Published in final edited form as:

Phytother Res. 2017 May ; 31(5): 778–782. doi:10.1002/ptr.5799.

A polyphenol-rich pomegranate fruit extract suppresses NF- κ B and IL-6 expression by blocking the activation of IKK β and NIK in primary human chondrocytes

Abdul Haseeb^{1,*}, Nazir M. Khan^{*}, Omer S. Ashruf, and Tariq M. Haqqi

Department of Anatomy & Neurobiology, Northeast Ohio Medical University, 4209 St Rt 44, Rootstown, OH 44272, USA

Abstract

Pomegranate fruit extract (PE) rich in polyphenols have been shown to exert chondroprotective effects but the mechanism is not established. Here, we used an *in vitro* model of inflammation in OA to investigate the potential of PE to suppress IL-1 β -stimulated expression of inflammatory cytokine IL-6, generation of reactive oxygen species (ROS) levels and investigated the mechanism of NF- κ B inhibition by analyzing the activation of the kinases upstream of I κ B α in primary human chondrocytes. Total and phosphorylated forms of kinases, and expression of IL-6 were determined at protein and mRNA levels by Western immunoblotting and Taqman assay respectively. DHR-123 staining estimated ROS generation. PE inhibited the mRNA and protein expression of IL-6, generation of ROS and inhibited the IL-1 β -mediated phosphorylation of IKK β , expression of IKK β mRNA, degradation of I κ B α , and activation and nuclear translocation of NF- κ B/p65 in human chondrocytes. Importantly, phosphorylation of NF- κ B-inducing kinase (NIK) was blocked by PE in IL-1 β -treated human OA chondrocytes. Taken together, these data suggest that PE exerts the chondroprotective effect(s) by suppressing the production of IL-6 and ROS levels. Inhibition of NF- κ B activation by PE was blocked via modulation of activation of upstream kinases in human OA chondrocytes.

Keywords

Pomegranate; Osteoarthritis; IL-1 β ; Chondrocytes; NF- κ B; I κ B; IKK β ; NIK

Introduction

Inflammation has emerged as an important contributing factor in the pathogenesis of osteoarthritis (OA), the most common degenerative disease of the joints. IL-6 is one of the major inflammatory cytokine implicated in cartilage damage due to its profound effect on

Address for correspondence: Tariq M. Haqqi, M.Phil., Ph.D., Professor, Department of Anatomy & Neurobiology, Northeast Ohio Medical University, 4209 St Rt 44, Rootstown, OH 44272, USA, thaqqi@neomed.edu, Phone: +13303256704.

*These authors contributed equally

¹Present address: Department of Cellular and Molecular Medicine, Lerner Research Institute, Cleveland Clinic, 9500 Euclid Ave, Cleveland, OH-44195

Competing interests

The authors declare that they have no competing interests.

both anabolic and catabolic cartilage physiology (Haseeb et al., 2016). Significant correlations have been reported between levels of IL-6 in the serum as well as in synovial fluid and OA severity (Stannus et al., 2010). Further, IL-6 has been shown as a crucial mediator of MMP-13 expression, a major protease involved in cartilage degradation (Suzuki et al., 2010; Haseeb et al., 2017). NF- κ B is an important regulator of IL-6 transcription in chondrocytes and has been identified as an important target of therapeutic strategies aimed at the treatment of OA (Roman-Blas and Jimenez, 2006; Haseeb and Haqqi, 2013). IL-1 β activates NF- κ B signaling pathway by binding its specific cell surface receptor, IL-1 receptor I (IL-1RI) that leads to activation of IKKs and subsequent phosphorylation and ubiquitin mediated proteosomal degradation of I κ B resulting into activation and nuclear translocation of NF- κ B. Recent studies suggest that some phytochemicals may prevent cartilage degradation by inhibiting the production of catabolic mediators in OA.

