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Leptin produced by joint white adipose tissue induces cartilage degradation via up-regulation and activation of matrix metalloproteinases

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Objectives: To investigate the effect of leptin on cartilage destruction.

Methods: Collagen release was assessed in bovine cartilage explant cultures, while collagenolytic and gelatinolytic activities in culture supernatants were determined by bioassay and gelatin zymography, respectively. The expression of matrix metalloproteinases (MMPs) was analysed by real-time RT-PCR. Signalling pathway activation was studied by immunoblotting. Leptin levels in cultured osteoarthritic joint infrapatellar fat pad or peri-enthesal deposit supernatants were measured by an immunoassay.

Results: Leptin, either alone or in synergy with IL-1, significantly induced collagen release from bovine cartilage by upregulation of collagenolytic and gelatinolytic activity. In chondrocytes, leptin induced *MMP1* and *MMP13* expression with a concomitant activation of STATs -1, -3, -5, MAPKs (JNK, Erk, p38), Akt and NF-κB signalling pathways. Selective inhibitor blockade of PI3K, p38, Erk and Akt pathways significantly reduced *MMP1* and *MMP13* expression in chondrocytes, and reduced cartilage-collagen release induced by leptin or leptin+IL-1. Interestingly, JNK inhibition had no effect on leptin-induced *MMP13* expression or leptin+IL-1-induced cartilage-collagen release. Conditioned media from cultured white adipose tissue (WAT) from OA knee joint fat pads, contained leptin, induced cartilage-collagen release and increased *MMP1* and *MMP13* expression in chondrocytes; the latter being partially blocked with an anti-leptin antibody.

Conclusions: This study demonstrates that leptin acts as a pro-inflammatory adipokine with a catabolic role on cartilage metabolism via the up-regulation of proteolytic enzymes and acts synergistically with other pro-inflammatory stimuli. Our data suggest that the infrapatellar fat pad, and other WAT, in arthritic joints is a local producer of leptin which may contribute to the inflammatory and degenerative processes in cartilage catabolism, providing a mechanistic link between obesity and OA.

Keywords

Leptin, osteoarthritis, cartilage, metalloproteinase

INTRODUCTION

Osteoarthritis (OA) is a complex disease with genetic, mechanical and environmental components leading to a number of changes within the joint but typified by the irreversible destruction of articular cartilage.(1) Articular cartilage is composed predominantly of an extracellular matrix (ECM) containing proteoglycan (aggrecan) and collagen (mainly type II collagen). Within this ECM chondrocytes control the turnover and remodelling of the cartilage matrix, through regulation of the expression of matrix components and matrix-degrading enzymes.(2) Type II collagen is cleaved by matrix metalloproteinases (MMPs), including MMP-1, -8, -13 and -14 whilst aggrecan is cleaved by 'A disintegrin and metalloproteinase with thrombospondin motifs' (ADAMTS) enzymes, most probably ADAMTS-5.(3) Together, these proteases release specific type II collagen or aggrecan fragments that can be measured *in vitro* and *in vivo.*(4) Whilst loss of aggrecan from cartilage is reversible, collagen breakdown is not and therefore represents an irreversible step in cartilage degradation.(5, 6)

A variety of cytokines and growth factors, such as the pro-inflammatory cytokines tumour necrosis factor α (TNFα) and interleukin-1 (IL-1) can induce the expression of many of the metalloproteinases described. Further, when in combination with oncostatin-M (OSM), an IL-6-family cytokine, these pro-inflammatory cytokines can synergistically enhance the production of catabolic MMPs.(7-9) These cytokines are key mediators of inflammation in rheumatoid arthritis but are increasingly being recognised as important in OA pathogenesis.(10)

One of the most significant risk factors for OA development is obesity (11, 12). The effects of obesity on OA can only partly be explained by an increase in biomechanical loading, since body mass index (BMI), or more specifically adiposity, is also a risk factor for OA in non-weight bearing joints, such as hands.(11, 13) These data suggest that an adipose-derived inflammatory factor acts as an obesity-related risk factor for OA. Adipose tissue secretes numerous cytokines and growth factors as well as the adipose-derived hormones, the adipokines (including adiponectin, resistin, visfatin and leptin)(14) to which chondrocytes are known to respond and in some cases produce. For example, adiponectin induces IL-8 expression by chondrocytes(15), OA chondrocytes are also responsive to both visfatin, which stimulates the expression of

ADAMTS4, *ADAMTS5*, *MMP3* and *MMP13* and induces prostaglandin E2 release, and resistin, that induces *MMP1*, *MMP13*, and *ADAMTS4*, both of which can thus be considered catabolic.(16, 17)

The best characterised role of leptin is to act as a signal for the central nervous system to inhibit food intake and to stimulate energy expenditure(18), however it also has a role in various physiological processes such as lipid metabolism, haematopoiesis, immune function, angiogenesis, reproduction, bone formation and inflammation.(19-25) It is mainly secreted by adipocytes and the levels of leptin within the circulation correlate with the amount of white adipose tissue (WAT).(18)

Articular cartilage produces leptin(26, 27) and expresses the functional leptin receptor Ob-R;(28) the expression of these two proteins is further increased in advanced OA and correlates with the BMI of OA patients.(29) Leptin has also been shown to have a pro-inflammatory effect on chondrocytes by inducing nitric oxide synthase.(30, 31)

The aim of the present study was to investigate the effects of leptin alone or in combination with pro-inflammatory cytokines on cartilage metabolism by measuring its effects on the release of cartilage collagen, expression and production of collagenases and gelatinases, and by evaluating the mechanisms involved in these effects. Importantly, we show that infrapatellar fat pad, and other WAT, produce significant amounts of leptin, which can contribute to the pro-inflammatory milieu of the joint, the induction of collagenase gene expression by chondrocytes, and potentially cartilage catabolism, providing a biological link between obesity and OA.

