Nitric oxide inhibits autophagy via suppression of JNK in meniscal cells

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Abstract

Objective. Autophagy is a potential protective mechanism that is involved in several degenerative diseases. Nitric oxide (NO) is associated with programmed cellular death in meniscal cells, but whether it can induce autophagy is still undetermined. This study aims to investigate the interaction between autophagy and NO in normal human meniscal cells.

Methods. Normal meniscal cells were harvested from female patients. NO donors and NO synthase inhibitors were used to regulate the level of NO. Changes in the incidence of autophagy and apoptosis were examined using flow cytometry, western blot and immunofluorescence methods. The effects of NO-mediated autophagy regulation of the expression of MMPs and aggrecanases (ADAMTS-4 and -5) were analysed by real-time PCR.

Results. NO donors inhibited autophagy as well as augmented apoptosis in human meniscal cells with serum deprivation. Conversely, treatment with NOS inhibitors resulted in up-regulation of the autophagy level while repressing apoptosis. NOS inhibitor treatment also resulted in down-regulation of MMPs and aggrecanase mRNA expression. This effect of NOS inhibitor was also blocked by autophagy inhibitors. Our results also showed that NOS inhibitor enhanced Jun-N-terminal kinase (JNK) activation. Furthermore, SP600125, a selective JNK inhibitor, blocked up-regulation of autophagy by NOS inhibitor.

Conclusion. Our results demonstrated that NO augmented serum deprivation-induced apoptosis of meniscal cells via inhibition of autophagy through inactivation of JNK. Up-regulation of autophagy may be a potential approach in the treatment of meniscal tissue degeneration.

Key words: meniscus, autophagy, apoptosis, JNK.

Introduction

The meniscus plays an important role in both load distribution and knee joint stability. It has been proved that articular degeneration of the knee joint is directly proportional to the amount of defective meniscus [1]. Because of the limited regenerative capacity of the fibrocartilage-like tissue, the preferred treatment methods are either meniscus grafting or tissue engineering of the meniscus [2, 3]. Moreover, studies aimed at programmed cell death (PCD) of meniscal cells have also been reported in recent years [4, 5].

PCD is believed to be a vital part in the pathological process of meniscus degeneration. However, autophagic cellular death in the meniscus has never been mentioned. The autophagic process includes the formation of double layers of the isolated membrane, sequestering the cargo, and later degrading with fusion of the lysosome. The microtubule-associated protein light chain 3 (LC3) as well as Beclin-1 are closely related to the formation of autophagosomes [6]. Thus LC3-II and Beclin-1 have been used as markers to detect autophagy flux [7]. Recently the autophagy of chondrocytes was thought to play a potential role in the development of OA [8–10].

Nitric oxide (NO) plays an important role as a molecular messenger in cellular activities. NO is synthesized by a family of NO synthases (NOS), including neuronal NOS, inducible NOS (iNOS) and endothelial NOS (eNOS).
iNOS and eNOS are expressed in the meniscus tissue [11]. During the pathological process of OA and RA, an increasing level of NO is detected in knee joints with a high number of apoptotic meniscal cells [12, 13]. It has also been shown that inflammatory cytokines such as IL-1 and TNF-α can increase the NO level in meniscal cells with overload strains [14–17]. Furthermore, IL-1 can suppress the repair process of the meniscus matrix through NO-mediated MMP up-regulation [18, 19]. Conversely, inhibition of NO reduces the pathological progression of OA and RA [20, 21]. Thus NO is thought to be one of the major causes in meniscus tissue degeneration and the pathological process of OA.

However, the interplay between autophagy and NO in meniscal cell has never been documented. The objective of this study was to determine whether NO can affect autophagy in human meniscal cells.

**Methods**

**Reagents and antibodies**

The Griess reagents and caspase-3 activity kit were purchased from Beyotime (Haimen, China). The Mitotracker kit, Lyso-Tracker kit and Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA, USA). Microtubule-associated protein light chain 3 (LC3), Beclin-1, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Jun-N-terminal kinase (JNK) and p-JNK antibodies were obtained from Abcam (Cambridge, UK). Hoechst 33258, 3-methyladenine (3-MA), rapamycin, monodansylcadaverine (MDC) and collagenases were purchased from Sigma-Aldrich (St Louis, MO, USA). The cell culture reagents were purchased from Gibco. The cell counting kit-8 (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan). The apoptosis detection kit was purchased from BD Pharmingen (San Diego, CA, USA). Specific small interfering RNAs (siRNAs) targeting human LC3B as well as control siRNA were purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

