Mitochondrial dysfunction triggers a catabolic response in chondrocytes via ROS-mediated activation of the JNK/AP1 pathway

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ABSTRACT
Mitochondrial function is impaired in osteoarthritis (OA) but its impact on cartilage catabolism is not fully understood. Here, we investigated the molecular mechanism of mitochondrial dysfunction-induced activation of the catabolic response in chondrocytes. Using cartilage slices from normal and OA cartilage, we showed that mitochondrial membrane potential was lower in OA cartilage, and that this was associated with increased production of mitochondrial superoxide and catabolic genes [interleukin 6 (IL-6), COX-2 (also known as PTGS2), MMP-3, -9, -13 and ADAMTS5]. Pharmacological induction of mitochondrial dysfunction in chondrocytes and cartilage explants using carbonyl cyanide 3-chlorophenylhydrazone increased mitochondrial superoxide production and the expression of IL-6, COX-2, MMP-3, -9, -13 and ADAMTS5, and cartilage matrix degradation. Mitochondrial dysfunction-induced expression of catabolic genes was dependent on the JNK (herein referring to the JNK family)/activator protein 1 (AP1) pathway but not the NFκB pathway. Scavenging of mitochondrial superoxide with MitoTEMPO, or pharmacological inhibition of JNK or cFos and cJun, blocked the mitochondrial dysfunction-induced expression of the catabolic genes in chondrocytes. We demonstrate here that mitochondrial dysfunction contributes to OA pathogenesis via JNK/AP1-mediated expression of catabolic genes. Our data shows that AP1 could be used as a therapeutic target for OA management.

This article has an associated First Person interview with the first author of the paper.

KEY WORDS: Osteoarthritis, Chondrocytes, Redox, Inflammation, JNK, cFos, AP1

INTRODUCTION
Mitochondria are complex organelles and the site of oxidative phosphorylation and cellular energy production under normal conditions, and are essential for the maintenance of redox balance and cellular homeostasis. However, under stress conditions, mitochondria are damaged and produce excessive amounts of reactive oxygen species (ROS) causing oxidative stress, and release pro-apoptotic proteins inducing catabolic pathways and cell death (Eckert et al., 2003). Defective mitochondrial function has been implicated in several diseases, including degenerative joint diseases, such as osteoarthritis (OA) (Blanco et al., 2011; Bratic and Larsson, 2013; Goetz et al., 2017; Nunnari and Suomalainen, 2012). Chondrocytes isolated from end stage OA cartilage have been shown to have reduced mitochondrial respiratory channel complex activity (Maneiro et al., 2003) and actively produce inflammatory mediators, such as interleukin (IL)-1β, TNFα and IL-6, which play a crucial role in cartilage extracellular matrix (ECM) degradation, chondrocyte hypertrophy and subchondral bone remodeling (Ansari et al., 2018a, 2019; Goldring and Otero, 2011; Greene and Loeser, 2015; Scanzello, 2017; Shen et al., 2017).

The major constituents of cartilage ECM are type II collagen and aggrecan, which are synthesized by the chondrocytes, and their degradation is at the center of OA pathogenesis (Goldring, 2012). Various in vivo and in vitro studies have shown high level expression of matrix metalloproteases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTSs) (Burrage et al., 2006). MMP-13 (collagenase) and ADAMTS5 (aggrecanase) are the two major proteases involved in the degradation of cartilage in OA, and the deletion of either the Mmp13 gene or the Adamts5 gene has been shown to have a protective effect in a mouse model of surgically induced OA (Glasson et al., 2005; Wang et al., 2013). However, it remains unclear how mitochondrial dysfunction in OA promotes the expression of inflammatory genes and cartilage ECM degrading proteases. We report here that mitochondrial dysfunction triggers the induction of OA-related catabolic genes in chondrocytes through a mechanism that involves ROS-dependent activation of the JNK (herein referring to the JNK family)/activator protein 1 (AP1) pathway.

RESULTS
Mitochondrial function is impaired in human OA cartilage compared to normal cartilage in vivo
Mitochondrial dysfunction in isolated chondrocytes has been described using end-stage disease cartilage (reviewed by Blanco et al., 2011). Here, we directly determined the mitochondrial membrane potential in chondrocytes embedded in cartilage obtained from normal (from donors with no known history of any rheumatic disorders) and OA donors, using JC-1 staining. Unfixed thin slices of normal and OA cartilage were prepared from freshly obtained samples and immediately stained with JC-1 dye. The dye successfully penetrated the cartilage ECM and stained the live chondrocytes in it. Compared to normal cartilage, OA cartilage sections showed increased green fluorescence (depolarized mitochondria) [Fig. 1A,B; Movie 1 (normal cartilage); Movie 2 (OA cartilage)]. Normal and OA cartilage sections were stained by Toluidine Blue to visualize the damaged and undamaged area (Fig. 1C). Mitochondrial dysfunction in OA chondrocytes was further correlated with reduced ATP production...
Interestingly, we also noted that the loss of mitochondrial membrane potential within OA cartilage was greater in the chondrocytes present in the superficial zone (damaged area) of OA cartilage compared to the chondrocytes present in the deep zone (undamaged area) (Fig. 1A), which was also observed in the surface plot (Fig. 1B). This showed that the mitochondrial function was not affected in the undamaged deep zone chondrocytes of the diseased cartilage. Uncultured chondrocytes prepared from the OA cartilage with high-grade OA [Mankin score ≥3; the higher the Mankin score, the more severe is the disease (Pauli et al., 2012)] showed a significantly increased population of dysfunctional mitochondria with loss of mitochondrial membrane potential compared to chondrocytes prepared from the OA cartilage with low-grade disease (Mankin score ≤2) (Fig. 1D,E). These results indicated that the severity of the disease correlates with increased mitochondrial dysfunction in OA. In addition, the mitochondrial mass in chondrocytes from high-grade OA was significantly increased compared to low-grade OA (Fig. 1F, G).
OA was drastically reduced compared to chondrocytes from low-grade OA cartilage, as determined by the ratio of mitochondrial to nuclear DNA content (Fig. 1G) and MitoTracker Deep Red staining (Fig. 1H, I). We also found a significant increase in the expression of interleukin 6 (IL-6), COX-2 (also known as PTGS2), MMP-3, -9, -13 and ADAMTS5 in uncultured chondrocytes from cartilage with high-grade OA compared to cartilage isolated from the cartilage with low-grade OA (Fig. 1J). The increase in the expression of the pathogenic genes positively correlated with the observed mitochondrial dysfunction in OA chondrocytes.

