (IKK β) expression vectors were co-transfected with -1395-bp CCL3 (a group III gene) and -1281-bp CCL4 (a group I gene) promoter constructs. These constructs contain several high probability candidate C/EBP β and NF- κ B binding sites (Figure 3B). The promoter activity of -1395-bp CCL3 and -1281-bp CCL4 constructs were up-regulated in a dose-dependent manner, suggesting that C/EBP β and IKK2 are both acting as activators (Figure 3C and D).

To confirm the potential role of NF- κ B in resistin-induced cytokine and chemokine activation, IKK-NBD, a specific NF- κ B inhibitor, was added to the human articular chondrocyte cultures before resistin treatment. The mRNA from these cells following 4 h of resistin treatment showed a modest but dose-dependent suppression of cytokine and chemokine activity (Figure 4A-C). As a control, we showed that the inhibitory effects of IKK-NBD following 4 h of IL-1 β stimulation, had a similar effect on well known NF- κ B-responsive genes like IL-1 β , IL-6, IL-8, CCL2, CCL5, CCL20 (Figure 4D). Therefore, this modest IKK-NBD suppression was not resistin specific. The modest suppression can be attributed to the use of primary chondrocytes in present study as opposed to previous experiments where only cell lines were used. To test this possibility, similar experiments were performed in the TC28 cell line where the inhibition was greater (Supplemental Figure 2B and 3).

DISCUSSION

Resistin, the adipocyte-derived cytokine, is a potent link between adipokines and inflammatory diseases (1, 11), including rheumatoid and osteoarthritic joint diseases (9, 11). To provide a view of the effect of resistin on human articular chondrocyte gene expression, we analyzed 25 genes related to the inflammatory cascade, including 6 cytokines, 14 chemokines and 5 matrix genes. We show the levels of the tested chemokines and cytokines are dramatically increased in human adult articular chondrocytes by exposure to the adipokine resistin. One exception was the lack of effect on CXCL12, also known as SDF-1. A similar pattern of expression was previously observed for chemokines induced by IL-1 β in human articular chondrocytes (12). The expression of mRNAs for MMP-1, MMP-13 and ADAMTS-4 was also increased while COL2A1 and aggrecan were down-regulated in response to the resistin. The expression of ADAMTS-5 was also monitored, and its expression was reduced by resistin (data not shown). Cytokines and chemokines are produced in inflamed joints by the synovium, macrophages and fibroblast-like synoviocytes, and they are thought to be key regulators of the inflammatory process (12, 13, 15, 22, 23). Cytokines both enhance the migration of cells into the joint and stimulate matrix metalloproteinase production in synovial fibroblasts and chondrocytes (22), and chemokines function in the recruitment of neutrophils, monocytes, immature dendritic cells, B cells and activated T cells (24). Furthermore, it has recently been reported that the CXC family of chemokines is important in the regulation of angiogenesis in rheumatoid arthritis, and CCL2, CCL3, and CCR2 stimulate osteoclastogenesis (25-27). Therefore, the production of chemokines and cytokines under the influence of resistin could significantly alter the metabolism of chondrocytes.

The cytokines and chemokines highly up-regulated by resistin have not been previously shown to be regulated by resistin in human chondrocytes. IL-1 α , IL-1 β , IL-6, IL-8, CCL2, CCL3, CCL4, and TNF- α have been identified in patient serum, synovial fluid and blood cells with resistin stimulation (9, 11, 16). Lee and colleagues also reported that resistin stimulated the secretion of CCL2 and IL-6 in mouse cartilage. The adipokines are expressed in the joint tissue and serum of patients with rheumatoid and osteoarthritic joint diseases (9, 10, 16, 28-31). Adiponectin is unable to modulate TNF- α or IL-1 β release in chondrocytes (30), but resistin can up-regulate them, especially IL-1 β which was increased over 100-fold in 100 ng/ml resistin treatment. As an important cytokine in inflammatory joint disease, IL-1 β can induce enzymes that degrade the extracellular matrix and reduce synthesis of the primary cartilage components COL2A1 and aggrecan (12).