MATERIALS AND METHODS

IL-1α was a generous gift from Dr. K. Ray (Glaxo-SmithKline, Stevenage, UK), and recombinant human OSM prepared in-house using expression vectors kindly provided by Prof. J. Heath (University of Birmingham, UK) and methods described.(32) TNFα, recombinant human leptin and adiponectin, anti-human leptin monoclonal antibody and human leptin ELISA kit were from R&D Systems (Abingdon, UK). Resistin and visfatin were from First Link (UK) Ltd (Birmingham, UK). The mouse IgG control was from Becton-Dickinson (UK). Primary antibodies against phospho-Erk1/2 (phospho-p44/42) (#9101), phospho-p38 (#9211), phospho-JNK1/2 (#9251), phospho-p65 (Ser 536; #3031) and phospho-STATs were all from Cell Signaling Technology (New England Biolabs, Hitchin, UK). The phospho-Akt antibodies used have previously been described.(33) A rabbit anti-β Tubulin antibody (ab6046) was from Abcam (Cambridge, UK). Secondary immunoglobulins/HRP were from Cytomation (Dako, Glostrup, Denmark). Chemical pathway inhibitors LY294002, U0126, SB203580, SP600125 and Akt inhibitors IV and VIII were from Merck Chemicals (Nottingham, UK). Tissue culture reagents were obtained from Lifetechnologies (Paisley, UK).

Cartilage degradation and enzyme activity assay

Bovine nasal cartilage has been previously validated as a model relevant to human disease(34) and was cultured as previously described.(7) Briefly, three discs (~2 mm^3 discs) of bovine nasal cartilage/well were incubated ± test reagents in serum-free DMEM medium for 14 days, with a medium change at day 7. Selective pathway inhibitors were added 30 min prior to test reagents at the concentrations indicated.Day 7 and day 14 supernatants were stored at -20°C. Cartilage collagen degradation was measured using a hydroxyproline assay (7) and collagenase activities in culture supernatants were determined by the ^{3}H-acetylated collagen diffuse fibril assay (35). Aminophenylmercuric acetate (0.67mM) was used to activate pro-collagenases. Gelatin zymography was used to measure gelatinase activity in the culture supernatants.(36)

Chondrocyte isolation and culture

Primary human articular chondrocytes (HACs) were derived from articular cartilage obtained from joint replacement patients diagnosed with OA. Normal cartilage was obtained from neck of femur fracture (NOF) patients (no history of OA) undergoing joint replacement surgery. All tissue was obtained with informed consent and Ethical Committee approval from the Newcastle and North Tyneside Health Authority. Enzymatic digestion of tissue and maintenance and culture of cells were as previously described.(9) When cells reached 80-90% confluence without passage they were serum-starved for 16 hours before pre-treatment with selective pathway inhibitors (for 30 min at the indicated concentrations) prior to the addition of leptin, cytokines or WAT conditioned media at the concentrations and duration indicated as previously described.(37) Bovine nasal chondrocytes were isolated and cultured as previously described.(7, 9)

RNA extraction and real-time reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from cultured HACs or bovine chondrocytes and reverse transcribed using the Ambion Cells-to-cDNA II Kit (Lifetechnologies). Oligonucleotides were purchased from Sigma-Genosys (Poole, UK) and were as previously described.(36, 38) Relative quantification of genes was performed using the ABI Prism 7900HT sequence detection system (Lifetechnologies). Bovine metalloproteinase expression profiles were determined using SYBR Green (Takara, Cambrex, Wokingham, UK) in accordance with the manufacturer's protocol and as described.(38) Throughout, the *18s* rRNA gene was used as an endogenous house-keeping control. TaqMan® Probe based assays were performed using TaqMan® Gene Expression mastermix (Lifetechnologies) according to the manufacturer's protocol as previously reported.(36) Adipokine receptor expression in NOF and OA hip cartilage patient samples was determined as part of a TaqMan® low-density array screen using assays ADIPOR1-Hs00360422_m1, ADIPOR2-Hs00226105_m1, ADIPOQ-Hs00605917_m1, LEPR-Hs00174497_m1 and LEP-Hs00174877_m1 (Lifetechnologies), again normalised to the housekeeping gene *18s* rRNA.

Immunoblotting

Cells were lysed after the indicated time-points with ice-cold buffer (50mM Tris-HCl, pH 7.5; 1.2M glycerol; 1mM EGTA; 1mM EDTA; 1mM Na_3VO_4; 10mM β-glycerophosphate; 50mM NaF; 5mM sodium pyrophosphate; 1% (v/v) Triton X-100; 1μM microcystin-LR; 0.1% (v/v) β-mercaptoethanol; Roche protease inhibitor complex (Roche, UK)), particulate matter removed by centrifugation at 13,000*g*, 5min at 4°C, and lysates stored at -80°C until required. Lysates were resolved by SDS-PAGE, transferred to PVDF membranes (Millipore, Watford, UK) and subsequently probed using the antibodies described. The optimum time-points for signalling pathway activation were based upon our previous work with HAC. (33, 36, 37, 39)

White adipose tissue (WAT)-conditioned media

The infrapatellar fat pad or peri-enthesal deposits from human OA knee joints were the source of WAT. This was separated from the rest of the joint and any fibrous material, such as connective tissue or blood vessels, prior to dissection into small pieces of approximately 50mg. The tissue was then washed twice with phosphate buffered saline and once with serum-free DMEM. After washing, the WAT was incubated for 24hours at 37°C in DMEM culture medium containing 5% foetal calf serum (FCS) and antibiotics (streptomycin (100μg/ml) and penicillin (100IU/ml)), which was replaced with serum-free DMEM (1ml/0.3g WAT) and incubated for 72hours at 37°C. After harvesting, the media were filter sterilised and stored at -80°C prior to use. To quantify the amount of leptin present in the WAT-conditioned media, the Human Leptin Quantikine ELISA Kit (R&D Systems, UK) was performed according to manufacturers' instructions.

Statistical analysis

For cartilage degradation assays and collagenase bioassays one-way analysis of variance (ANOVA) with Bonferroni post-hoc statistical tests were performed. Statistical differences between sample groups in the real-time RT-PCR experiments were assessed using the two-tailed Student's *t*-test. SPSS 15.0 software was used for all statistical analyses. Significance levels were indicated as * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

RESULTS

Leptin induces collagenase gene expression and cartilage resorption

To study the effects of leptin on cartilage catabolism, bovine cartilage was stimulated with leptin in the absence or presence of several pro-inflammatory cytokines; IL-1, TNFα or OSM. Leptin alone induced low but significant ($p<0.05$) collagen release (approximately 5%) compared to control (Fig. 1A). IL-1 or TNFα alone induced ~30% collagen release but when combined with leptin this synergistically increased to >60% (Fig. 1A). This increase in collagen release was accompanied by an up-regulation of total and active collagenolytic and gelatinolytic (MMP-2 and MMP-9) activities (Fig. 1B and 1C), demonstrating a role of leptin in the activation of a number of proMMPs. OSM alone or in combination with leptin did not effect cartilage resorption.