Mitochondrial depolarization is associated with increased ROS production causing oxidative stress. Unfixed thin slices of low- and high-grade human OA cartilage with live chondrocytes were prepared from freshly obtained cartilage samples and immediately stained with either DHR123 or MitoSOX Red to determine the ROS levels. Both dyes successfully penetrated the cartilage ECM and stained the live chondrocytes. Compared to cartilage with a lower Mankin score, chondrocytes in the cartilage with a higher Mankin score showed high levels of DHR123 (Fig. 2A,B) and MitoSOX fluorescence (Fig. 2C,D), indicating high levels of ROS in the cartilage with more severe disease. Using MitoSOX staining, we also quantified the production of mitochondrial superoxide and found a significant increase in the production of mitochondrial superoxide in chondrocytes prepared from cartilage with high-grade disease (higher Mankin score) compared to chondrocytes prepared from cartilage with low-grade disease (Fig. 2E,F).

**Pharmacological induction of mitochondrial dysfunction induces an OA-like phenotype in chondrocytes and cartilage explants in vitro**

As chondrocyte is the only cell type present in the cartilage tissue, we determined the consequences of induced mitochondrial dysfunction on catabolic gene expression and investigated the associated mechanism using a well-known inducer of mitochondrial dysfunction, carbonyl cyanide 3-chlorophenylhydrazone (CCCP). When treated with CCCP, chondrocytes from low-grade OA cartilage (Makin score ≤2) produced high levels of mitochondrial superoxide, as determined by fluorescence microscopy (Fig. 3A) and flow cytometry (Fig. 3B,C). We also found significant loss of mitochondrial membrane potential in chondrocytes upon CCCP treatment (Fig. 3D,E). The loss of mitochondrial membrane potential was dependent on CCCP concentration in the beginning (10 min) but after 1 h almost 90% of the mitochondrial population was depolarized at all concentrations tested in this study (10-50 µM) (Fig. 3F). Moreover, live-cell imaging showed a rapid increase in the red fluorescence and an increase in green fluorescence of the JC-1 dye within 6 min of CCCP (50 µM) treatment (Fig. 3G). Also, live-cell imaging revealed mitochondrial membrane potential loss in chondrocytes upon IL-1β (5 ng/ml) stimulation (Fig. 3H), as reported previously (Ansari et al., 2018b). The depolarization of mitochondria was associated with reduced ATP production in normal and OA chondrocytes treated with either IL-1β or CCCP (Fig. 3I). Interestingly, the reduction in ATP production was severe in chondrocytes isolated from the diseased tissue compared to chondrocytes prepared from normal healthy tissue (Fig. 3J). The CCCP-induced oxidative stress was also confirmed by the estimation of reduced glutathione (GSH) in primary mouse chondrocytes and was found to be significantly reduced (Fig. 3J).

Osteoarthritic cartilage and chondrocytes expressed high levels of catabolic genes, including inflammatory mediators and cartilage ECM-degrading proteases (Fig. 1J). In order to confirm that mitochondrial dysfunction is directly associated with the increased expression of the catabolic genes, we treated chondrocytes with CCCP and found a significant increase in the expression of IL-6, MMP-3, -9, -13 and ADAMTS5 at mRNA (Fig. 4A) and protein levels (Fig. 4B). IL-1β- and H2O2-treated chondrocytes were used as positive controls (Fig. 4A,B). These results were further validated by treating chondrocytes with different concentrations of CCCP (10-50 µM) and with 50 µM CCCP at different time points, and the data showed a dose- and time-dependent effect of CCCP on the expression of the catabolic genes in chondrocytes (Fig. 4C-E). The viability of chondrocytes treated with CCCP up to 72 h post-treatment was determined by an MTT assay and no significant cell death was observed at 24 h post treatment (Fig. S1). We also treated human normal chondrocytes with CCCP and found that the expression of MMP-3, -9, -13 and COX-2 was significantly increased in CCCP-treated normal chondrocytes (Fig. 4F). Taken together, these results highlight a direct association between mitochondrial dysfunction in chondrocytes and high-level expression of catabolic genes involved in OA pathogenesis.

**Pharmacological induction of mitochondrial dysfunction promotes cartilage ECM degradation in cartilage explants**

To study the effect of mitochondrial dysfunction on cartilage matrix integrity, cartilage explants prepared from the preserved OA cartilage were treated with CCCP for 72 h and the expression of MMP-13 and ADAMTS5 was determined by immunohistochemistry (IHC) staining. We found increased expression of MMP-13 and ADAMTS5 upon induction of mitochondrial dysfunction by CCCP (Fig. 5A,B). The increase in the expression of MMP-13 and ADAMTS5 was associated with increased degradation of proteoglycans (Fig. 5C,D) and type II collagen (Fig. 5E). Furthermore, when mouse femoral head explants were treated with CCCP, loss of mitochondrial membrane potential was observed through increased JC-1 green fluorescence (Fig. 5F,G). Histological analyses of these explants showed decreased Safranin O/Fast Green (S/F) staining (Fig. 5H) and increased soluble glycosaminoglycans (sGAG) released in the culture supernatant (Fig. 5I). We also found increased expression of MMP-13 in mouse femoral head explants treated with CCCP (Fig. 5J; Fig. S2A).