The pomegranate fruit extract (PE) is a rich source of polyphenols and possess potent antioxidant properties (Noda et al., 2002). The bioavailable constituents of PE were reported as potent suppressors of COX-2 activity and IL-1 β -induced PGE₂ production in human chondrocytes *in vitro* (Shukla et al., 2008). We have also shown that PE and one of its constituent delphinidin exhibit strong chondroprotective effects and act by inhibiting the IL-1 β induced proteoglycan breakdown, expression of MMPs and IL-1 β induced activation of NF- κ B in human chondrocytes (Ahmed et al., 2005; Haseeb et al., 2013). Here, we investigated the effect of PE on expression of inflammatory cytokine IL-6, and its regulatory transcription factor NF- κ B in IL-1 β -stimulated primary human OA chondrocytes by determining the effect on the activation of the kinases upstream of I κ B α including inhibitor of nuclear factor kappa-B kinase subunit beta (IKK- β) and NF- κ B-inducing kinase (NIK). We also determined effects of PE on IL-1 β -induced oxidative stress by measuring the levels of reactive oxygen species (ROS) in OA chondrocytes.

Materials and Methods

Reagents

Media and reagents for cell culture were purchased either from HyClone Laboratories (Logan, UT) or from Invitrogen (Carlsbad, CA). Pronase and Collagenase were purchased from Roche Diagnostics (Indianapolis, IN). Recombinant human IL-1 β was purchased from R&D Systems (St. Paul, MN, USA). Primary antibodies against I κ B, phospho-I κ B, IKK β , Phospho-NIK and β -actin were from Santa Cruz Biotechnologies (Santa Cruz, CA) and antibodies against IKK α , phospho-IKK α / β , NIK and horseradish peroxidase conjugated secondary antibodies were purchased from Cell Signaling Technology (Beverly, MA).

Preparation and chemical composition of the polyphenol-rich pomegranate fruit extract used in the study have been described earlier (Rasheed et al., 2010; Shukla et al., 2008). The pomegranate fruit extract is a rich source of two types of polyphenolic compounds: anthocyanins (such as delphinidin, cyanidin, and pelargonidin), which give the fruit and juice its red color, and hydrolyzable tannins (such as punicalin, pedunculagin, punicalagin, gallagic, and ellagic acid), which account for 92% of the antioxidant activity of the whole fruit (Malik et al., 2005; Akhtar et al., 2017).

Preparation of primary human chondrocytes

The study protocol was reviewed and approved by the Institutional Review Board of Northeast Ohio Medical University, Rootstown, OH, as “non-human subject study under 45 CFR” and that no informed consent was needed. Discarded and de-identified knee or hip joint cartilage samples were collected through the NIH supported National Disease Research Interchange (NDRI) per the IRB approved protocol. The unaffected areas of the cartilage were used as source to isolate OA chondrocytes by enzymatic digestion as previously described (Haseeb et al., 2016). At about 80% confluence, OA chondrocytes were serum starved overnight and were then treated with 50 µg/ml PE for 2 hrs followed by treatment with human recombinant IL-1β (10 ng/ml) for different times.

Western immunoblotting

After treatments, OA chondrocytes were harvested, washed with cold PBS and lysed in ice-cold RIPA buffer as described previously (Haseeb et al., 2017). Equivalent amounts of lysate protein (20 µg) were resolved by SDS-PAGE, transferred and blots were developed as described earlier (Haseeb et al., 2017).

Measurement of NF-κB/p65 activation by ELISA

Activation of NF-κB and DNA-binding activity assay was performed using Trans-AM ELISA kit (Active Motif, Carlsbad, CA). Briefly, chondrocytes were treated as described above for the indicated times and nuclear extracts were prepared using the nuclear extract kit (Active Motif). A total 15 µg of the nuclear extract was used to determine the activity of NF-κB as per manufacturer's instruction.

RNA isolation and Real time PCR analysis of IL-6 and IKKβ gene expression

Chondrocytes treated as above were harvested and total RNA was isolated using Trizol® reagent and cDNA was synthesized as described previously (Haseeb et al., 2017). The mRNA expression of IL-6 and IKKβ was quantified using TaqMan Gene Expression Assays (Integrated DNA technology) as previously described (Haseeb et al., 2017). Relative expression levels were calculated using the 2^{-C_T} method.