The level of gene expression is regulated at both transcriptional and post-transcriptional levels in eukaryotic cells, fibroblasts and chondrocytes (17, 21, 32). Modulation of the mRNA decay rate is a strategy widely used by cells to adjust the intensity of expression (33). Recently, Baltimore and Hao (17) reported that mRNA stability influences the levels of genes encoding inflammatory molecules in mouse fibroblasts, providing a temporally controlled process of protein expression. The same trend is observed in our human chondrocytes in a 24 h time period for cytokine and chemokine genes, including TNF- α , IL-1 β , IL-6, CXCL1, CXCL2, CCL2, CCL20, CCL5, CX3CL1, and CXCL5. As they reported, the expression of genes from group I that were highly related to mRNA stability contained a large number of AU-rich tracts (AREs, Table 1), which are known to destabilize mRNA. The effect of mRNA stability was also important in genes from group II, but mRNAs from group III were more stable and mRNA stability did not significantly affect their expression. In the present study, although IL-1 β and CXCL1 were not in group I in human articular chondrocytes, the extension of mRNA stability in these genes indicated that the mRNA stability is also involved in the steady state level of mRNA. For BMP-2, Fukui and colleagues showed the up-regulation of BMP-2 in chondrocytes via both transcription

and mRNA stability (21). Furthermore, the results of mRNA stability revealed that mRNA stability is also involved in the up-regulation in group II and group III. Together, these studies support the view that the mRNA stability is an important determinant in the resistin-induced gene expression.

To explore potential transcriptional regulation of the chemokines and cytokines, they were sub-classified based on the extent of their up-regulation at 24 h, and subjected to a computational analysis for transcription factor binding sites that are highly represented in each set. Chang et al. (19) have demonstrated that the computed scores are highly correlated with binding probability, such that promoters with higher combined scores were more likely to be bound by the transcription factor than promoters with lower scores. In the genes highly up-regulated, binding sites for factors related to NF- κ B had very high scores (>90%). The importance of NF- κ B signaling pathway for resistin-induced inflammation has been reported for blood cells (11). We also show that the activity of pNF- κ B luciferase reporter was increased significantly after resistin treatment in human chondrocytes. Co-transfection of the IKK2 expression vector established that IKK2 could enhance the promoter activity of CCL3 and CCL4 with resistin stimulation. Together, these observations showed that NF- κ B signaling is involved in cytokine and chemokine expression in human chondrocytes with resistin treatment.

It has been reported that the NF- κ B inhibitor, hypoxostoxide, reduced fibronectin fragment induction of IL-6, IL-8, CCL2, CXCL1, CXCL2, and CXCL3 in human articular chondrocytes (34). Amos and colleagues also demonstrated that inhibition of NF- κ B activity inhibited most inflammatory mediators, but not all (35). Thus, to address the role of NF- κ B in resistin-mediated cytokine and chemokine expression, we used the cell-permeable IKK-NBD peptide that prevents NEMO/IKK γ association with IKK α and IKK β required for NF- κ B activation (36). We showed that IKK-NBD modestly inhibited the resistin-induced cytokine and chemokine mRNA expression, but did not inhibit all of the mRNA expression. However, since IKK-NBD is a potent inhibitor of only canonical IKK signaling, the resistin-induced cytokine and chemokine mRNA up-regulation could be also activating NF- κ B subunits by an IKK independent mechanism, which could be important in further studies. To begin to account for the additional expression, we investigated the role of another transcription factor with a high binding score, C/EBP β . Co-transfection of C/EBP β expression vector indicated that C/EBP β could also enhance the promoter activity of CCL3 (group III) and CCL4 (group I) (37, 38). As IKK-NBD inhibited approximately 40% of the CCL3 and CCL4 mRNA expression, C/EBP β could also be an important regulator.