These results thus link the mitochondrial dysfunction with the active production of matrix degrading proteases and cartilage ECM...
degradation. ROS generation in cartilage explants treated with CCCP was confirmed by immunostaining with anti-DNA/RNA oxidative damage marker (nucleic acid oxi dam) antibody (Fig. 5K; Fig. S2B) and with 3-nitrotyrosine antibody (Fig. 5L,M). The immunostaining data showed ROS caused damage to DNA/RNA and proteins in CCCP-treated mouse cartilage explants, and support the hypothesis that pro-oxidants generated upon induction of mitochondrial dysfunction damage the macromolecules in chondrocytes, which may be contributory to disease severity and pathogenesis by disrupting their physiological activity.

To investigate the mechanism of mitochondrial dysfunction-induced expression of catabolic genes in chondrocytes, we used MitoTEMPO to scavenge mitochondrial superoxide produced due to mitochondrial damage induced by treatment with CCCP, and support the hypothesis that pro-oxidants generated upon induction of mitochondrial dysfunction damage the macromolecules in chondrocytes, which may be contributory to disease severity and pathogenesis by disrupting their physiological activity.

To investigate the mechanism of mitochondrial dysfunction-induced expression of catabolic genes in chondrocytes, we used MitoTEMPO to scavenge mitochondrial superoxide produced due to mitochondrial damage induced by treatment with CCCP, and determined the expression of the catabolic genes. First, we prepared human OA chondrocytes from high-grade OA cartilage (Mankin score $\geq 3$) and stained them with MitoSOX in the presence of different concentrations of MitoTEMPO. Chondrocytes prepared from high grade OA cartilage produced high levels of mitochondrial superoxide that were reduced by treatment with MitoTEMPO (Fig. S3A,B). Treatment of chondrocytes prepared from low-grade OA cartilage with MitoTEMPO also suppressed the CCCP-induced production of mitochondrial superoxide (Fig. 6A,B) and the expression of COX-2, IL-6, MMP-3, -9, -13 and ADAMTS5 (Fig. 6C). We also determined the secreted levels of IL-6 and MMP-13 in culture supernatant and found a significant reduction in the secreted levels of IL-6 and MMP-13 (Fig. 6D). These results indicated that ROS generated due to mitochondrial dysfunction in chondrocytes induce the expression of COX-2, IL-6, MMP-3, -9, -13 and ADAMTS5.

Mitochondrial dysfunction or treatment with H$_2$O$_2$ failed to activate the NF$\kappa$B pathway in human primary OA chondrocytes

We next investigated the signaling pathways involved in mitochondrial dysfunction-induced expression of catabolic genes in chondrocytes. We first determined the activation of the NF$\kappa$B pathway by analyzing the degradation of I$\kappa$B$\alpha$, the most abundant endogenous inhibitor of NF$\kappa$B. Interestingly, CCCP treatment of chondrocytes did not induce degradation of I$\kappa$B$\alpha$, indicating that mitochondrial dysfunction in chondrocytes does not activate gene expression through the activation of NF$\kappa$B as shown by us previously (Singh et al., 2002), but both H$_2$O$_2$ and CCCP treatment failed to induce I$\kappa$B$\alpha$ degradation (Fig. 7B). Similarly, in human primary chondrocytes transfected with NF$\kappa$B luciferase reporter, activation of NF$\kappa$B was not observed upon CCCP treatment (Fig. 7C). Taken together, these data demonstrated that

Fig. 2. High-grade human OA cartilage produces high levels of mitochondrial superoxide in vivo. (A,C) Slices of human OA cartilage from low-grade (n=5) and high-grade (n=5) OA samples were stained with DHR123 (A) and MitoSOX (C), and the images were captured using a confocal microscope. (B,D) DHR123 and MitoSOX fluorescence intensity in low- and high-grade OA cartilage, respectively. Scale bars: 200 µm. (E,F) Human primary chondrocytes isolated from low-grade and high-grade OA cartilage (n=5 in each group) were immediately stained with MitoSOX, and the fluorescence intensity was determined using a flow cytometer. Data are means±95% c.i. **P<0.005 (Mann–Whitney test).
Fig. 3. See next page for legend.
Fig. 3. Pharmacological induction of mitochondrial dysfunction increased the levels of mitochondrial superoxide in human primary OA chondrocytes. (A) Human primary chondrocytes (n=5) were stained with MitoSOX (10 µM) followed by treatment with CCCP (50 µM). The cells were fixed and nuclei were counterstained with DAPI. Images were captured using a confocal microscope. Scale bars: 10 µm. (B,C) Human primary chondrocytes (n=5) were stained with MitoSOX followed by treatment with CCCP, and the level of mitochondrial superoxide was determined using a flow cytometer. (D,E) Human primary OA chondrocytes (n=5) were stained with JC-1 dye followed by CCCP treatment (D). The JC-1 fluorescence was determined using a flow cytometer. DMSO-alone treatment was used as a control. The cells in Q3 (proportion is indicated by percentage) have increased green fluorescence, an indicator of mitochondrial membrane potential loss, in CCCP-treated chondrocytes (FL1, green fluorescence; FL2, red fluorescence). The ratio of red to green fluorescence was calculated to determine mitochondrial membrane potential loss (E). (F) Chondrocytes (n=3) were stained with JC-1 and treated with different concentrations (10-50 µM) of CCCP for the indicated times. The ratio of red and green JC-1 fluorescence was calculated to determine the mitochondrial membrane potential loss. (G) Human OA chondrocytes were stained with JC-1 followed by treatment with CCCP (50 µM), and the red and green fluorescence in live chondrocytes was recorded by confocal microscopy at 1 min intervals. Images are representative of three independent experiments. Scale bars: 10 µm. (H) Human OA chondrocytes were stained with JC-1 followed by treatment with IL-1β, and the red and green fluorescence was recorded by live-cell imaging as above. Images are representative of three independent experiments. Scale bars: 10 µm. (I) ATP levels were determined in CCCP- and IL-1β-treated normal and OA chondrocytes (n=3 in each group). (J) GSH levels were determined in primary mouse chondrocytes (n=5) treated with DMSO (control) or CCCP. Data are mean±SD (c.i. *P<0.05, **P<0.005, ***P<0.0005 [Mann–Whitney test (C); Student’s t-test (E,I,J)].