Measurement of ROS levels

OA chondrocytes were pretreated with PE (50 µg/ml) for 2 hrs and then labeled with DHR123 (5 µM) for 0.5 hr followed by stimulation with IL-1β (1 ng/ml) for 5 minutes. Cell suspension of chondrocytes was prepared and ROS levels were estimated by measuring the oxidation of DHR123 using BD Accuri C6 Flowcytometer by analyzing DHR positive cells using FlowJo software as described previously (Khan et al., 2017).

Statistical analysis

Each experiment was repeated on primary chondrocytes obtained from different patients to ensure reproducibility of the data. Values shown are mean±SD and were compared using two tailed Student's t-test. A P -value<0.05 was considered significant. Data were plotted using the Origin 8.1 software.

Results

Inhibition of IL-1 β -induced mRNA and protein expression of IL-6 by PE in human OA chondrocytes

Since IL-6 has emerged as an important inflammatory mediator associated with the pathogenesis of OA, we determined the effect of PE on the expression of IL-6 in IL-1 β -stimulated OA chondrocytes. Our data shown in Fig. 1A, demonstrate that stimulation of human OA chondrocytes with IL-1 β results in profound induction of IL-6 mRNA expression, whereas pre-treatment of OA chondrocytes with PE significantly blocked the IL-1 β -mediated high levels of IL-6 mRNA expression in a dose dependent manner. Inhibitory effect on IL-6 mRNA expression was also mimicked at the protein levels which were also significantly suppressed by pre-treatment of human OA chondrocytes with PE (Fig. 1B). These results indicated that PE exerts a potent anti-inflammatory effect through the effective suppression of IL-6 mRNA and protein expression in OA chondrocytes under pathological conditions.

PE suppressed the IL-1 β -mediated generation of ROS in human OA chondrocytes

Since oxidative stress play an important role in OA pathogenesis, we determined whether PE has suppressive effect on IL-1 β -induced oxidative stress in OA chondrocytes. The level of oxidative stress was determined by measuring the ROS levels using oxidation-sensitive dye DHR-123 and results showed that IL-1 β treatment significantly increased the ROS production in OA chondrocytes which was significantly suppressed by pretreatment of OA chondrocytes with PE (Fig. 1C). These results indicated that PE was effective in relieving IL-1 β -induced oxidative stress by suppressing the production of excessive ROS in OA chondrocytes.

PE inhibited the IL-1 β -induced activation and DNA binding activity of NF- κ B/p65 in human OA chondrocytes

Since IL-6 is transcriptionally regulated by NF- κ B (Roman-Blas and Jimenez, 2006; Haseeb et al., 2013), we determined the effect of PE on the activation and activity of NF- κ B in IL-1 β -stimulated OA chondrocytes. As shown in Fig. 2A, IL-1 β -stimulation results in 7-fold activation of NF- κ B/p65 within 30 minutes of treatments compared to untreated control. Interestingly, pretreatment with PE significantly inhibited IL-1 β -mediated activation and DNA binding of NF- κ B/p65 ($p < 0.05$) (Fig. 2A).

Since IL-1 β -stimulation results in proteasomal degradation of I κ B α , the inhibitory unit of NF- κ B, we evaluated the effect of PE on the degradation of I κ B α after stimulation with IL-1 β in OA chondrocytes. As shown in Fig. 2B, IL-1 β caused a rapid degradation of I κ B α (within 15 minutes) which was partially restored after 1 hr. Pretreatment of OA chondrocytes with PE significantly inhibited the IL-1 β -mediated I κ B α degradation ($p < 0.05$) at 15 and 30 minutes' post stimulation (Fig. 2B). These results suggest that PE exert its IL-6 suppressive effect through the inhibition of NF- κ B activation in OA chondrocytes.