Our previous work has focussed on synergy between various inflammatory mediators, especially with IL-1, and the potent regulation of the collagenases.(7-9, 33, 36, 40) We therefore assessed the synergy between leptin and IL-1. A concentration-dependent increase in cartilage-collagen degradation was observed with leptin concentrations exceeding 10μg/ml although when in combination with IL-1 (0.25ng/ml) even the lowest concentration of leptin tested (1μg/ml) elicited a synergistic increase in cartilage collagen release (Fig. 1D). We also investigated whether other adipokines (visfatin, resistin and adiponectin) could induce or potentiate cartilage resorption but, surprisingly only leptin showed such efficacy (Sup. Fig. 1A) despite previous literature demonstrating their ability to induce a catabolic phenotype in chondrocytes(15-17) and significant expression by chondrocytes of adiponectin receptors (Sup. Fig. 2).

When added to primary HAC, leptin induced the gene expression of the major collagenases *MMP1* and *MMP13*. Again, when in combination with IL-1 (0.25ng/ml) higher concentrations of leptin (>1 μg/ml) synergistically induced these collagenases. (Fig. 1E and 1F). A similar induction of *MMP1* and *MMP13* by leptin and leptin + IL-1 was also observed in isolated bovine chondrocytes (Sup. Fig 1B and C), emphasising the suitability of the bovine cartilage model.

Leptin activates STAT, MAPK and Akt signalling in primary HAC

Stimulation of primary HAC with leptin ± IL-1 activated multiple signalling pathways. Leptin alone induced STAT1, -3 and -5 tyrosine phosphorylation (at positions Y701, Y705 and Y694, respectively) but failed to induce robust STAT serine phosphorylation with the exception of a modest increase in STAT3 S727 phosphorylation. This was in contrast to IL-1 which only induced STAT serine phosphorylation. Some synergy between leptin and IL-1 occurred with the phosphorylation of STAT1 and STAT3 at S727. These activation events declined by 60 min post-stimulation (Fig. 2A). Leptin alone activated all three mitogen-activated protein kinase (MAPK) pathways, evidenced by phosphorylation of the stress-activated protein kinase/cJun-amino-terminal kinase JNK (T183/Y185), Erk (T202/Y204 of Erk1) and p38 (T180/Y182). When combined with IL-1, an increase in phosphorylated p38 and JNK1/2 were detected (Fig. 2B), the latter only becoming obvious by 60 min. Leptin also led to the phosphorylation of Akt (at S473 and Y308) although no additional phosphorylation was observed when combined with IL-1 (Fig. 2C). Finally, leptin led to a modest phosphorylation of the NF-κB subunit p65 at 20 min but, as with the serine phosphorylation of STAT-1/3, this was not as pronounced as that elicited by IL-1 alone (Fig. 2C).

Different signalling pathways control leptin-induced collagenase expression and cartilage-collagen release

Next, to test whether leptin regulated the expression of *MMP1* and *MMP13* via the identified signalling pathways, HAC were cultured in the presence of selective pathway inhibitors. All the inhibitors, with the exception of Akt VIII, significantly suppressed leptin-induced expression of *MMP1*, while only the JNK inhibitor, SP600125, failed to inhibit leptin-induced *MMP13* (Fig. 2D and E).

In the bovine cartilage resorption assay all the inhibitors significantly reduced the modest collagen release elicited by leptin (Fig. 3A). When the combination of IL-1 and leptin was used as the stimulus, only the JNK and Akt VIII inhibitors were ineffective at blocking resorption (Fig. 3B). This inhibitor profile was reminiscent to that seen for the *MMP13* gene expression experiment (compare Fig. 2E and 3B).

WAT-conditioned culture media induce MMPs and cartilage resorption

In order to assess whether WAT-derived leptin WAT might have an impact on cartilage degradation we initially confirmed that WAT-, not control, -conditioned media were able to induce *MMP1* and *MMP13* by primary bovine chondrocytes (Sup. Fig. 3A). Next, WAT-conditioned culture media (using fat pads from 4 different OA patients) were incubated with bovine nasal cartilage ± IL-1. Alone, the WAT-conditioned media induced modest, but significant, collagen release compared with control-conditioned medium (Fig. 3C) and when combined with IL-1, a further synergistic release of proteoglycan (not shown) and collagen was observed (Fig. 3D).

Leptin in WAT-conditioned media contributes to the induction of *MMP1* and *MMP13* expression

Next, HAC were cultured with WAT-conditioned media taken from fat pads of 14 OA patients. Ten WAT-media samples significantly up-regulated *MMP13,* while the same ten samples, plus N1688, also significantly up-regulated *MMP1* expression. The levels of induction of *MMP1* and *MMP13* obtained were comparable to that induced by leptin (25μg/ml) alone (Fig. 4A). To determine if the induction of the collagenases induced by the WAT-media was in part mediated by secreted leptin we first quantified the amount of leptin present in the WAT-conditioned media using a specific leptin immunoassay (Fig. 4B). After the removal of outliers, 12 WAT-conditioned media contained a wide-range of leptin levels from 99 to 1777pg/ml (Fig. 4B). Using regression analysis, the amount of leptin showed a trend towards a positive correlation with the *MMP1* and *MMP13* fold-induction by these media (R^2 = 0.1798 and 0.4897, respectively) which approached but importantly did not reach significance, suggestive of a role for leptin in combination with other WAT-produced pro-inflammatory factors in collagenase induction. To attempt to conclusively test if the leptin produced by the WAT was involved in the up-regulation of the collagenases induced by the conditioned media, HAC were incubated with WAT-conditioned media ± an anti-leptin antibody. When analysing the WAT-conditioned media that induced each collagenase >5 fold, the leptin blocking antibody led to a significant reduction in the induced collagenase levels (Fig. 4C and D). Finally, a

similar experiment was repeated where HAC were again incubated with WAT-conditioned media ± an anti-leptin or control (IgG) antibody. As expected, the anti-leptin but not the control antibody significantly reduced the level of induction of both collagenases by leptin, and moreover did so in 4/5 WAT-conditioned media examined (Sup. Fig. 3B and C).