Mitochondrial dysfunction induces catabolic gene expression through the AP1 pathway

We further investigated the effect of induced mitochondrial dysfunction on the downstream transcription factor(s) involved in the regulation of catabolic gene expression in chondrocytes. The AP1 transcription factor family is composed of homodimeric and heterodimeric complexes of cJun and cFos proteins. CCCP-induced mitochondrial dysfunction increased the phosphorylation of cFos and cJun in human primary chondrocytes (Fig. 8A). In addition to CCCP, oligomycin and rotenone failed to activate the NFκB pathway but activated the AP1 pathway, as evidenced by the increased phosphorylation of cFos (Fig. 8B), suggesting this phenomenon is associated with mitochondrial dysfunction and not the dysfunction inducer. The activation of AP1 transcription factor activity in chondrocytes with induced mitochondrial dysfunction was further confirmed by using an AP1 luciferase reporter activity assay in human primary OA chondrocytes (Fig. 8B) and in primary mouse chondrocytes (Fig. 8C). IL-1β treatment of mouse chondrocytes was used as positive control (Fig. 8C). CCCP-induced mitochondrial dysfunction also triggered the nuclear translocation of cFos (Fig. 8D). Activation of cFos and AP1 was also confirmed by an ELISA-based cFos–AP1 activity assay in the nuclear extract from human primary chondrocytes (Fig. 8E). To validate the role of AP1 in vivo in human OA cartilage, we determined the expression of cFos in low- and high-grade human OA cartilage by IHC. Our results showed a significant increase in the expression levels of cFos (Fig. 8F,G) in the high-grade OA cartilage compared to low-grade OA cartilage. To further confirm the role of the AP1 pathway in mitochondrial dysfunction-induced expression of pro-inflammatory and cartilage ECM degrading proteases, we pretreated primary chondrocytes with the cFos and cJun inhibitor (T5224) followed by CCCP treatment, and the expression of the catabolic genes was found to be significantly reduced (Fig. 8H). These results demonstrate that mitochondrial dysfunction-induced expression of pro-inflammatory and cartilage ECM degrading proteases in chondrocytes is mediated via the JNK-mediated activation of the AP1 pathway (Fig. S5).

DISCUSSION

Mitochondrial dysfunction and oxidative stress have been shown to be important contributors to aging and many age-related human disorders (Bratic and Larsson, 2013). Several in vitro studies have established a significant role for mitochondrial dysfunction in catabolic gene expression in chondrocytes (Blanco et al., 2011; Cillero-Pastor et al., 2008, 2013). In this study, we showed that, compared to normal cartilage, mitochondrial function is impaired in OA cartilage in vivo. Our data also showed a significant increase in the mitochondrial superoxide levels in human OA cartilage in vivo, which correlated with catabolic gene expression in OA cartilage. Using CCCP as a mitochondrial dysfunction inducer, we determined the effect of mitochondrial dysfunction on the expression of OA-related catabolic genes in chondrocytes and uncovered the associated signaling pathway. We found that mitochondrial dysfunction increased the protein expression of inflammatory...
mediators COX-2 and IL-6, and matrix-degrading proteases MMP-3, -9, -13 and ADAMTS5, in human and mouse cartilage explants, and correspondingly, increases in type II collagen and proteoglycan degradation were observed upon CCCP-induced mitochondrial dysfunction in vitro, which is in agreement with the increase in their gene expression in vivo. Treatment with mitochondria-targeted antioxidant MitoTEMPO blocked the mitochondrial dysfunction-induced increase in ROS levels and the expression of catabolic genes, thus demonstrating the direct role of mitochondrial superoxide in the expression of inflammatory and matrix-degrading molecules in cartilage. We also discovered that the effect of mitochondrial dysfunction on catabolic gene expression was chiefly mediated through the activation of the JNK/AP1 pathway in chondrocytes. Our data also showed an increase in the expression of the cFos component of AP1, which correlated with the increase in the severity of the disease. This study provides a detailed mechanism of mitochondrial dysfunction-induced expression of catabolic genes in human primary OA chondrocytes.

Mitochondria are the center of cellular homeostasis and are the major source of cellular energy requirements. Impairment of mitochondrial function may induce oxidative stress with adverse effects on cellular homeostasis (Blanco et al., 2004). We show here that mitochondrial dysfunction and oxidative stress are increased with the severity of OA. It is important to note that the chondrocytes in the deep zone of the undamaged human OA cartilage showed relatively higher mitochondrial membrane potential compared to chondrocytes present in the superficial damaged cartilage, indicating that chondrocytes in deep zone cartilage have a phenotype similar to normal cartilage chondrocytes. This was also supported by Toluidine Blue staining, which showed near normal matrix in the deep zone. A limitation of this
Fig. 5. See next page for legend.
study was the lack of comparison of mitochondrial membrane potential between normal low-grade and high-grade OA human cartilage/chondrocytes and its association with catabolic gene expression. Such studies in the future may help us understand the progression of disease with increased mitochondrial dysfunction. Oxygen tension is an important player and plays a key role in chondrocyte dedifferentiation and redifferentiation, and in the regulation of gene expression in chondrocytes (Li et al., 2014; Liu et al., 2019; Malda et al., 2004). There exists a gradient of oxygen in the cartilage tissue in vivo with decreasing oxygen tension with increasing depth (Liu et al., 2019). The experiments in this study were performed in the presence of 5% CO2 and 95% air (~20% oxygen). The higher oxygen tension may contribute to increased ROS production in chondrocyte monolayer culture, which may affect chondrocyte gene expression. Future studies in controlled oxygen tension atmosphere may provide additional insight into mitochondrial dysfunction in chondrocytes and its contribution to catabolic gene expression.