PE inhibited the phosphorylation of IKK α / β and downregulated the expression of IKK β at protein and mRNA levels in human OA chondrocytes

IKK α was degraded by its phosphorylation by the upstream IKKs complex (O'Neill and Greene, 1998), we investigated the effect of PE on the activation of IKKs in IL-1 β -stimulated OA chondrocytes. As shown in Fig. 2C, IL-1 β caused maximum phosphorylation of IKK α / β at 15 minutes' post-stimulation, whereas pre-treatment with PE completely blocked the IL-1 β -induced phosphorylation of IKK α / β . To determine whether the phosphorylated subunit was IKK α or IKK β , we immunoprecipitated the respective proteins from the cell lysate using validated anti-IKK α or anti-IKK β antibodies and then probed the immunoprecipitated proteins by Western blotting using anti-phospho-IKK α / β antibody. These data showed that IKK β was phosphorylated (activated) but phosphorylation of immunoprecipitated IKK α protein was not detected using the anti-phospho-IKK α / β antibody and Western blotting (data not shown). Interestingly, pre-treatment of OA chondrocytes with PE also significantly downregulated the expression level of total IKK β (Fig. 2C). To further investigate whether this downregulation was at transcriptional level, we performed TaqMan assay and indeed found a decrease in IKK β mRNA levels in OA chondrocytes upon PE treatment in a dose-dependent manner (Fig. 2D).

PE inhibited the intrinsic as well as IL-1 β induced phosphorylation of NIK in human OA chondrocytes

IL-1 β mediated phosphorylation of IKKs is mediated by activated upstream kinase NIK (O'Neill and Greene, 1998), therefore to determine if the observed inhibition of IKK phosphorylation was due to the inhibition of NIK, we analyzed the activation of NIK in human OA chondrocytes stimulated with IL-1 β in the presence or absence of PE. Our results showed that PE significantly inhibited the IL-1 β induced phosphorylation of NIK in human OA chondrocytes (Fig. 2E). Taken together these results suggest that PE suppressed NF- κ B activation by inhibiting the activation of the upstream kinase NIK in IL-1 β stimulated OA chondrocytes.

Discussion

Inflammation has now emerged as a characteristic feature of OA pathophysiology. Inflammatory cytokines such as IL-1 β , TNF- α and IL-6 are present at high levels in OA joints and are the major mediators of disturbed chondrocyte function and cartilage degeneration. IL-1 β is believed to be an important cytokine involved in the pathogenesis of OA because of its ability to stimulate other inflammatory cytokines and chemokines. In the present study, we show that IL-1 β strongly stimulated the expression of inflammatory cytokine IL-6 in cultured primary human OA chondrocytes and PE treatment significantly suppressed these stimulatory effects of IL-1 β . Since we found that expression of IL-6 mRNA was suppressed, we suspected that PE might be acting through the suppression of its transcription. Earlier studies have established the role of NF- κ B in the transcriptional regulation of IL-6 (Roman-Blas and Jimenez, 2006; Haseeb et al., 2013) and our data showed that treatment of OA chondrocytes with PE significantly suppressed the activation of NF- κ B in IL-1 β stimulated OA chondrocytes (Fig. 2A–E).

PE is a rich source of bioavailable flavonoids rich in antioxidant activity (Noda et al., 2002). Our data showing strong inhibitory effects of PE on IL-1 β mediated induction of oxidative stress confirms the previous findings (Fig. 1C). Since NF- κ B is an oxidant-sensitive transcription factor and therefore suppression of oxidative stress by PE may contribute to its inhibitory effect on the activation of NF- κ B. We have earlier shown that PE inhibited IL-1 β -induced activation of MKK-3, p38 α and transcription factor RUNX-2 and NF- κ B in human OA chondrocytes (Ahmed et al., 2005; Rasheed et al., 2010). Data presented in this study are in agreement with our publication (Ahmed et al., 2005; Rasheed et al., 2010) and further extend our knowledge about the mechanism of chondroprotection by PE in OA chondrocytes by indicating a novel mechanism of inhibition of NF- κ B activation through inhibition of NIK and activation and expression of IKK β , both at mRNA and protein levels (Fig. 2C, D). It will be interesting to further investigate the mechanism of downregulation of IKK β expression by PE to determine if the antioxidant effect of the flavonoids present in PE has some role to play. PE also blocked both the basal and IL-1 β induced phosphorylation of NIK in human chondrocytes derived from OA cartilage (Fig. 2E). Our earlier data showed that delphinidin blocks the NF- κ B activation by inhibiting the phosphorylation of IRAK1^{Ser376} and NIK in primary human chondrocytes (Haseeb et al., 2013). Since delphinidin is one of the constituents of PE, we may speculate that delphinidin in PE may be responsible for the molecular effects observed in our study, or it may be a synergistic effect of several other flavonoids present in PE.

