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Research Article

Production of reactive oxygen species by withaferin A causes loss of type collagen expression and COX-2 expression through the PI3K/Akt, p38, and JNK pathways in rabbit articular chondrocytes

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ABSTRACT

Withaferin A (WFA) is a major chemical constituent of *Withania somnifera*, also known as Indian ginseng. Many recent reports have provided evidence of its anti-tumor, anti-inflammation, anti-oxidant, and immune modulatory activities. Although the compound appears to have a large number of effects, its defined mechanisms of action have not yet been determined.

We investigated the effects of WFA on loss of type collagen expression and inflammation in rabbit articular chondrocytes. WFA increased the production of reactive oxygen species, suggesting the induction of oxidative stress, in a dose-dependent manner. Also, we confirmed that WFA causes loss of type collagen expression and inflammation as determined by a decrease of type II collagen expression and an increase of cyclooxygenase-2 (COX-2) expression via western blot analysis in a dose- and time- dependent manner. WFA also reduced the synthesis of sulfated proteoglycan via Alcian blue staining and caused the synthesis of prostaglandin E_2 (PGE_2) via assay kit in dose- and time-dependent manners.

Treatment with N-acetyl-L-cysteine (NAC), an antioxidant, inhibited WFA-induced loss of type II collagen expression and increase in COX-2 expression, accompanied by inhibition of reactive oxygen species production. WFA increased phosphorylation of both Akt and p38. Inhibition of PI3K/Akt, p38, and JNK with LY294002 (LY), SB203580 (SB), or SP600125 (SP) in WFA-treated cells rescued the expression of type II collagen and suppressed the expression of COX-2. These results demonstrate that WFA induces loss of type collagen expression and inflammation via PI3K/Akt, p38, and JNK by generating reactive oxygen species in rabbit articular chondrocytes.

Abbreviations: WFA, withaferin A; ROS, reactive oxygen species; COX-2, cyclooxygenase-2; PGE_2, prostaglandin E_2; NAC, N-acetyl-L-cysteine; OA, osteoarthritis; ECM, extracellular matrix; PI3K/Akt, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase; JNK, c-jun N-terminal kinase; L-NMMA, NG-monomethyl-L-arginine, monoacetate salt; DCF-DA, 2′,7′-dichlorodihydrofluorescein diacetate; DIDS, 4,4′ diisothio-cyanatostilbene-2,2′-disulfonic acid

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Introduction

Withaferin A (WFA), a steroidal lactone found in the leaves and roots of *Withania somnifera*, reportedly inhibits immunologically induced inflammation and exhibits a variety of pharmacological effects in experimental animals [1,2]. Although the biological properties of crude root extracts have been largely reported, only a few of them concern with the pure compound withaferin A.

Osteoarthritis (OA) is a progressive and dynamic process in joint tissues, including cartilage, underlying bone, the entire synovial joint, synovium, and muscle. It is the most common clinical syndrome of joint pain associated with significant functional disability and with the signs and symptoms of inflammation, including pain, stiffness, and limited movement. Chondrocytes are the unique cells of the articular cartilage extracellular matrix (ECM) and are responsible for the synthesis and degradation of the cartilage consisting of type II collagen and sulfated proteoglycans [3,4]. The normal function of articular cartilage depends on the ECM, which needs to exert specific mechanical properties [4]. The disruption of homeostasis decreases type II collagen and sulfated proteoglycan and increases apoptosis [5]. The mechanism that controls the degradation of cartilage in OA, which is characterized by an imbalance between anabolism and catabolism of cartilage-specific ECM, remains poorly understood, but anabolic and catabolic factors appear to have crucial roles [4,6]. Specifically, OA is caused by the failure of chondrocytes to maintain homeostasis between the synthesis and breakdown of ECM components.

Although OA is largely recognized as a non-inflammatory disease, inflammation is known to contribute the symptoms and progression of OA [7,8]. The pro-inflammatory cytokines interleukin (IL)-1β, IL-6, and tumor necrosis factor-α play pivotal roles in this metabolic dysfunction and are known to display catabolic properties and orchestrate pathophysiological processes in OA. Prostaglandins (PGs) are important lipid mediators produced at evaluated levels in inflamed tissues and formed by COX-2; they are important mediators of inflammation in arthritis.