C/EBP β has been previously associated with IL-1 β -induced and TNF- α -induced changes in chondrocyte gene expression. C/EBP β is increased in chondrocytes by IL-1 β and TNF- α , and down-regulates COL2A1 and cartilage-derived retinoic acid-sensitive protein (cd-rap) (37-39). In addition, C/EBP β plays an important role in repressing cartilage gene expression in non-cartilaginous tissues (40). Hirata and colleagues reported C/EBP β promoted the transition from proliferation to hypertrophy in growth plate chondrocytes (41). A cooperative interaction of C/EBP β and NF- κ B has been demonstrated in other genes. The involvement of both C/EBP β and NF- κ B was recently shown in the expression of IL-1 β and IL-8 (42, 43). C/EBP β regulates the basal transcriptional activity of IL-8, and C/EBP β and NF- κ B together mediate the IL-8 response to infection by *Pseudomonas aeruginosa* (43).

In summary, we have shown that many cytokines and chemokines are up-regulated by the adipokine resistin in human articular chondrocytes. These findings begin to provide a molecular mechanism by which the increased resistin after traumatic joint injury (16) could lead to matrix degradation. The mRNA stability of some cytokines and chemokines was increased by resistin, which indicated the potential involvement of a post-transcriptional

mechanism in the induction of these genes in human chondrocytes. By computational analysis and experimental studies, NF- κ B is the most highly represented transcription factor binding site, but we demonstrate that C/EBP β is also involved. Combined with our IL-1 β results on human chondrocytes (12), it can be expected that this high level increase in such a wide range of cytokines and chemokines will have a significant impact on cartilage cells and should be considered in the pathophysiology of rheumatoid and osteoarthritic joint diseases. These studies provide the basis for further investigation into the function and regulation of chemokines in synovial joint disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Response of proinflammatory cytokines, chemokines and matrix molecules to resistin. Chondrocytes from normal human knee cartilage (**A**, **B**), and chondrocytes from the preserved area of OA cartilage (**C**, **D**) were treated with various doses of resistin for 24 h. **A**, Genes with relative change of mRNA less than 10-fold. **B**, Genes with relative change of mRNA over 10-fold. Dose response of cytokines and chemokines to resistin: **C**, transcripts that continue increase over 100 ng/ml; **D**, transcripts that were stable or decrease after 100 ng/ml. Quantitative real-time PCR was performed with indicated primers (Supplemental Table 1) and the change of mRNA was normalized to GAPDH mRNA, and compared with no resistin treatment (set as 1). Each *bar* of (A, B) represents the mean $\pm$ S.D. from three experiments with cells from the same patient. Each *bar* of (C, D) represents the mean $\pm$ S.D. from three patient pools, and each was repeated three times.

Figure 2.

Time course of response of cytokines, chemokines and matrix molecules to resistin. Chondrocytes from the preserved areas of cartilage from knees of OA patients were treated with 100 ng/ml of resistin for 0, 1, 4, 8, and 24 h. Quantitative real-time PCR was performed with indicated primers (Supplemental Table 1) and the change of mRNA was normalized to GAPDH mRNA. Each *bar* represents the mean $\pm$ S.D. of change compared with zero time (set as 1) from three patient pools, and each was repeated three times.

Figure 3.

Involvement of transcriptional regulation in the expression of cytokines and chemokines to resistin. **A**, Resistin stimulates the activity of pNF-κB Luc reporter in TC28 human chondrocytes. Relative luciferase activity indicates the fold expression relative to the activity of zero time (set as 1) in the presence of resistin (100 ng/ml). **B**, The candidate C/EBPβ and NF-κB binding sites in human CCL3 (-1395) and CCL4 (-1281) constructs. C/EBPβ and IKK2 stimulate the expression of CCL3 (**C**) and CCL4 (**D**) promoter in TC28 human chondrocytes. The CCL3 and CCL4 promoter constructs were co-transfected with C/EBPβ and IKK2 expression plasmids into T/C-28a2 cells without or with resistin (100 ng/ml) 8 h (CCL4) or 24 h (CCL3). The luciferase activity of the empty vector in the absence of resistin is set as 1. Each bar represents the mean ± S.D.