In summary, data presented in this report show a potent inhibitory effect of polyphenol rich PE on IL-6 expression and oxidative stress and identify the molecular mechanism of NF- κ B activation through inhibition of the phosphorylation of multiple upstream kinases including NIK and IKK β as well as downregulation of IKK β expression in human OA chondrocytes.

Acknowledgments

Grant Sponsor: USPHS/National Institute of Health and Northeast Ohio Medical University

Grant Number: RO1-AT-005520; RO1-AT-007373; RO1-AR-067056; R21-AR-064890

This work was supported in part by National Institute of Health grants (RO1-AT-005520; RO1-AT-007373; RO1-AR-067056; R21-AR-064890) and funds from the Northeast Ohio Medical University to TMH.

References

- Ahmed S, Wang N, Hafeez BB, Cheruvu VK, Haqqi TM. Punica granatum L. extract inhibits IL-1 beta-induced expression of matrix metalloproteinases by inhibiting the activation of MAP kinases and NF-kappa B in human chondrocytes in vitro. *J Nutr.* 2005; 135(9):2096–2102. [PubMed: 16140882]
- Akhtar N, Khan NM, Ashruf OS, Haqqi TM. Inhibition of cartilage degradation and suppression of PGE₂ and MMPs expression by pomegranate fruit extract in a model of posttraumatic osteoarthritis. *Nutrition.* 2017; 33:1–13. [PubMed: 27908544]
- Haseeb A, Ansari MY, Haqqi TM. Harpagoside suppresses IL-6 expression in primary human osteoarthritis chondrocytes. *J Orthop Res.* 2017; 35(2):311–320. [PubMed: 27082319]
- Haseeb A, Chen D, Haqqi TM. Delphinidin inhibits IL-1 beta-induced activation of NF-kappaB by modulating the phosphorylation of IRAK-1(Ser376) in human articular chondrocytes. *Rheumatology (Oxford).* 2013; 52(6):998–1008. [PubMed: 23392593]