**Cell culture**

With institutional review board approval (Xinhua Hospital, School of Medicine, Shanghai Jiao Tong University), normal meniscal specimens were collected from four osteosarcoma patients who underwent amputation by hip disarticulation at our hospital. Informed consent was obtained from each patient before surgery. The meniscal tissues were from four female patients [mean age 21 years (s.d. 9.4)]. The average modified Mankin score of the meniscal tissue was 0.25 (s.d. 0.5) [22]. All the cells used in the current study were only isolated from the inner one third of the non-degenerative meniscus, in which the meniscal cells have higher chondrogenic phenotypes [23]. The tissue was then minced into pieces and digested in 0.1% collagenase type II for 6 h at 37°C. The debris was seeded in DMEM/F-12 medium with 10% fetal bovine serum (FBS) in a 37°C, 5% CO₂ environment. After reaching 90% confluence, the primary passage cells were harvested and replanted. First-passage cells maintained in a monolayer were used for further experiments. After they reached 90% confluence, the medium of first-passage cells was changed to DMEM/F-12 with 1% FBS and antibiotics for 12 h in order to synchronize the cells before the next experiments. The NO donors (10 μM DETA NONOate [2,2’-(hydroxynitrosohydrazino)bis-ethanamine] and 200 μM sodium nitroprusside (SNP)) and NOS inhibitors (200 μM L-NAME (NG nitro-L-arginine methyl ester, hydrochloride) and 1 mM L-NMMA (NG monomethyl-L-arginine, monoacetate salt)) were pre-treated for 24 h in the presence or absence of autophagy inducer (rapamycin 10 μM) or inhibitor (3-MA 10 nM).

**Cell viability and NO concentration measurement**

The cell viability was assessed using CCK-8. The NO concentration was detected using Griess reagents. Meniscal cells were incubated in a 96-well plate at a density of 1 × 10⁵ cells/well and then exposed to NO donors (10 μM DETA NONOate and 200 μM SNP) or NOS inhibitors (200 μM L-NAME and 1 mM L-NMMA) in serum-free medium for 24 h. After that the culture media of every well was changed to a combination of 180 μl DMEM/F-12 and 20 μl CCK-8. The cells were incubated at 37°C for 2 h. The cell viability was assessed by measuring absorbance at 450 nm with a microplate reader. For NO concentration detection, 50 μl of Griess reagent I and II were added to each well. The NO concentration was assessed by measuring the absorbance at 540 nm. For cell viability and NO concentration evaluation, the data were expressed as fold changes relative to that of meniscal cells cultured in 10% FBS.

**Flow cytometry**

The meniscal cells were incubated in six-well plates at the density of 1 × 10⁵ cells/well with 10% FBS and DMEM/F-12 medium. After treatment with NO donors (10 μM DETA NONOate and 200 μM SNP), NOS inhibitors (200 μM L-NAME and 1 mM L-NMMA), JNK inhibitor (SP600125, 20 μM), autophagy inducer (rapamycin, 10 μM) or inhibitor (3-MA, 10 nM) in serum-free medium for 24 h, the cells were collected to examine the autophagy and apoptosis incidence using flow cytometry. The autophagy incidence was measured by detecting the MDC-positive cells. Apoptosis incidence was detected by using the Annexin V-FITC apoptosis detection kit. The samples were then analysed by a fluorescence-activated cell sorter (Beckman Coulter, Miami, FL, USA).

**Caspase-3 activity**

The meniscal cells were incubated in six-well plates at the density of 1 × 10⁵ cells. A caspase-3 activity kit, which is based on the ability of caspase-3 to convert acetyl-Asp-Glu-Val-Asp p-nitroanilide into p-nitroaniline, was used. According to the manufacturer’s protocol, after being treated with NO donors (10 μM DETA NONOate and 200 μM SNP), NOS inhibitors (200 μM L-NAME and 1 mM L-NMMA) or JNK inhibitor, cells were lysed with lysis buffer (100 μl per 2 × 10⁵ cells) on ice. Then a mixture of 10 μl of cell lysate, 80 μl of reaction buffer and 10 μl of...
2 mM caspase-3 substrate was added to each well. Caspase-3 activity was quantified in the samples with a microplate spectrophotometer at an absorbance of 405 nm. Caspase-3 activity was qualified as fold enzyme activity compared with that of the meniscal cells cultured in 10% FBS.

Lyso-Tracker, Mito-Tracker and Hoechst 33258 staining

The cells were prepared at a density of 25 000 cells/well in a 24-well plate. After treatment with SNP (200 μM) or L-NMMA (1 mM) for 24 h in serum-free media, the meniscal cells were treated with Lyso-Tracker (75 nM), Mito-Tracker (100 nM), or Hoechst 33258 (2 μg/ml) at 37°C for 2 h. Morphological changes were evaluated under a fluorescence microscope.