DISCUSSION

Several studies have linked leptin, obesity and OA.(27, 41-47) For example, serum leptin concentrations not only correlate with BMI but are also closely related to the radiographic severity, joint inflammation and disease severity of OA. Others have reported a marked increase in leptin protein expression in OA cartilage compared to normal cartilage.(26) However, the most compelling evidence that leptin plays a direct, rather than correlative, role in OA pathogenesis comes from studies using leptin-signalling deficient mice. These mice are severely obese yet exhibited the same (increased) level of OA incidence as that of obese wild-type mice.(48)

In the current study, we demonstrated for the first time that leptin, but surprisingly not the other adipokines visfatin, resistin or adiponectin, can directly induce cartilage collagen degradation, alone and when combined with pro-inflammatory cytokines (IL-1 or TNFα). Adiponectin has previously been shown to induce the expression of IL-8 by chondrocytes (15) but also to have a chondroprotective role.(49) We found little response to this adipokine in our assays even though one of its receptors (ADIPOR2) was highly, and moreover, differentially expressed in diseased cartilage. We also found bovine cartilage to be unresponsive to both resistin and visfatin (sup. Fig. 1), both of which have previously been reported to induce a number of metalloproteinases when added to chondrocytes.(16, 17) These discrepancies could be due to a number of factors, including tissue examined (cartilage vs. cells), species, source of adipokine and culture conditions. The effects of leptin upon cartilage collagen release were accompanied with an up-regulation of collagenase and gelatinase activities in the culture medium. A recent study has similarly shown an induction of the collagenases from human OA cartilage following leptin or leptin + IL-1 stimulation.(50) The same group also showed that leptin alone or in combination with IL-1 enhanced the expression of the pro-inflammatory mediators iNOS and COX-2, and production of NO, PGE2, IL-6 and IL-8.(43)

Leptin signalling through leptin receptors (Ob-R/LEPR) is mediated via the JAK/STAT (janus kinase/signal transducers and activators of transcription) pathway, but additionally the phosphatidylinositol 3-kinase (PI3K)/Akt, MAPK and NF-κB pathways, all of which have been reported to be important in leptin signalling by chondrocytes (30, 43), in accordance with the data presented here. Interestingly, our data show

that leptin alone is a better inducer of STAT phosphorylation at the tyrosine position rather than serine, the latter only becoming prominent when leptin acts synergistically with IL-1. This is consistent with the role of the tyrosine kinase family JAKs, probably JAK2, in Ob-R (LEPR) signalling.(51) We also showed for the first time that the MAPK and PI3K pathways are important mediators of leptin signalling in chondrocytes and cartilage since chemical blockade of any of the these pathways prevented cartilage collagen release. Interestingly, the JNK inhibitor, SP600125, did not inhibit either leptin+IL-1-induced cartilage-collagen release or leptin-induced *MMP13* by chondrocytes but did block *MMP1* levels, supporting the dogma that MMP-13 is the major type-II collagen degrading enzyme(52) and validating the bovine cartilage model.

Gradual loss of articular cartilage is a major characteristic of OA, with initial changes including the loss of proteoglycan. With this in mind, the *in vivo* injection of leptin into the joints of rats induces cartilage proteoglycan loss and increases the expression of MMP-2 and -9 in articular cartilage.(46) The destructive process in OA is determined by an imbalance between anabolic and catabolic mediators (and their regulators), in the joint, and the local distribution of these mediators in the cartilage.(10, 52) Leptin appears to be an important local and systemic factor influencing cartilage/bone homeostasis. It is likely that locally produced, rather than circulatory, leptin may be more important in regulating cartilage homeostasis since the concentration of leptin in synovial fluid is higher than in the corresponding sera of OA patients (29), and these levels are associated with disease activity.(26, 53) Moreover, expression of leptin and LEPR have previously been detected in human cartilage with levels significantly increased in end-stage OA cartilage.(26, 41) The endogenous leptin produced by chondrocytes has also been shown to induce *MMP13* expression.(54) However, although we could detect leptin and LEPR expression in cartilage, neither were differentially expressed in OA in our experiments and the level of leptin expression detected was deemed negligible (Sup. Fig. 2). Therefore, we focussed on leptin production by the infrapatellar fat pad, a joint tissue reported to be a site of leptin production.(41) For the first time to our knowledge we have shown that WAT-conditioned media, collected from cultured infrapatellar fat pad and other WAT of OA knee joints, contains significant quantities

of leptin, the amount of which correlated with the ability of the WAT-conditioned media to stimulate *MMP1* and *MMP13* expression in chondrocytes. These WAT-conditioned media were also able to induce collagen release from cartilage cultures. Furthermore, the ability of these WAT-conditioned media to stimulate *MMP1* and *MMP13* expression was partially abolished by an anti-leptin antibody. Together, these data suggest that leptin produced by the WAT-conditioned media was in part responsible for the cartilage degradation observed, but importantly that these media contain other pro-inflammatory cytokines, such as IL-1, whose expression by WAT-tissue we could detect (data not shown), that synergise with leptin to induce collagenase expression.

Taken together our findings suggests that the infrapatellar fat pad of arthritic joints can be a significant local producer of leptin and may contribute to inflammatory and degenerative processes in OA cartilage catabolism via the up-regulation of the collagenolytic metalloproteinases MMP-1 and MMP-13, thus providing a direct mechanistic link between obesity and OA.

AUTHOR CONTRIBUTIONS

GJL, TEC, ADR and DAY were responsible for the conception and design of the study and analysis and interpretation of the data. WH, ME, GJL and GIK collected and assembled the data. WH, GJL, ADR and DAY were responsible for drafting of the article. ADR, TEC and DAY obtained funding for the study. All authors approved the final article.