Oxidative stress in chondrocytes has been implicated in the increased expression of inflammatory mediators and matrix-degrading proteases (Ahmad et al., 2020b; Ansari et al., 2020; Lepetsos and Papavassiliou, 2016), and targeting of ROS suppresses the severity of OA in vivo (Coleman et al., 2018; Khan et al., 2017; Martin et al., 2009). Mitochondria are the most common site of ROS production in the cells and previous studies have demonstrated increased ROS production and inflammatory gene expression in primary chondrocytes upon induction of mitochondrial dysfunction by oligomycin and antimycin A.
Fig. 7. See next page for legend.
Mitochondrial damage and dysfunction in the activation and suppression of catabolic gene expression in chondrocytes. (A) I kBx levels in human primary OA chondrocytes treated with CCCP for the indicated times. (B) I kBx levels in human primary OA chondrocytes treated with CCCP, H2O2 and IL-1β for 15 and 30 min. IL-1β treatment was used as a positive control. (C) NFκB luciferase reporter activity assay in chondrocytes (n=3) treated with DMSO (control) and CCCP. Renilla luciferase plasmid transfection was used for normalization. (D) JNK, p38 and ERK phosphorylation in CCCP, IL-1β- and H2O2-treated chondrocytes was determined by immunoblotting. (E) JNK, p38 and ERK phosphorylation in CCCP-treated human primary OA chondrocytes. (F) COX-2, ADAMTS5, MMP-13 and IL-6 mRNA levels in human primary OA chondrocytes (n=3) treated with CCCP in the presence or absence of JNK inhibitor (SP600125). (G) MMP-13 and IL-6 levels in the culture supernatant of CCCP-treated human primary OA chondrocytes (n=3) in the presence and absence of JNK inhibitor (SP600125). (H) Expression of MMP-13 in human primary chondrocytes treated with CCCP in the presence of either the NFκB inhibitor (MG132), JNK inhibitor (SP600125), p38 inhibitor (SB202190) or ERK inhibitor (PD98059) was determined by immunoblotting. (I) Expression of IL-6 and MMP-13, and mouse primary chondrocytes treated with CCCP in the presence of JNK-IN-8 was determined by immunoblotting. (J) NFκB luciferase reporter activity (FF, firefly luciferase) was determined in mouse chondrocytes (n=5) in response to CCCP treatment. IL-1β-treated chondrocytes were used as a positive control. Renilla luciferase (RL) was used for normalization. Data are mean±95% c.i. *P<0.05, **P<0.005, ***P<0.0005, ns, not significant.

Our data thus suggest that mitochondrial dysfunction-induced oxidative stress is a crucial modulator of inflammatory gene expression in OA chondrocytes.

Targeting of many pro-inflammatory signaling pathways, such as NFκB, MAPK and Wnt, have been shown to exert chondroprotective effects in in vitro and in vivo studies (Ahmed et al., 2005; Deshmukh et al., 2018; Lietman et al., 2018; Loeser et al., 2008; Rasheed et al., 2010; Roman-Blas and Jimenez, 2006). Our data are distinct, as we show here that mitochondrial dysfunction-induced expression of catabolic genes occurred independently of the NFκB pathway in human primary OA chondrocytes. Interestingly, treatment of human primary chondrocytes with H2O2 also did not activate the NFκB pathway, establishing that ROS is not an activator of NFκB in primary chondrocytes. It has been reported that ROS exert the inflammatory effects through the activation of the JNK pathway (Lo et al., 1996). The role of the JNK pathway in the regulation of catabolic gene expression in chondrocytes has also been confirmed previously by using a JNK-specific inhibitor (Akhtar and Haqqi, 2011; Haseeb et al., 2017) and by others using a knockout mouse model of JNK (Ismail et al., 2016; Loeser et al., 2020). In this study, we demonstrate, for the first time, that mitochondrial dysfunction increases the expression of COX-2, IL-6, MMPs and ADAMTS5 through the activation of the JNK pathway in human chondrocytes.

AP1 transcription factors represent the dimeric transcription factor complex of Fos, Jun or the activating transcription factor family that bind to the AP1 DNA-binding site found in the promoter and regulate the expression of various cytokines and MMPs. Inhibition of cFos and cJun AP1 transcription factors using T-5224 has been shown to exert a protective effect in a mouse model of collagen-induced arthritis and intervertebral disc degeneration (Aikawa et al., 2008; Makino et al., 2017). Our data presented here demonstrate that ROS produced as a result of mitochondrial dysfunction induce the phosphorylation of the cFos and cJun components of AP1, and increased its nuclear translocation in chondrocytes. Moreover, use of the cFos and cJun inhibitor T-5224 suppressed the expression of mitochondrial dysfunction-induced catabolic gene expression in chondrocytes, thus providing further support to our results as this demonstrates that JNK pathway activation by ROS plays a crucial role in catabolic gene regulation in OA. These findings were further supported by the observation that cFos expression was highly upregulated in the high-grade human OA cartilage, which also showed higher levels of ROS compared to low-grade OA cartilage in vitro. Taken together, these data indicate that mitochondrial dysfunction-induced activation of AP1 transcription factors is directly associated with disease severity and progression in OA.

In summary, we have identified a functional mechanism for the expression of catabolic genes in OA by mitochondrial dysfunction-induced oxidative stress in chondrocytes. We demonstrate here that disruption of mitochondrial function activates the JNK/cFos/AP1 pathway, leading to the expression of the catabolic genes and ECM degradation in vivo and in vitro, and is in agreement with recent reports that impairment of mitochondria may constitute an important factor in the pathogenesis of OA.

**MATERIALS AND METHODS**

**Cartilage and chondrocytes**

Studies for the use of discarded de-identified human cartilage were approved as a non-human subject study under 45 Code of Federal Regulations, Exemption 4, by the Northeast Ohio Medical University and Summa Health Institutional Review Boards. Discarded and de-identified OA cartilage was used in this study.