Western blot analysis

The cells were collected, lysed and sonicated in the lysis buffer on ice. After being centrifuged, equal amounts of protein (25 μg/lane) were subjected to SDS-PAGE. Samples were transferred to a polyvinylidene difluoride membrane after being separated by electrophoresis. The membranes were blocked with 5% BSA followed by immunoblotting with LC3 (1:3000), Beclin-1 (1:300), JNK (1:500), p-JNK (1:1000) and GAPDH (1:2500) antibodies overnight at 4°C and horseradish peroxidase-conjugated secondary antibodies were incubated for 1 h at room temperature. Immunoreactive bands were visualized by chemiluminescence (Pierce ECL). The resulting autoradiograms were then analysed by densitometry. Equal loading of proteins was confirmed by detecting GAPDH levels. Quantification was performed with ImageJ software. To investigate the effect of NO on autophagy, the LC3 level was determined in the presence or absence of bafilomycin A1. To quantify the steady-state level of LC3 protein, bafilomycin A1 (100 nM, Sigma-Aldrich, St Louis, MO, USA) was used to block the degradation of the protein in this study.

siRNA transfection

The meniscal cells grown to 40% confluence in a six-well plate were transfected with siRNA using the Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer’s instructions. After incubating the cells for 6 h at 37°C in a CO2 incubator, the siRNA transfection mixture was replaced with DMEM-F12 and 10% FBS. The experiments were carried out 48 h after transfection. Knockdown of LC3 was confirmed by immunoblotting with anti-LC3 antibody. The transfected cells were subsequently exposed to serum-free medium for 24 h in the presence or absence of L-NMMA. The cell viability and apoptosis incidence were measured by CCK-8 or flow cytometry.

Immunofluorescence

Meniscal cells were prepared at a density of 50 000 cells/well in a 24-well plate. After being treated with SNP or L-NMMA in the absence of FBS for 24 h, the cells were fixed with 4% paraformaldehyde in PBS (pH 8.0) for 10 min. The cells were then permeabilized with 0.25% Triton-X 100 in PBS for 15 min. Antigenic sites were blocked in 5% BSA and then incubated with LC3 antibody at a dilution of 1:100 overnight at 4°C. Subsequently the treated cells were washed and incubated with a fluorescein-labelled secondary antibody for 1 h at room temperature. Protein localization was visualized by confocal microscopy (Olympus Fluoview, Tokyo, Japan).

Real-time PCR

The RNA of cells was isolated using a Trizol reagent (Invitrogen, Carlsbad, CA, USA), then 400 ng of total RNA was reverse-transcribed into complementary DNA using the PrimeScript RT reagent kit (Takara RR036A, Shiga, Japan) according to the manufacturer’s instructions. The expression of MMPs and aggrecanase genes was determined by real-time PCR using SYBR Premix Ex Taq (Takara, Shiga, Japan) and an ABI Prism 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA). The thermal cycling was performed as previously described [24]. The fold changes in aggrecanase and MMP mRNA expression relative to the control was calculated as $2^{-\Delta \Delta CT}$. The primers are listed in supplementary Table S1, available at Rheumatology Online.

Statistical analysis

Results are expressed as the mean (±S.D.) of six independent experiments. The results of western blots were repeatedly measured three times. Statistical analyses were performed using the SPSS 15 statistical software program (IBM, Armonk, NY, USA). Statistically significant differences between the groups were determined by two-way repeated measure analysis of variance (ANOVA). $P$-values < 0.05 were considered significant.

Results

The effect of autophagy on NO production

To investigate the effect of NO on autophagy, the meniscal cells were treated with different concentrations of SNP in serum-free medium for 24 h. Western blot analysis indicated that the SNP treatment led to a concentration-dependent decrease in the LC3 protein level of meniscal cells (Fig. 1A). To determine the effect of autophagy on NO production, the meniscal cells were treated with autophagy inducer or inhibitor in serum-free medium for 24 h. Rapamycin, a common inducer of autophagy, significantly decreased the production of intracellular NO ($P < 0.05$). Conversely, 3-MA, which is known as a common inhibitor of autophagy, increased the production of NO in human meniscal cells ($P < 0.05$) (Fig. 1B). All the results suggested that induction of autophagy might decrease the production of intracellular NO.

NO donors reduced autophagy levels in meniscal cells

To determine whether NO can regulate autophagy in meniscal cells, two NO-releasing chemical compounds,
Fig. 1 Nitric oxide donors (DETA NONOate and SNP) inhibited autophagy in human meniscal cells

(A) SNP inhibited LC3-II protein expression in a concentration-dependent manner. The meniscal cells were treated with different SNPs (100, 200 and 500 μM) in serum-deprived media for 24 h in the presence of bafilomycin A1 (100 nM).