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FIGURE LEGENDS:

Fig. 1. Leptin in combination with pro-inflammatory cytokines induces cartilage-collagen release from bovine cartilage by enhancing collagenolytic and gelatinolytic activities. Bovine nasal cartilage was treated with leptin (25μg/ml unless indicated otherwise) with OSM (10ng/ml), IL-1α (0.25ng/ml) or TNFα (10ng/ml) in serum-free medium for 14 days. The release of collagen and enzyme activities were assayed as described in Material and Methods. **A** and **D,** Cumulative collagen release by day 14 is expressed as a percentage of the total (mean ± SD, n=4). Inset shows an expansion of the leptin alone data for clarity. **B**, Active (open bars) and total (solid bars) collagenolytic activity values (mean ± SD, n=4). **C**, Gelatin zymography of day 14 media. Pro and active MMP-2 and MMP-9 are indicated. *p< 0.05; **p< 0.01; ***p<0.001 vs. each respective control. Data are representative of 3 separate cartilage experiments. **E** and **F**, primary human articular chondrocytes were stimulated with leptin as indicated ± IL-1 (0.25ng/ml) for 24 hours. The expression of *MMP1* and *MMP13* and *18s* were assayed by real time RT-PCR. The levels of *MMP1* (solid bars; **E**) and *MMP13* (grey bars; **F**) were expressed as fold against 18s rRNA. *p< 0.05; **p< 0.01 vs. control or cytokine alone. Experiments were performed as n=4 and are representative of 3 independent experiments.

Fig. 2. Signalling pathways activated after leptin and leptin+IL-1 stimulation of chondrocytes and their role in collagenase expression. Primary HAC were serum-starved overnight and then stimulated for 20 and 60 min with either leptin (25μg/ml) or IL-1 (0.25ng/ml) alone or in combination. Cells were lysed, and extracts immunoblotted with antibodies against (**A**) phospho-STAT1 (Y701 or S727), phospho-STAT3 (Y705 and S727), phospho-STAT5 (Y694) and β-tubulin as loading control; (**B**) phospho-Erk (p44/42) (T202/Y204 of Erk1), phospho-JNK (SAPK)(T183/Y185), phospho-p38 (T180/Y182); or (**C**) phospho-Akt (S473 and Y308) and phospho-p65 (S536). **D** and **E**. Primary human chondrocytes were stimulated with leptin (25μg/ml) ± the inhibitors; LY (LY249002, 5μM), U0126 (5μM), SB (SB203580, 10μM), SP (SP600125, 10μM), Akt IV (3μM) or Akt VIII (3μM) for 20 hours. Cells were pre-treated for 30 min with inhibitors or vehicle control prior to the addition of leptin.

The expression of *MMP1* (**D**) and *MMP13* (**E**) was assayed by real time RT-PCR and expressed as fold relative to the control after normalisation to *18s* rRNA. *p<0.05; **p<0.01; ***p<0.001 vs. control. Data are representative of 3 separate experiments.

Fig. 3. Effects of signalling pathway inhibitors on leptin induced cartilage-collagen release and WAT-conditioned media cartilage degradation. **A**. Bovine cartilage was stimulated with leptin (25μg/ml) ± inhibitors; LY (LY249002, 5μM), U0126 (5μM), SB (SB203580, 10μM), SP (SP600125, 10μM), Akt IV (3μM) or Akt VIII (3μM) or (**B**) leptin+IL-1 (0.25ng/ml) ± the pathway inhibitors described above, in serum-free medium for 14 days. Cartilage was pre-treated for 30 min with inhibitors or vehicle control prior to the addition of leptin or leptin+IL-1. **C** and **D.** WAT samples were collected from the infrapatellar fat pad of OA patients as described and incubated in 5% FCS/DMEM culture medium (1ml medium/0.3g WAT) at 37°C for 24 hours, then incubated in serum-free DMEM for 3 days. 20% (v/v) WAT-conditioned media from four patients were then added to bovine cartilage alone (**C**) or +IL-1 (0.25ng/ml) (**D**) in serum-free medium for 14 days. In all experiments on day 7, media were collected and replaced with identical test reagents. The release of collagen was assayed as described in Material and Methods. Cumulative collagen release by day 14 is expressed as the percentage of total (n=4). *p<0.05; **p<0.01; ***p<0.001 vs. control. Data are representative of 3 separate experiments.

Fig. 4. Leptin in WAT-conditioned media and its effect on *MMP1* and *MMP13* expression. A, Primary HAC were cultured in serum-free medium for 24 hours and then incubated with 20% (v/v) WAT-conditioned media in DMEM for 24 hours. The expression of *MMP1* (black) and *MMP13* (grey), normalised to *18s* rRNA, were assayed by real time RT-PCR (n=4/WAT-conditioned medium). **B**, The levels of leptin in WAT-conditioned media collected from OA fat pad were assayed by ELISA then expressed relative to the fold induction of *MMP1* (o) or *MMP13* (■) for each WAT-medium sample. Best fit lines are leptin vs. *MMP1*, broken; leptin vs. *MMP13*, solid. **C** and **D,** Primary HAC were co-incubated with an anti-leptin antibody (20μg/ml) and 20% (v/v) WAT-conditioned media in DMEM for 24 hours, then the expression of *MMP1* (**C**) and *MMP13* (**D**) assayed by real time RT-PCR and normalised to *18s* rRNA