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References:

(Ahmed et al., 2005; Deshmukh et al., 2018; Lietman et al., 2018; Loeser et al., 2008; Rasheed et al., 2010; Roman-Blas and Jimenez, 2006).

(Akhtar and Haqqi, 2011; Haseeb et al., 2017).

(Aikawa et al., 2008; Makino et al., 2017).

(Ansari et al., 2019). Oxidative stress induced by IL-1β treatment in human primary OA chondrocytes is associated with increased apoptosis (Ansari et al., 2018b; Collins et al., 2016). We have previously shown that IL-1β-induced ROS generation promotes IL-6 expression through ROS-mediated activation of the cFos-AP1 pathway in chondrocytes and scavenging of ROS by N-acetyl-L-cysteine or diphenylidonium inhibited the IL-1β-induced activation of cFos and suppressed IL-6 expression (Haseeb et al., 2017). Previous *in vitro* studies have shown the effect of mitochondrial dysfunction on the expression of MMPs (Cillero-Pastor et al., 2013; Reed et al., 2014); however, the mechanism is not fully understood. We show here that scavenging of mitochondrial superoxide by a mitochondria-specific ROS scavenger, MitoTEMPO, suppressed the expression of catabolic genes, indicating the potential role for ROS generated due to mitochondrial damage and dysfunction in the activation and enhanced expression of catabolic genes in chondrocytes in OA.
Fig. 8. Mitochondrial dysfunction activated the cFos-AP1 pathway in human primary chondrocytes. (A) Human primary chondrocytes were treated with CCCP (50 µM) for the indicated times and phosphorylation of cFos and cJun was determined by immunoblotting. (B) AP1 luciferase reporter activity in CCCP-treated chondrocytes (n=3). Renilla luciferase transfection of chondrocytes was used for normalization. (C) AP1 luciferase reporter activity in CCCP-treated mouse chondrocytes (n=5). IL-1β-treated mouse chondrocytes were used as a positive control. Renilla luciferase transfection was used for normalization. (D) Nuclear translocation of cFos in CCCP-treated human primary OA chondrocytes. Scale bars: 5 µm. (E) cFos-AP1 activity in the nuclear extract prepared from chondrocytes (n=5) treated with different concentrations of CCCP (10, 30 and 50 µM). (F) S/F staining (top panel) and cFos IHC (bottom panel) in low-grade and high-grade human OA cartilage. Scale bars: 200 µm (top panel); 100 µm (bottom panel). (G) Quantification of the cFos IHC. (H) Human primary chondrocytes (n=3) were treated with AP1 inhibitor (T-5224) for 2 h followed by CCCP for 24 h, and the expression of IL-6, MMP-13, ADAMTS5 and COX-2 was determined by qPCR. Data are mean±95% c.i. *P<0.05; **P<0.005; ns, not significant [Student’s t-test (B,G) and ANOVA (C,E,H)].
derived from OA patients who underwent total knee arthroplasty at the Summa Health System Barberton Hospital, Barberton, Ohio. Normal cartilage (no known history of any rheumatic diseases) was procured commercially from the National Disease Research Interchange. The tissue was shipped the same day of collection for overnight delivery in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with antibiotics and was processed immediately upon receipt. The animal studies were approved by the Institutional Animal Care and Use Committee of the Northeast Ohio Medical University. Chondrocytes from human cartilage and from 6-week-old male C57BL/6 mouse knee joints were prepared by enzymatic digestion, and cultured as described previously in several of our publications (Ahmad et al., 2020a; Ahmed et al., 2005; Akhtar and Haqqi, 2011; Ansari et al., 2019; Green et al., 2020; Rasheed et al., 2010). Human primary OA chondrocytes (10^6/well in a six-well plate) were cultured in DMEM/F-12 (1:1) supplemented with 10% fetal bovine serum (FBS). Human OA cartilage explants were prepared using a punch biopsy (3 mm) and cultured in DMEM/F-12 plus 10% FBS. Femoral heads from 8-week-old male C57BL/6 mice were collected and used as explants for in vitro studies.

Reagents and antibodies

CCCP was procured from Sigma-Aldrich (C2759), DMEM from GE Healthcare (SH30243.02) and F-12 medium from Thermo Fisher Scientific (11760562). Pronase and Collagenase were purchased from Sigma-Aldrich (1145964001 and 11088793001, respectively). TaqMan assays for mRNA quantification were obtained from Integrated DNA Technologies. Validated antibodies against human IL-6 (sc-103026, 1:1000 for western blotting) and β-actin (sc-47778, 1:2000 for western blotting) were obtained from Santa Cruz Biotechnology. Antibodies against MMP-13 (ab39012, 1:1000 for western blotting and 1:200 for IHC/immunofluorescence and ADAMTS5 (ab41037, 1:1100 for western blotting and 1:200 for IHC) were obtained from Abcam. Antibodies against Phospho-JNK (ab 4668, 1:1000 for western blotting) and total JNK (9252S, 1:1000 for western blotting) were purchased from Cell Signaling Technology. Antibodies against P-Cfos (sc-81485, 1:4850 for western blotting) and total cFos (sc-166940, 1:500 for western blotting and 1:100 for IHC) were obtained from Santa Cruz Biotechnology. The DNA/RNA oxidative damage marker antibody (sc12501, 1:100 for IHC) was purchased from QED Bioscience, and 3-nitrotyrosine antibody (sc-32757, 1:100 for IHC) was obtained from Santa Cruz Biotechnology. Horseradish peroxidase (HRP)-conjugated anti-mouse IgG (7076S) and anti-rabbit IgG (7074S) antibodies were obtained from Cell Signaling Technology. We used the HRP-conjugated secondary antibodies at a dilution of 1:3000. Anti-rabbit IgG Alexa Fluor-488 (A-11034), anti-rabbit Alexa Fluor-594 (A-11012), anti-mouse Alexa Fluor-488 (A-11059) and anti-mouse Alexa Fluor-594 (A-11005) were procured from Invitrogen and each of these antibodies was used at a dilution of 1:100. MitoTracker (SML0737, Sigma-Aldrich), JNK-In-8 (S4901, Selleck Chemicals), TS224 (HY-12270, MedChemExpress), ERK inhibitor PD98059 (513000, Calbiochem), JNK inhibitor SP600125 (420119, Calbiochem) and NF-kB inhibitor MG132 (474790, Calbiochem) were also used in these studies.

