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Meniscus is more susceptible than cartilage to catabolic and anti-anabolic effects of adipokines

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SUMMARY

Objective: This study compared the effects on cartilage and meniscus matrix catabolism and biosynthesis of several adipokines implicated in osteoarthritis (OA).
Design: Bovine cartilage and meniscus explants were cultured for 1 or 9 days in serum-free medium alone or with 0.02, 0.2, or 2 μg/ml of leptin, visfatin, adiponectin, or resistin. Media were supplemented with ^{3}H-proline or ^{35}S-sodium sulfate to evaluate protein and sulfated glycosaminoglycan (sGAG) accumulation on the last day of culture. Explants were assayed for radiolabel, sGAG, and DNA contents. Cultured media were assayed for sGAG, nitrite and lactate dehydrogenase.
Results: Cartilage tissue was minimally affected by adipokines, with only the highest resistin dose increasing sGAG release and nitrite production compared to controls. In sharp contrast, meniscus tissue was responsive to several adipokines, with elevated sGAG and nitrite release following treatment with resistin, leptin, or visfatin. Cartilage sGAG content was unaltered by adipokine treatment whereas meniscal sGAG content significantly decreased with resistin dosage. Protein (^{3}H) incorporation was unaffected by adipokine treatment in both tissues. sGAG (^{35}S) incorporation did not significantly vary with adipokine treatment in cartilage but was inhibited by treatment with leptin, visfatin, and resistin in meniscus.
Conclusion: Our results indicate that meniscal tissue is more susceptible to adipokine-stimulated catabolism than is cartilage. Resistin had the strongest effect of the adipokines tested, inducing sGAG release in both tissues and depleting sGAG content in meniscus. These results suggest that increased adipokine levels due to obesity or joint injury may alter the mechanical integrity of the knee joint through biological pathways.

Introduction

Obesity in developed nations is at epidemic levels, with prevalence among US adults estimated at 36% in 2009–10[1] and predicted to reach about 50% by 2030[2]. Obesity is a major risk factor for hip and knee osteoarthritis (OA), with every 5 unit increase in body mass index (BMI) doubling the risk of OA[3]. The association between obesity and OA has been viewed as predominantly biomechanical[4], but overloading may not be the only link. Knee OA incidence and progression were more strongly associated with fat mass than with BMI or total mass[5], and obesity has been identified as a strong risk factor for hand OA[6,7], suggesting that systemic, biologic links between obesity and OA may exist in addition to direct mechanical effects.

Adipose tissue is now recognized as a metabolically active organ[8] that secretes biologically active factors collectively described as adipokines. Altered adipokine levels have been implicated in diabetes, cardiovascular disease, metabolic syndrome, and inflammatory disorders including rheumatoid arthritis[9]. With observations of adipokines including leptin[10], visfatin[11], adiponectin and resistin[12] in the synovial fluid of osteoarthritic joints, roles for adipokines in the onset and progression of OA have been hypothesized[13]. In the knee, the synovium and infrapatellar fat pad have been identified as major adipokine producers[14,15]. Elevated leptin[10,16], resistin[17,18], and visfatin[19,20] levels in both serum and synovial fluid have been associated with increased OA progression or

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severity, but the association of adiponectin with OA remains unclear[21,22].

In OA joints, synovial fluid concentrations range around 10–20 ng/mL for leptin[10,14,16], 10–40 ng/mL for visfatin[19,20], 0.3–2.3 μg/ml for adiponectin[12,14,23], and 3–50 ng/mL for resistin[12,14,17]. Increased BMI correlates with elevated synovial fluid concentrations of leptin[10] but lower synovial fluid concentrations of adiponectin[21,24]. While studies have reported no correlation between synovial fluid concentrations of visfatin[20] or resistin[24] and BMI, both correlated with OA severity.

Various adipokines have induced cell-mediated cartilage catabolism *in vitro*. Leptin induced production of proinflammatory factors and enhanced matrix metalloproteinase (MMP) expression in OA cartilage[25,26]. Visfatin stimulated chondrocyte aggrecanase expression and MMP production[11], while adiponectin induced production and expression of several MMPs and proinflammatory factors[22,27]. Resistin induced production of proinflammatory factors and inhibited matrix synthesis in mouse cartilage[17] and increased aggrecanase and MMP expression by human chondrocytes[28]. Particularly relevant to the potential involvement of adipokines in OA development, serum and synovial fluid level of resistin were elevated following traumatic joint injury (median resistin level approximately 3 ng/ml 1 week following injury)[17].

However, little is known about the effects of adipokines on joint tissues other than articular cartilage. A recent investigation demonstrated that 100 ng/ml visfatin increased nitric oxide production and MMP activity or matrix degradation in adult porcine cartilage and meniscus[29]. Particularly given growing evidence that meniscal degeneration is an early event in knee OA that accompanies or precedes cartilage degeneration[30,31], a more detailed understanding of the effects of catabolic mediators on meniscal metabolism is desirable. The goals of this study were to evaluate dose-dependent catabolic and anti-anabolic effects of leptin, visfatin, adiponectin and resistin on cartilage and meniscus tissue explants, and to examine the relative susceptibility of these tissues to each adipokine.