- Haseeb A, Haqqi TM. Immunopathogenesis of osteoarthritis. *Clinical Immunology*. 2013; 146:185–196. [PubMed: 23360836]
- Khan NM, Ansari MY, Haqqi TM. Sucrose, But Not Glucose, Blocks IL1-beta-Induced Inflammatory Response in Human Chondrocytes by Inducing Autophagy via AKT/mTOR Pathway. *J Cell Biochem*. 2017; 118(3):629–639. [PubMed: 27669541]
- Malik A, Afaq F, Sarfaraz S, Adhami VM, Syed DN, Mukhtar H. Pomegranate fruit juice for chemoprevention and chemotherapy of prostate cancer. *Proc Natl Acad Sci U S A*. 2005; 102:4813–4818. [PubMed: 15781865]
- Noda Y, Kaneyuki T, Mori A, Packer L. Antioxidant activities of pomegranate fruit extract and its anthocyanidins: delphinidin, cyanidin, and pelargonidin. *J Agric Food Chem*. 2002; 50(1):166–171. [PubMed: 11754562]
- O'Neill LA, Greene C. Signal transduction pathways activated by the IL-1 receptor family: ancient signaling machinery in mammals, insects, and plants. *J Leukoc Biol*. 1998; 63(6):650–7. [PubMed: 9620655]
- Rasheed Z, Akhtar N, Haqqi TM. Pomegranate extract inhibits the interleukin-1beta-induced activation of MKK-3, p38alpha-MAPK and transcription factor RUNX-2 in human osteoarthritis chondrocytes. *Arthritis Res Ther*. 2010; 12(5):R195. [PubMed: 20955562]
- Roman-Blas JA, Jimenez SA. NF-kappaB as a potential therapeutic target in osteoarthritis and rheumatoid arthritis. *Osteoarthritis Cartilage*. 2006; 14(9):839–848. Epub 2006 May 2026. [PubMed: 16730463]
- Shukla M, Gupta K, Rasheed Z, Khan KA, Haqqi TM. Bioavailable constituents/metabolites of pomegranate (*Punica granatum* L) preferentially inhibit COX2 activity ex vivo and IL-1beta-induced PGE2 production in human chondrocytes in vitro. *J Inflamm (Lond)*. 2008; 5:9.doi: 10.1186/1476-9255-1185-1189 [PubMed: 18554383]
- Stannus O, Jones G, Cicuttini F, Parameswaran V, Quinn S, Burgess J, et al. Circulating levels of IL-6 and TNF-alpha are associated with knee radiographic osteoarthritis and knee cartilage loss in older adults. *Osteoarthritis Cartilage*. 2010; 18(11):1441–1447. [PubMed: 20816981]
- Suzuki M, Hashizume M, Yoshida H, Shiina M, Mihara M. IL-6 and IL-1 synergistically enhanced the production of MMPs from synovial cells by up-regulating IL-6 production and IL-1 receptor I expression. *Cytokine*. 2010; 51:178–183. [PubMed: 20403707]

Figure 1. PE inhibited IL-1 β -induced expression of IL-6 and generation of ROS in human OA chondrocytes

OA chondrocytes were pretreated with PE (1–50 μ g/ml) followed by treatment with IL-1 β (10 ng/ml) for indicated times. Cell lysate were prepared using RIPA buffer for immunoblot analysis or RNA were isolated for real time PCR analysis. **(A)** Expression of IL-6 was measured by quantitative PCR using the TaqMan assay system (Life Technologies). β -actin was used as endogenous expression control. **(B)** Protein expression of IL-6 was investigated by immunoblotting using antibodies against indicated protein. β -actin was used as a control for equal loading. **(C)** Chondrocytes treated with PE and stained with DHR-123 (5 μ M, 0.5 h) were stimulated with IL-1 β (1 ng/ml) for 5 minutes at 37°C and twenty thousand cells in each group were acquired using a flowcytometer. Flow cytometric histograms showing DHR +ve cells were analyzed using FlowJo software. Data points represent mean $\pm$ SD from three replicates. *p 0.01, as compared to control, # 0.01, as compared to IL-1 β .

Figure 2. PE inhibited IL-1β-induced activation of NF-κB in human OA chondrocytes

OA chondrocytes were pretreated with PE (50 μg/ml) followed by treatment with IL-1β (10 ng/ml) for indicated times. Cell lysate were prepared using RIPA buffer for immunoblot analysis or RNA were isolated for real time PCR analysis. (A) Nuclear cell lysate (15 μg) was used for the ELISA based p65 DNA-binding activity assay using Trans-AM kit (Active Motif). (B) IκBα degradation was investigated by immunoblotting using antibodies against indicated protein. β-actin was used as a control for equal loading. (C) Protein expression of phospho-IKKα/β and total IKKα/β were investigated by immunoblotting using specific antibodies (D) mRNA expression of IKKβ was measured by quantitative PCR using the TaqMan assay system (Life Technologies). β-actin was used as endogenous expression control (E) Protein levels of phospho-NIK was investigated by immunoblotting using specific antibody. Immunoblot results are representatives of two blots performed on samples obtained from two individuals *p 0.01, as compared to control.# 0.01, as compared to IL-1β.