

Osteoarthritis and Cartilage



Free fatty acid palmitate activates unfolded protein response pathway and promotes apoptosis in meniscus cells

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SUMMARY

Introduction: Obesity is the major risk factor for the development of osteoarthritis (OA); however, the mechanisms involved are not clearly understood. Obesity is associated with increased production of adipokine and elevated levels of circulating free fatty acids (FFA). A recent study has shown that saturated fatty acid palmitate induced pro-inflammatory and pro-apoptotic pathways in chondrocytes. Meniscus has been shown to be more susceptible than articular cartilage to catabolic stimuli. Thus, the aim of this study was to determine the effect of FFA (specifically, palmitate) on meniscus cells.

Methods: Cultured primary porcine meniscus cells were stimulated with 500 μ M FFA (palmitate and oleate) for 24 h to induce endoplasmic reticulum (ER) stress. After treatment, cell lysates were prepared and immunoblotted for C/EBP homologous protein (CHOP). To determine the activation of unfolded protein response (UPR) signaling, cell lysates were probed for cJun N-terminal kinase (JNK), cleaved caspase -3 and Xbp-1s, an alternative mRNA splicing product generated due to Ire1 α activation.

Results: Treatment of isolated primary meniscus cells with palmitate but not oleate induced expression of CHOP and Xbp-1s. Palmitate treatment of meniscus cells also activated JNK and increased expression of caspase-3, thus promoting apoptosis in meniscus cells.

Conclusions: Palmitate induces ER stress and promotes apoptotic pathways in meniscus cells. This is the first study to establish ER stress as a key metabolic mechanistic link between obesity and OA, in addition to (or operating with) biomechanical factors.

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Introduction

Obesity is one of the major risk factors for the development of osteoarthritis (OA)¹; however, the mechanisms involved are not clearly understood. Obesity is associated with elevated levels of free fatty acids (FFA)², resulting in lipid accumulation in non-adipose tissues. This process leads to lipotoxicity, characterized by cell dysfunction, inflammation, and cell death. Induction of endoplasmic reticulum (ER) stress is one mechanism proposed for lipid toxicity. ER is an important cell organelle involved in protein and lipid biosynthesis. Disruption of this highly synchronized process leads to accumulation of unfolded proteins, resulting in ER stress and triggering ER signaling or the unfolded protein response (UPR). The UPR pathway is activated to restore ER homeostasis by

upregulating ER chaperones, attenuating protein translation, and degrading misfolded proteins³. UPR is mediated by the ER stress transducers IRE1 α (inositol requiring ER-to-nucleus signal kinase-1), ATF-6 (activating transcription factor-6), and PERK (PKR [double-stranded-RNA dependent protein kinase]-like ER kinase). Normally, these proteins are inactive and are bound to the ER chaperone, GRP 78/BiP. However, under stress conditions, BiP disassociates itself from these proteins and bind to unfolded proteins, thus activating the ER stress transducers and UPR signaling (4 and references therein).

Studies have shown that joint tissue and synovial fluid accumulates FFA and OA severity correlates with FFA levels⁴. A recent study showed that saturated fatty acid palmitate induced pro-apoptotic and pro-inflammatory pathways in chondrocytes⁵. OA is a disease of the whole joint; multiple tissues (articular cartilage, bone, ligaments, and meniscus) are involved in its pathology. Meniscus tissue, like articular cartilage, is exposed to similar biomechanical forces and biochemical environment, yet its role in obesity-linked OA is not clearly understood. A recent study showed that increase in body mass index (BMI) negatively regulated gene transcripts associated with extracellular matrix in human

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meniscus⁶. In addition, meniscus was more susceptible than articular cartilage to catabolic effects of adipokines⁷. These studies suggest that meniscus could be a primary tissue affected in obesity-linked OA. Thus, in the current study we examined the effect of FFAs (specifically, palmitate) on meniscus cells. Palmitate induced ER stress and activated UPR signaling in meniscus cells, and activation of UPR (Ire1 α) signaling by palmitate induced apoptotic pathways in these cells.

Materials and methods

Collagenase-P was purchased from Roche Applied Science. Pronase was purchased from Calbiochem. Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 (1:1), antibiotics, fetal bovine serum and BCA reagent were obtained from Thermo Fisher (Rockford, IL). Nitrocellulose membranes and ECL chemiluminescence detection kits were purchased from GE Life Sciences (Pittsburgh, PA). 4-phenylbutyric acid (PBA), low-endotoxin, fatty acid-free bovine serum albumin, FFA (palmitate & oleate), tunicamycin, and thapsigargin were purchased from Sigma–Aldrich (St. Louis, MO). Antibodies to CHOP, Xbp1s, cleaved caspase-3, phospho-JNK, total JNK, and GAPDH were from Cell Signaling Technology (Danvers, MA). 4 μ 8c, an Ire1 α inhibitor, was purchased from Axon Medchem (Reston, VA).