Method

Materials

Immature bovine stifles were from Research 87 (Marlborough, MA). High glucose Dulbecco's modified Eagle's medium (DMEM) was from HyClone (Logan, UT). Non-essential amino acids (NEAA) and proteinase K were from Life Technologies (Carlsbad, CA). N-(2-hydroxyethyl)-piperazine-N′-2-ethanesulfonic acid (HEPES), gentamicin and phosphate buffered saline (PBS) were from Mediatech (Manassas, VA). Insulin-transferrin-selenous acid premix (ITS+) was from BD Biosciences (Franklin Lakes, NJ). L-ascorbic acid 2-phosphate, proline, ammonium acetate, 1,9-dimethyl-methylene blue (DMMB), shark chondroitin sulfate, sodium nitrite, bisbenzimide (Hoechst 33258), and calf thymus DNA were from Sigma (St. Louis, MO). Sulfanilamide reagent and naphthylethylenediamine dihydrochloride solution were from Ricca Chemical (Arlington, TX). The CytoTox-ONE™ Homogeneous Membrane Integrity assay kit was from Promega (Madison, WI). ^{3}H-proline and ^{35}S-sodium sulfate were from PerkinElmer (Waltham, MA). Recombinant human leptin was from Shenandoah Biotechnology (Warwick, PA), recombinant human visfatin was from Novus Biologicals (Littleton, CO), and recombinant human adiponectin and resistin were from BioVendor (Candler, NC).

Experimental design

Independent experiments evaluated the effects of initial (1 day) and sustained (9 day) exposure to adipokines. Both experiments involved two substudies, each consisting of an untreated control group and three doses (0.02, 0.2 and 2 μg/ml) of two adipokines: leptin and visfatin in one substudy, adiponectin and resistin in the other. Tissue explants for each substudy were isolated from four immature bovine stifles (one each from four animals, total of sixteen donors), and samples from different donors and relative positions (anterior, central, posterior) were distributed across experimental groups ($n = 6$–8 explants/tissue/condition).

Tissue culture

Using a 4 mm biopsy punch, full thickness explants were isolated from the lateral femoral condylar cartilage and the caudal surface of the radially middle region of lateral menisci from bovine stifles. Explants were cultured at 37°C, 5% CO_2, and 95% relative humidity in basal, serum-free medium consisting of high glucose DMEM with 0.1 mM NEAA, 10 mM HEPES buffer, 50 μg/ml L-ascorbic acid-2-PO_4, 50 μg/ml gentamicin, 0.4 mM proline, and 1% ITS+ premix. After overnight culture, explants were trimmed to 2 mm thickness retaining intact articular surfaces.

Radiolabeled cultured media were prepared with 20 μCi/ml of ^{3}H-proline and 10 μCi/ml of ^{35}S-sodium sulfate to label biosynthesis of proteins and sulfated glycosaminoglycans (sGAGs), respectively. In the first experiment, explants were cultured for 22 h in radiolabeled basal medium alone (control) or supplemented with 0.02, 0.2, or 2 μg/ml of adipokines, as described above. In the second experiment, explants were cultured for 8 days in radiolabel-free media supplemented with adipokines, with media exchanges every 48 h. Explants were then cultured for an additional 22 h in media supplemented with radiolabels to assess chronic effects of adipokines on biosynthesis. Conditioned media were collected and stored at −20°C for later analysis.

Biochemistry

Explants were washed four times for 30 min each at 4°C in PBS supplemented with 0.8 mM sodium sulfate and 1.0 mM L-proline to remove unincorporated radiolabels. Explants were weighed, lyophilized overnight and re-weighed dry, then digested overnight at 60°C in 1 ml proteinase K (1 mg/40 mg cartilage wet mass, 1 mg/20 mg meniscus wet mass) buffered with 100 mM ammonium acetate. Digest radiolabel contents were measured using a liquid scintillation counter. Explant DNA contents were measured using the Hoechst 33258 assay[32] with calf thymus DNA standards. Sulfated glycosaminoglycan (sGAG) contents of digested explants and conditioned media were assayed using the dimethylmethylene blue (DMMB) assay[33] with chondroitin sulfate standards ranging from 0 to 100 μg/ml. To estimate production of nitric oxide (NO), a cell signaling mediator in inflammation[34], nitrite contents of conditioned media were measured using the Griess assay[35] with sodium nitrite standards ranging from 0 to 100 μM. Lactate dehydrogenase (LDH), a surrogate indicator of cell lysis, was measured in conditioned media from the 9-day culture studies using the CytoTox-ONE™ kit with purified LDH from fibrocytes as standards. As necessary, samples were diluted to remain in the linear region of each assay.

Data analysis

Radiolabel incorporation of each explant was normalized to its DNA content. All data were log transformed to improve normality. Statistical analyses were performed using Minitab 17 (Minitab, Inc., State College, PA). For each experiment, responses for each adipokine were analyzed using general linear models (GLMs) and Bonferroni's test for planned pairwise comparisons. Tissue and