Figure 4.

IKK-NBD peptide inhibits the expression of cytokines and chemokines in human articular chondrocytes with resistin treatment. Human articular chondrocytes were pretreated with vehicle (DMSO), IKK-NBD peptide 100 μM or 200 μM, or IKK-NBD control peptide (100 μM) for 2 h and then exposed to resistin (100 ng/ml) (**A**, **B**, **C**) or IL-1β (1 ng/ml) (**D**) for 4 h. After resistin or IL-1β treatment, total RNA was isolated, and real-time quantitative PCR was performed with indicated primers (Supplemental Table 1). Each bar represents the mean ± S.D. of change compared with resistin alone or IL-1β alone (set as 1) from three patient pools, and each was repeated three times. The *p* value of 100 μM IKK-NBD (°) or 200 μM IKK-NBD (*) was compared with resistin alone based on student's *t*-test (° and *, means *p* < 0.05; °° and **, means *p* < 0.01; ***, means *p* < 0.001).

Table 1

Half-life of mRNA in human articular chondrocytes in response to resistin.

	Genes	AREs in 3' UTR	Half Life (hr)	
			W/O resistin	W/ resistin
Group I	TNF- α	7	ND	ND
	BMP-2	11	ND	ND
	IL-6	6	ND	ND
	CCL4	2	ND	ND
	CXCL2	6	ND	ND
	CXCL3	7	ND	ND
Group II	IL-8	6	3.04 $\pm$ 0.02	16.09 $\pm$ 3.84
	CCL2	1	3.48 $\pm$ 1.17	7.40 $\pm$ 1.14
	CCL20	3	4.43 $\pm$ 1.61	44.11 $\pm$ 4.10
	CXCL1	3	1.70 $\pm$ 0.49	3.25 $\pm$ 0.66
	CXCL5	10	3.60 $\pm$ 0.02	8.92 $\pm$ 0.09
	CXCL6	5	1.57 $\pm$ 0.42	5.91 $\pm$ 2.00
	CX3CL1	2	1.50 $\pm$ 0.73	2.78 $\pm$ 0.71
Group III	IL-1 α	5	2.49 $\pm$ 0.08	15.66 $\pm$ 0.86
	IL-1 β	4	2.32 $\pm$ 0.71	7.01 $\pm$ 2.22
	CCL3	3	1.71 $\pm$ 0.37	2.39 $\pm$ 0.004
	CCL3L1	3	5.93 $\pm$ 3.17	6.16 $\pm$ 3.04
	CCL5	0	7.89 $\pm$ 0.40	8.22 $\pm$ 0.85
	CCL8	5	5.47 $\pm$ 0.69	16.35 $\pm$ 1.62

Data are the mean $\pm$ S.D. from three to five patient pools, and each was repeated three times. (ND: not determined)

Table 2

Transcription factor binding motif prevalence in genes with over 10-fold change (*R*-score is the average *R*-score for this whole group promoters).

TRANSFAC motif Accession number	Transcription factor	<i>R</i> -Score (Average)
M00774	NF -kappaB	3.40983
M00208	NF -kappaB	3.21679
M00052	NF -kappaB (p65)	2.78441
MA0107	p65	2.77573
M00054	NF -kappaB	2.65063
MA0061	NF -kappaB	2.64151
M00053	c-Rel	2.56274
MA0101	c-REL	2.51207
M00319	MEF -3	2.4851
M00194	NF -kappaB	2.36984
M00086	I κ -1	2.25059
M00109	C/EBPbeta	2.14304
M00453	IRF -7	1.91043

Group A genes in this analysis were: IL-1 α , IL-1 β , IL-6, IL-8, CCL3, CCL3L1, CCL4, CCL5, CCL8, CCL20, CXCL1, CXCL2, CXCL3, CXCL5, and CXCL6.