Determination of mitochondrial membrane potential by JC-1 dye staining in human OA cartilage

Loss of mitochondrial membrane potential in cartilage was assessed using the mitochondria-specific fluorescent probe JC-1 (5,5′,6,6′-tetrachloro-1,1′,3′,3′-tetraethyl-benzimidazolylcarbocyanine iodide) (T3168, Invitrogen) as described previously (Ansari et al., 2018b). Multiple (5-10) hand-cut thin slices (70-120 µm) of normal (Invitrogen) as described previously (Ansari et al., 2018b). Briefly, chondrocytes prepared from the low-grade and high-grade human OA cartilage (n=5 per group) by sequential enzymatic digestion, were directly (no further culture) stained with JC-1 (5 µM) for 20 min at 37°C. The cells were fixed with 10% NBF, and red and green fluorescence of the JC-1 dye was recorded using a flow cytometer (BD Accuri C6). For pharmacological induction of mitochondrial dysfunction, chondrocytes were stained with JC-1 (5 µM) for 20 min followed by treatment with CCCP or other mitochondrial dysfunction inducers for 5 min, or for the indicated time, and analyzed using a flow cytometer. Flow cytometry data were analyzed using FlowJo software (Tree Star Inc). For live-cell imaging, chondrocytes were stained with JC-1 and treated with CCCP or IL-1β. The images were captured at every 1 min interval using a confocal microscope.

Measurement of mitochondrial superoxide with MitoSOX or DHR123 in chondrocytes and cartilage

Mitochondrial superoxide levels in chondrocytes were determined using MitoSOX Red dye (Life Technologies, M36008) or DHR123 (Life Technologies, D23806) as described previously (Ansari et al., 2018b). Briefly, human primary OA chondrocytes were stained with MitoSOX Red (5 µM) or DHR123 (5 µM) for 10 min at 37°C in DMEM (Phenol Red Free), followed by CCCP (50 µM) or DMSO (0.1%) treatment for 30 min. Chondrocytes were washed with PBS, and MitoSOX or DHR123 fluorescence was determined by flow cytometry. To determine mitochondrial superoxide levels in OA cartilage, thin slices of low-grade (Mankin score ≤2) and high-grade (Mankin score ≥3) OA cartilage (n=5 in each group) were stained with MitoSOX (5 µM) or DHR123 (5 µM) for 1 h. Cartilage sections were washed with PBS, fixed and permeabilized with 0.3% Triton X-100 in PBS. The nuclei were counterstained with DAPI and images were captured using a confocal microscope at 10× magnification. We also quantified mitochondrial superoxide in low-grade (Mankin score ≤2) and high-grade (Mankin score ≥3) OA cartilage by staining the chondrocytes with MitoSOX immediately after digestion of the cartilage, followed by flow cytometry.

Treatment of chondrocytes and cartilage explants with CCCP

All the experiments were performed with human primary chondrocytes or primary mouse chondrocytes. Human primary OA chondrocytes were prepared from low-grade OA cartilage as described above and seeded in a six-well plate (10^5 per well) in complete medium (DMEM-F12 supplemented with 10% FBS), cultured for 48-72 h and then treated with CCCP (50 µM or indicated concentration) for the indicated time in complete medium to avoid any unwanted stress conditions. DMSO (0.1%) treatment on its own of chondrocytes served as a control. Loss of mitochondrial membrane potential was analyzed by flow cytometry. Chondrocytes were harvested at the end of the experiment to prepare RNA for quantitative (q)PCR or cell lysate for immunoblotting. Human cartilage explants were collected from the undamaged areas of low-grade (Mankin score ≤2) OA cartilage using a punch biopsy (3 mm) and treated with CCCP (50 µM) for 72 h. A small volume of culture supernatant was collected at 24, 48 and 72 h to determine sGAG levels, and cartilage explants were harvested at 72 h for histology. Mouse femoral heads were harvested from 8-week-old mice (n=6 per group) and used as cartilage explants, and treated with DMSO or CCCP (50 µM) for 48 h. The explants were stained with JC-1 for mitochondrial membrane potential analysis and fixed with 10% NBF for histological analyses.

Determination of accumulation of dysfunctional mitochondria

The accumulation of dysfunctional mitochondria in OA chondrocytes was determined by either MitoTracker Deep Red (Life Technologies, M22426) staining or by isolation of total DNA and taking the ratio of mitochondria gene versus nuclear gene as described previously (Rooney et al., 2015) using the following primers: mitochondrial gene, tRNA-Leu(UUR), forward, and
endogenous normalization control.

sGAG was determined against a standard prepared using chondroitin antibody for 2 h followed by washing with 1× TBS, and developed using a HRP detection reagent (Millipore, WBLUF0500) and imaged using a PXi-4 gel imaging system (Syngene).