Methods

Meniscus cell isolation and culture conditions

Meniscus tissue was harvested from the knee joints of 3-month-old pigs ($n = 16$), donated by the Departments of Cardiovascular Surgery and Plastic and Reconstructive Surgery at Wake Forest School of Medicine. Meniscus cells were isolated under aseptic conditions by sequential enzymatic digestion at 37°C using pronase 2 mg/ml in serum-free DMEM/F-12/antibiotics for 1 h, followed by overnight digestion with collagenase-P at 0.25 mg/ml in DMEM/F-12 (5% fetal bovine serum). Viability of isolated cells was determined using trypan blue and cells were counted using a hemocytometer. Monolayer cultures were established by plating cells in six-well plates at 2×10^6 cells/ml in DMEM/F-12 medium supplemented with 10% fetal bovine serum. Cells were maintained for approximately 3–5 days with feedings every 2 days until they reached 100% confluency prior to experimental use.

BSA-free fatty acid conjugates

FFA (palmitate and oleate) were conjugated to low-endotoxin, fatty acid-free bovine serum albumin as described previously⁸. For experiments, BSA-FFA conjugate was diluted in culture media.

Meniscus cell stimulation and immunoblotting

Confluent monolayers of porcine meniscus cells were made serum-free overnight before treating with FFA (500 μ M) or 2 μ M of thapsigargin and 1 μ g/ml tunicamycin overnight. In some experiments, cells were pretreated with 1 mM of PBA or Ire1 α inhibitor (4 μ 8c) for 30 min followed by treatment with palmitate or oleate. Treatment of cells with inhibitor did not affect meniscus cell viability (data not shown). After incubation, cells were washed with PBS and lysed with lysis buffer that contained 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM tetrapyrrophosphate, 1 mM glycerol phosphate, 1 mM Na₃VO₄, 1 μ l/ml leupeptin, and 1 mM phenyl methyl sulfonyl fluoride. Lysates were centrifuged to remove insoluble material, and the soluble protein concentration was determined with BCA reagent. Samples

containing equal amounts of total protein were separated by SDS-PAGE, transferred to nitrocellulose, and probed for signaling proteins. Immunoreactive bands were detected using the ECL system. All immunoblotting experiments were repeated at least three times with similar results.

Statistics

The data presented in study are representative of at least three independent experiments performed with cells obtained from different pigs.

Results

Palmitate-induced ER stress in meniscus cells

Meniscus cells responded to palmitate, but not to oleate treatment. Treatment of meniscus cells with palmitate increased the expression of C/EBP homologous protein (CHOP), a marker for ER stress [Fig. 1(A)]. Tunicamycin and thapsigargin are known ER stress inducers, which we have used as positive controls in our experiments. Pretreatment of cells with 4-phenylbutyric acid (PBA), a small molecule chemical chaperone, reduced the palmitate-induced expression of CHOP [Fig. 1(B)]. These data suggest that palmitate induced ER stress in meniscus cells.

Palmitate promotes apoptosis via the Ire1 α UPR pathway

UPR is mediated by the ER resident proteins ATF6, PERK, and Ire1 α . Ire1 α plays an important role in ER stress-induced apoptosis. Hence, we were interested in examining if palmitate activates Ire1 α signaling. Palmitate treatment of meniscus cells induced expression of Xbp1s, an alternative mRNA splicing product generated due to Ire1 α activation [Fig. 2(A)]. In addition, stimulation of cells with palmitate also induced both increased cjun n-terminal kinase (JNK)

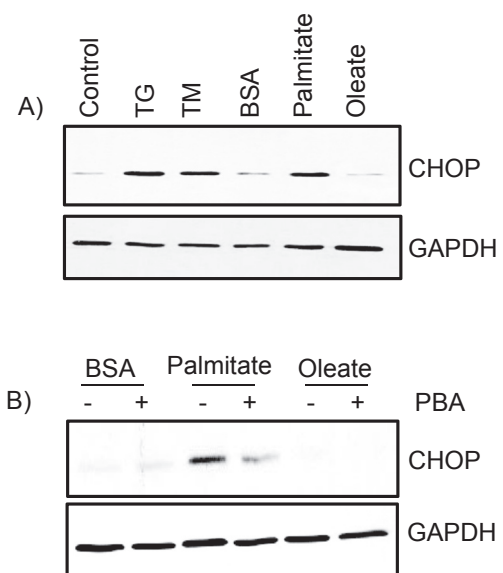


Fig. 1. The free fatty acid palmitate induces ER stress. A) Porcine primary meniscus cells were stimulated with 500 μ M FFA (palmitate and oleate), 2 μ M of thapsigargin, and 1 μ g/ml tunicamycin overnight. B) In other experiments, cells were pretreated with or without 1 mM PBA for 30 min followed by treatment with BSA-conjugated palmitate or oleate (500 μ M) overnight. After incubation, cell lysates were immunoblotted with antibodies to CHOP. Blots were stripped and reprobed with GAPDH as a loading control. Data are representative of at least three independent experiments performed with cells obtained from different pigs.

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