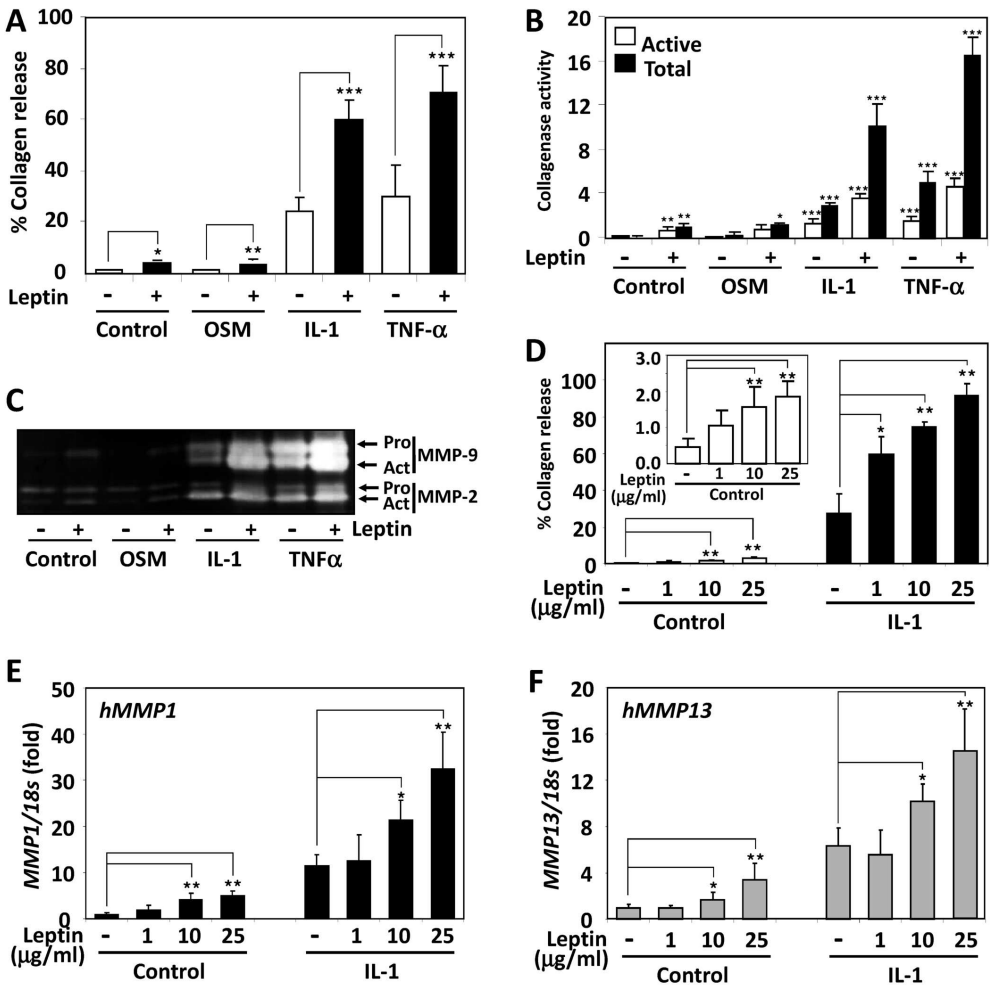
levels (n=3). Only WAT-media which induced a >5 fold increase in collagenase expression are included. *p< 0.05; **p< 0.01 and ***p<0.001 vs. basal control or WAT-conditioned medium induced levels.

Supplementary Figure 1. A. Other adipokines ±IL-1 do not induces cartilage-collagen release from bovine cartilage. Bovine nasal cartilage was treated with resistin (R), visfatin (V) or adiponectin (Ad) (all at 25µg/ml) ± IL-1 (0.25ng/ml) in serum-free medium for 14 days. The release of collagen from the cartilage was assayed as described in Material and Methods. Data are representative of 3 separate cartilage experiments. **B** and **C**, Primary bovine chondrocytes stimulated with leptin as indicated ± IL-1 (0.25ng/ml) for 24 hours. The expression of *MMP1* and *MMP13* and 18s were assayed by real time RT-PCR. The levels of *MMP1* (solid bars; **B**) and *MMP13* (grey bars; **C**) were expressed as fold against 18s rRNA. **p< 0.01 vs. control or cytokine alone. Experiments were performed as n=4 and are representative of 3 independent experiments.

Supplementary Figure 2. Comparative gene expression between cartilages from fracture to neck of femur (NOF) and osteoarthritis (OA) patients. The expression of the genes indicated were determined by real-time RT-PCR from total RNA isolated from OA (open) and NOF (shaded) cartilage samples. Gene expression data are presented as a ratio of the gene levels to those of the housekeeping gene *18s* rRNA using the calculation $2^{-\Delta C}{}_{T}$. Lines within the boxes represent the median, the boxes represent the 25th and 75th percentiles, and the lines outside the boxes are the minimum and maximum values. Only the expression of *ADIPOR2* was significantly differentially expressed where ***p<0.001 (Mann-Whitney U-test).

Supplementary Figure 3. Effects of WAT-conditioned media on collagenase expression. WAT samples were collected from the infrapatellar fat pad of OA patients and processed as described. After culture in serum-free DMEM for 3 days, harvested media were then added to cell cultures at 20% (v/v) WAT-conditioned media in DMEM for 24 hours. B. Primary bovine nasal chondrocytes were incubated with WAT-conditioned media and *MMP1* and *MMP13* expression measured by real-time RT-PCR and normalised to *18s* rRNA in six separate experiments (with six replicates/WAT sample). The average fold-induction for each WAT sample (n=19 for *MMP1* and n=15 for *MMP13*) is plotted with the lines within the boxes representing

the median, the boxes represent the 25th and 75th percentiles, and lines outside the boxes are the minimum and maximum values. **B** and **C**. Primary HAC were co-incubated with an anti-leptin antibody (20µg/ml) or irrelevant control (IgG) and WAT-conditioned media for 24 hours, then the expression of *MMP1* (**C**) and *MMP13* (**D**) assayed by real time RT-PCR (n=3) and normalised to *18s*. *p< 0.05; **p< 0.01 and ***p<0.001 vs. basal control or WAT-conditioned medium induced levels.