Histology and IHC

Histology and IHC in human and mouse cartilage tissue was carried out as described previously (Ansari and Haqqi, 2016). Briefly, primary chondrocytes were harvested for cell lysate preparation for immunoblot analysis of protein expression or total RNA preparation for gene expression analysis, and the cell debris, and protein concentration was determined using a Bradford assay (Bio-Rad, 500-0006). The cell lysate was resolved on 10% or 12% SDS-PAGE, transferred to a polyvinylidene membrane (Bio-Rad, 170-0027), and protein levels were determined using a chemiluminescent detection system (Roche, 11697498001) and phosphatase inhibitor (Thermo Scientific, PI-78420). The cell lysate was centrifuged at 20,000 μg for 10 min at 4°C to remove cell debris, and protein concentration was determined using a Bradford assay (Bio-Rad, 500-0006). The cell lysate was resolved on 10% or 12% SDS-PAGE, transferred to a polyvinylidene membrane (Bio-Rad, 170-0027), and protein levels were determined using a chemiluminescent detection system (Roche, 11697498001) and phosphatase inhibitor (Thermo Scientific, PI-78420). The cell lysate was centrifuged at 20,000 μg for 10 min at 4°C to remove cell debris, and protein concentration was determined using a Bradford assay (Bio-Rad, 500-0006). The cell lysate was resolved on 10% or 12% SDS-PAGE, transferred to a polyvinylidene membrane (Bio-Rad, 170-0027), and protein levels were determined using a chemiluminescent detection system (Roche, 11697498001) and phosphatase inhibitor (Thermo Scientific, PI-78420). The cell lysate was centrifuged at 20,000 μg for 10 min at 4°C to remove cell debris, and protein concentration was determined using a Bradford assay (Bio-Rad, 500-0006). The cell lysate was resolved on 10% or 12% SDS-PAGE, transferred to a polyvinylidene membrane (Bio-Rad, 170-0027), and protein levels were determined using a chemiluminescent detection system (Roche, 11697498001) and phosphatase inhibitor (Thermo Scientific, PI-78420). The cell lysate was centrifuged at 20,000 μg for 10 min at 4°C to remove cell debris, and protein concentration was determined using a Bradford assay (Bio-Rad, 500-0006).

RNA isolation and qPCR

Total RNA from cartilage or chondrocytes treated with CCCP or DMSO control was isolated using TRIzol (Life Technologies, 15596018). Chloroform extraction and ethanol precipitation was performed as described previously (Ansari et al., 2019). Total RNA (1 μg) was used to prepare cDNA using a high capacity cDNA synthesis kit (Life Technologies, 4368813) and mRNA expression was analyzed using TaqMan gene expression assays (Integrated DNA Technologies). β-actin was used as an endogenous normalization control.

Western blotting

Chondrocyte lysate preparation and western blotting of chondrocytes treated with CCCP or DMSO control were performed as described previously (Ansari et al., 2017). Human primary chondrocytes were harvested at the end of the experiment and lysed in RIPA buffer supplemented with protease inhibitors (Roche, 11697498001) and phosphatase inhibitor (Thermo Scientific, PI-78420). The cell lysate was centrifuged at 20,000 μg for 10 min at 4°C to remove cell debris, and protein concentration was determined using a Bradford assay (Bio-Rad, 500-0006). The cell lysate was resolved on 10% or 12% SDS-PAGE gels and transferred to a polyvinylidene membrane (Bio-Rad, 1704272), blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline with Tween 20 (TBS-T, 0.1% Tween 20) and incubated with target protein antibody (2% BSA in TBS-T) overnight at 4°C. Next day, the blot was washed three times with TBS-T, incubated with appropriate HRP-conjugated secondary antibody at room temperature for 2 h, developed using the chemiluminescent HRP detection reagent (Millipore, WBLUF0500) and imaged using a PXi-4 gel imaging system (Syngene).

Histology and IHC in human and mouse cartilage tissue was carried out as described previously (Ansari et al., 2018a, 2019). Briefly, human normal, OA cartilage tissue or explants treated with CCCP or DMSO control were fixed in 10% NBF, decalcified and processed for paraffin embedding and 5 μm sections were prepared for S/F or Toluidine Blue staining. For IHC, cartilage sections were deparaffinized by three changes in xylene (5 min each) and rehydrated by passing through a series of alcohol solutions of decreasing concentrations (100%, 90%, 70% and 50%), and finally washed with 1× TBS for 10 min. The antigen retrieval was performed by heating the sections in 10 mM citrate buffer (pH 6.0) using a microwave oven for 3 min and sections in 10 mM citrate buffer (pH 6.0) using a microwave oven for 3 min. The sections were then washed with 1× TBS and incubated with 5% goat serum for 30 min at room temperature, and incubated in primary antibody overnight at 4°C. Sections were washed with 1× TBS and incubated with HRP-conjugated secondary antibody for 2 h followed by washing with 1× TBS, and developed using a DAB substrate kit (Pierce, 34002).

GSH estimation

Primary mouse chondrocytes (n=5) were treated with DMSO (control) or CCCP overnight and the GSH levels were determined using an Amplitite Rapid Fluorometric Glutathione Assay Kit (AAT Bioquest, 10060) following the instructions provided by the manufacturer.

Statistical analyses

All experiments were performed with chondrocytes or cartilage explants from five or the indicated number of donor samples. Statistical significance between two groups was calculated using a two-tailed unpaired Student's t-test.
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**i-test for parametric data and a Mann–Whitney U-test for non-parametric data. Statistical significance between more than two groups was analyzed using one-way ANOVA, followed by Dunnett’s test for post-hoc analysis. All statistical analysis was performed using GraphPad Prism software (version 7.04). The data are presented as dot plot with mean±95% c.i. The dots represent the number of biological replicates in each experiment. In all experiments, P values less than 0.05 were considered significant (⁎P<0.05, ⁎⁎P<0.005 and ⁎⁎⁎P<0.0005).**

**Competing interests**

The authors declare no competing or financial interests.

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**Author contributions**


**Funding**

This work was supported by the National Institutes of Health (NIH)/National Institute of Arthritis and Musculoskeletal and Skin Diseases (R01-AR067056 to T.M.H.); the National Center for Complementary and Integrative Health (R01-AT007373 to T.M.H.); and the Northeast Ohio Medical University to T.M.H.. Deposited in PMC for release after 12 months.

**Supplementary information**

Supplementary information available online at https://jcs.biologists.org/lookup/doi/10.1242/jcs.247353.supplemental

**Peer review history**

The peer review history is available at https://dev.biologists.org/lookup/doi/10.1242/jcs.247353 reviewer-comments.pdf

**References**


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