COX catalyzes the first committed step in the synthesis of prostanoids and is rapidly expressed in several cell types after exposure to pro-inflammatory molecules. Specifically, when chondrocytes and synovial tissue are stimulated with cytokines and endotoxin, they release COX-2, which spontaneously releases substantial amounts of PGs [9,10]. Various PGs are produced by chondrocytes, and the synthesis of PGs by chondrocytes can be stimulated by biologically relevant factors such as IL-1β, tumor necrosis factor-α, and reactive oxygen species (ROS) [11].

Oxidative stress-induced ROS act as secondary messengers in cell signaling transduction and elicit an inflammatory response. The response is generated via signal transduction cascades of a range of growth factors, cytokines, and extracellular signaling pathways [12,13]. Uncontrolled ROS may cause oxidative modification of cellular macromolecules, DNA, lipids, and proteins and is believed to be the mechanism through which these molecules medicate their effect on the inflammatory process [14].

Previous reports have shown that ROS destroy the biochemical integrity of proteoglycans, hyaluronic acid, and collagens [15,16]. Cartilage and chondrocytes are highly sensitive to ROS, as the latter contribute to proteoglycan degradation [17,18]. ROS are connected to the inflammatory response and commonly contribute to the tissue-injuring effects of inflammatory reactions [19]. Antioxidants prevent ROS-caused COX-2 expression in chondrocytes [20].

Increasing evidence implies that the activation of the phosphatidylinositol 3-kinase (PI3K/Akt) and mitogen-activated protein kinase (MAPK) families is directly influenced by ROS [21–24]. Activation of PI3K/Akt and MAPK families, p38, extracellular signal-regulated kinases, and c-jun N-terminal kinase (JNK) by ROS are typically associated with differentiation and inflammation in a variety of cells [25,26]. In the present study, we examined the effects of WFA on loss of type collagen expression and inflammation in rabbit articular chondrocytes. Moreover, we determined a possible role of ROS in loss of type collagen expression and inflammation of chondrocytes via activation of the PI3K/Akt and MAPK families, p38, and JNK, by ROS.

Materials and methods

Materials

WFA was purchased from Calbiochem (San Diego, CA, USA), and SP600125 was obtained from ENZO Life science (Plymouth Meeting, PA, USA). The other chemicals used, SB203580 and LY294002, were purchased from BIOMOL (Plymouth Meeting, PA, USA). The anti-collagen type II and β-actin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-COX-2 antibody was obtained from Cayman Chemical (Ann Arbor, MI, USA). The anti-phospho-p38, anti-phospho-Akt, anti-phospho-JNK, and anti-phospho-c-Jun antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA).

Culture of primary chondrocytes and experimental conditions

Rabbit articular chondrocytes released from cartilage slices of 2-week-old New Zealand white rabbits were dissociated for 6 h in 0.2% collagenase type II (381 units/mL of solid, Sigma Aldrich, St. Louis, MO, USA) in Dulbecco's modified Eagle's medium (Invitrogen, CA, USA). After collecting individual cells via brief centrifugation at $300 \times g$ at 25 °C for 10 min, the cells were suspended in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) bovine calf serum (Invitrogen, Carlsbad, CA, USA), 50 μg/mL streptomycin (Sigma Aldrich), and 50 units/mL penicillin (Sigma Aldrich) and plated on culture dishes at a density of 5×10^4 cells/cm^2. After 3 days of culture, the medium was replaced with fresh medium and cells were treated with the indicated pharmacological reagents.

Western blot analysis

Whole-cell lysates were prepared by extracting protein using a buffer containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, and 0.1% sodium dodecyl sulfate (SDS) supplemented with inhibitors for proteases and phosphatases. Proteins were size-fractionated using SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The nitrocellulose sheet was blocked with 5% non-fat dry milk in Tris-buffered saline. Blots were developed using a peroxidase-conjugated secondary antibody and an image-Quant LAS 4000 (Amersham Biosciences Corp, Piscataway, NJ, USA). Protein levels were