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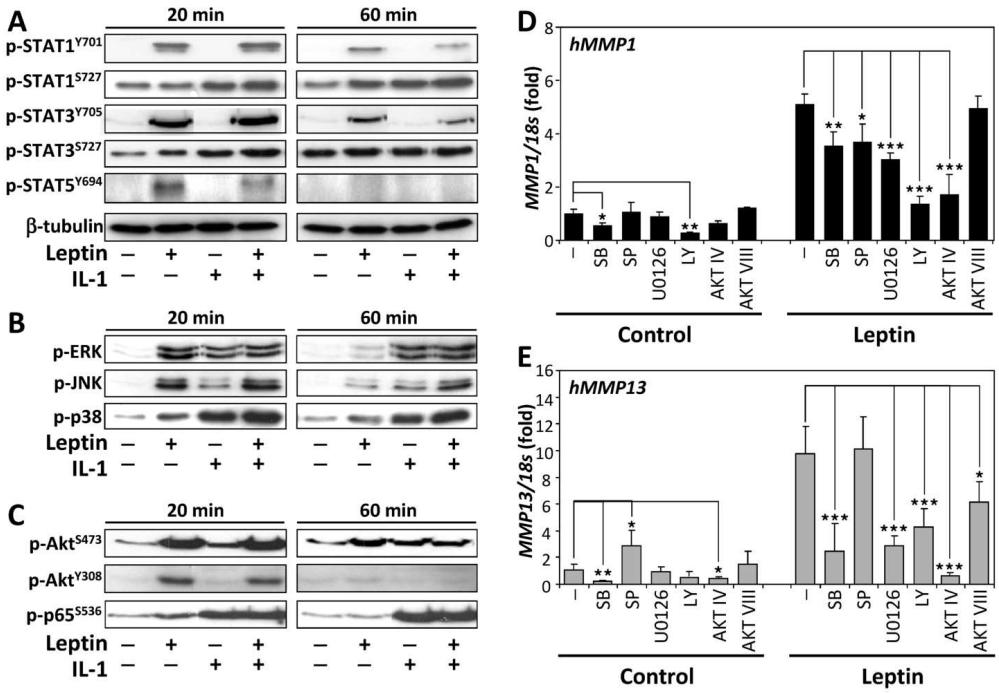

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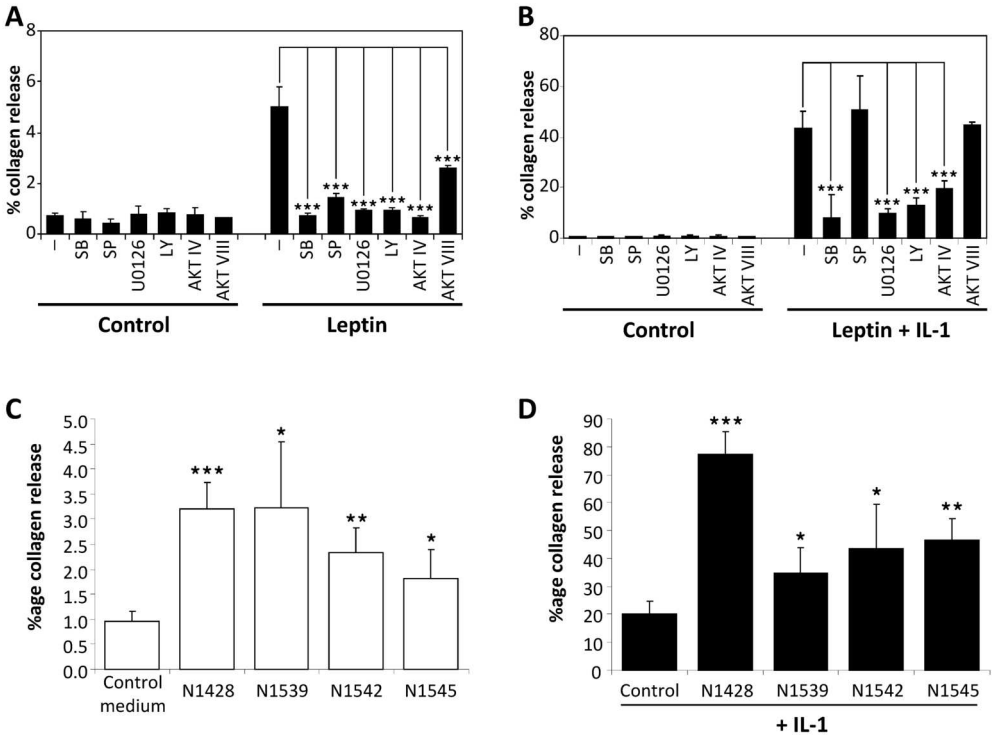

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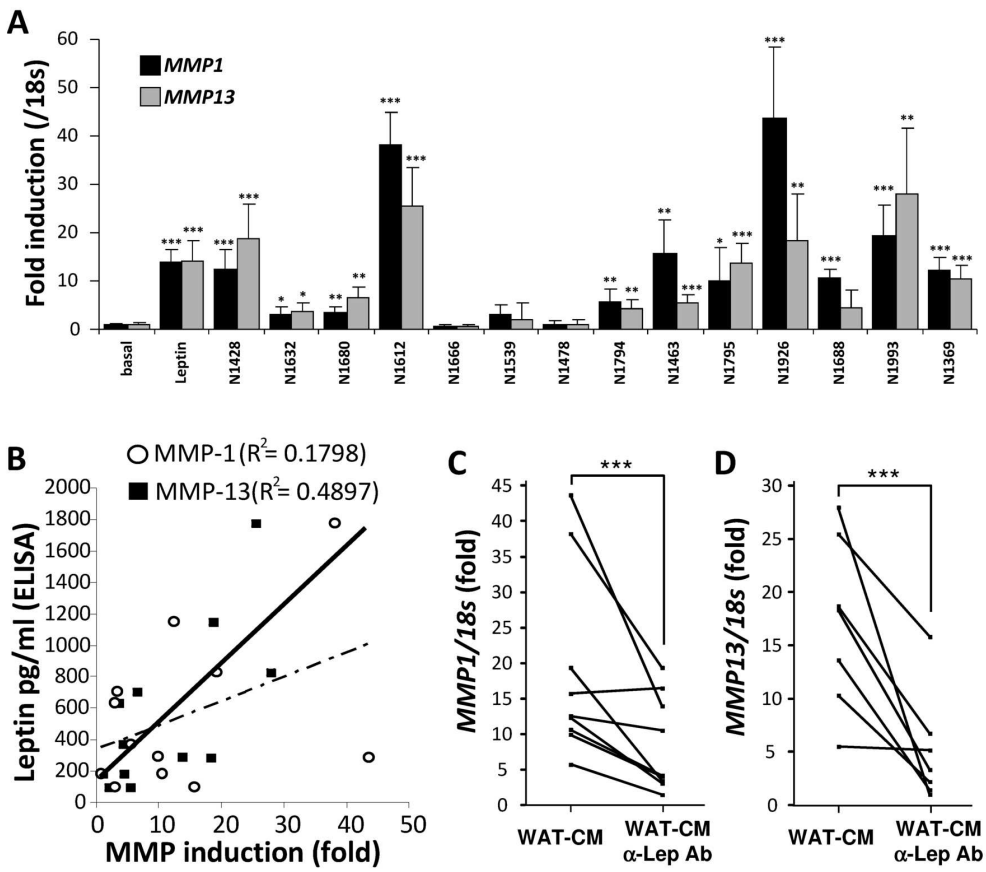

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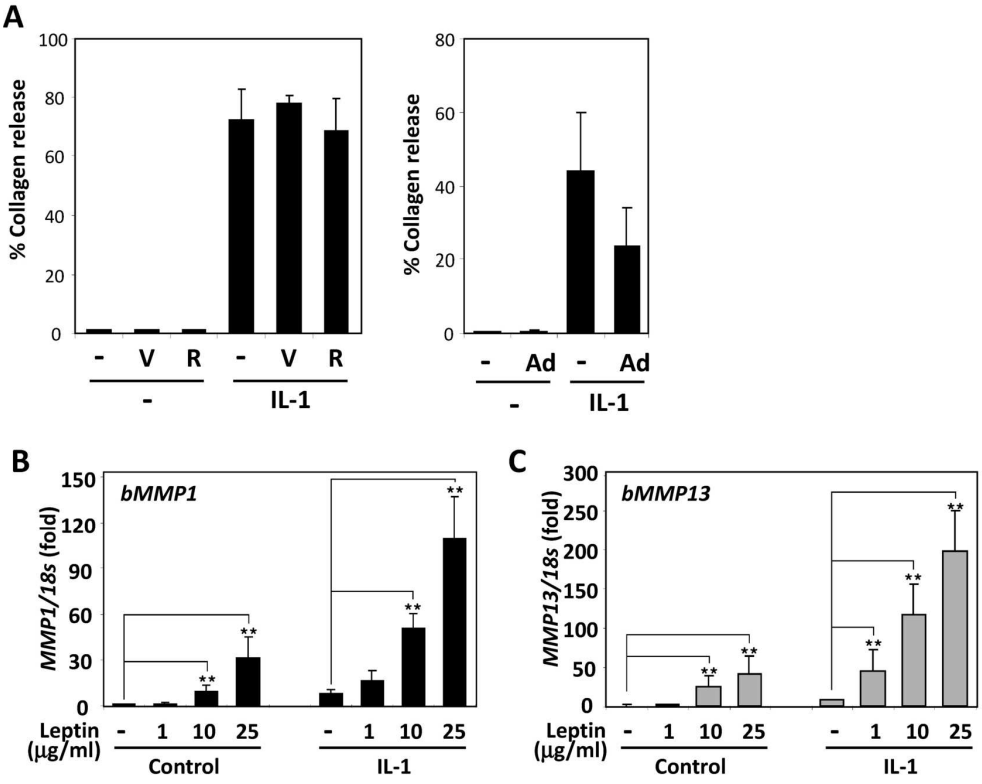

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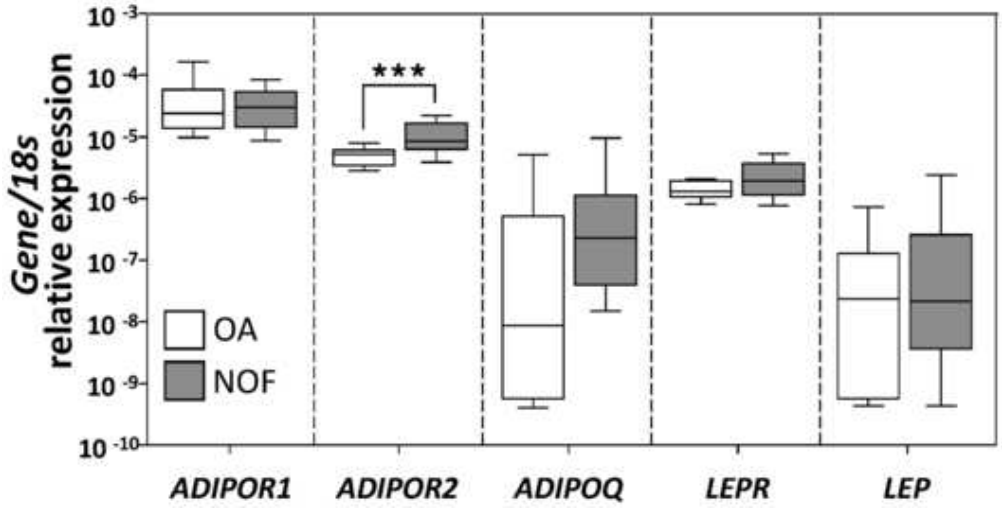

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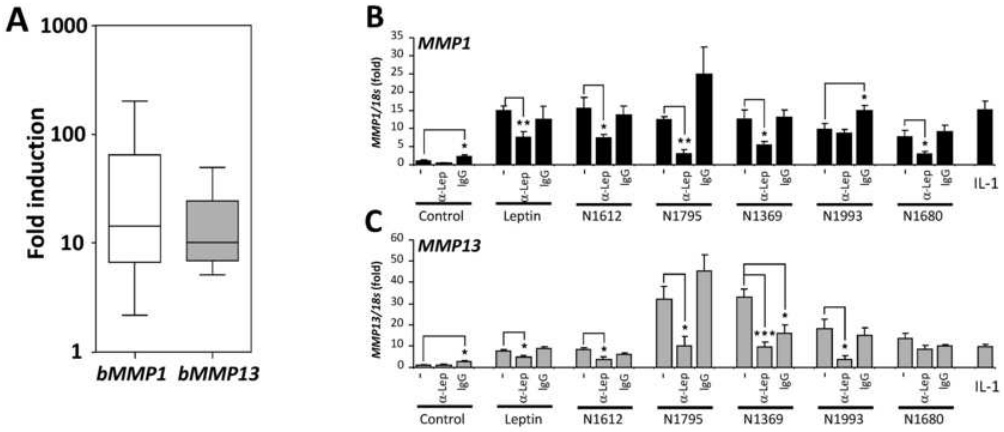

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