(continued)
DETA NONOate and SNP, were used in this study. The cells cultured in 10% FBS were used as the control. After 24 h stimulation, NO donors increased NO levels of meniscal cells ($P < 0.05$) in serum deprivation medium (Fig. 1C). Meanwhile, NO donors evidently decreased the number of meniscal cells ($P < 0.05$) (Fig. 1D). The activity of caspase-3 and apoptosis incidence was increased by the treatment of NO donors ($P < 0.05$) (Fig. 1E and F). Meanwhile, NO donors significantly reduced LC3-II and Beclin-1 levels as well as the incidence of autophagy ($P < 0.05$) (Fig. 1G and H). All the results suggested that NO inhibited autophagy in human meniscal cells as well as cell viability.

**NOS inhibitors enhanced autophagy levels in meniscal cells**

We used NOS inhibitors to investigate whether inhibition of the NO level can enhance autophagy in meniscal cells. Two NOS inhibitors, $\text{L-NAME and L-NMMA}$, were used in the study. The cell survival rate increased with the reduction of the NO level ($P < 0.05$) (Fig. 2A and B). Meanwhile, the apoptosis incidence and caspase-3 activity were down-regulated by the treatment of NOS inhibitors ($P < 0.05$) (Fig. 2C and D). Interestingly, $\text{L-NAME and L-NMMA increased LC3-II and Beclin-1}$ as well as the autophagy incidence in meniscal cells (Fig. 2E and F). Thus all the results suggested that NOS inhibitors could increase human meniscal cell survival via up-regulation of autophagy. Because mitochondria was targets for autophagic degradation [25], we used Mito-Tracker and Lysotracker to determine the changes of autophagy in human meniscal cells. The expression of LC3 protein was also investigated by immunofluorescence. The meniscal cells cultured in 10% FBS were used as controls. The apoptosis of the cells was recorded by Hoechst staining. The images showed that $\text{L-NMMA}$ increased the density of the lysosome as well as LC3 expression. Increased density of the lysosomes was associated with a reduced density of mitochondria in some areas of the cytoplasm. Meanwhile, SNP augmented the numbers of Hoechst-positive cells (Fig. 3). The results of lysosome density and LC3 expression agreed with those from flow cytometry and western blot measurement.

**Inhibition of autophagy promoted serum deprivation-induced apoptosis**

To determine the interplay between autophagy and apoptosis in meniscal cells, we regulated autophagy pharmacologically. The results of flow cytometry showed that treatment with rapamycin significantly reduced the incidence of apoptosis in serum-deprived meniscal cells ($P < 0.05$) (Fig. 4A). The cell viability also increased after pre-treatment with rapamycin ($P < 0.05$) (Fig. 4B). To further determine the role of autophagy in meniscal cells, knockdown of LC3B by siRNA was used to repress autophagic activity (Fig. 4C). The results suggested that knockdown of LC3 expression increased the apoptotic incidence in meniscal cells ($P < 0.05$) (Fig. 4D). Meanwhile, cell viability significantly decreased ($P < 0.05$) (Fig. 4E). All the results indicated that induction of autophagy could reduce the incidence of apoptosis in serum-deprived meniscal cells.

To further investigate whether NOS inhibitor rescued meniscal cells through up-regulation of autophagy, we inhibited autophagy activity by knockdown of LC3 by siRNA. After being transfected of LC3 siRNA or control siRNA, the meniscal cells were exposed to serum-free medium with $\text{L-NMMA}$ for 24 h. The results suggested that $\text{L-NMMA}$ failed to rescue the LC3 knockdown cells from serum deprivation-induced apoptosis ($P < 0.05$) (Fig. 4F). Meanwhile, L-NMMA increased the cell viability of control siRNA transfected cells ($P < 0.05$) (Fig. 4G). Taken together, the data showed that NOS inhibitor rescued meniscal cells from apoptosis through up-regulation of autophagy.

**NOS inhibitor down-regulated the mRNA expression of catabolic markers**

To investigate whether NO-mediated autophagy regulation affects the pathological process of meniscus degeneration, we detected the mRNA expression of catabolic markers in human meniscal cells. The cells cultured in 10% FBS were used as controls. We found that $\text{L-NMMA}$ decreased the mRNA expression of most catabolic markers ($P < 0.05$) (Fig. 5). However, this effect was blocked by 3-MA (10 nM), which is a selective autophagy inhibitor [7, 24]. These results suggest that autophagy maintained the homeostasis of the meniscus extracellular matrix (ECM).

**NO inhibited autophagy via inactivation of JNK**

To investigate whether JNK was involved in NO-mediated autophagy regulation, we measured the expression of JNK and p-JNK when the meniscal cells were treated with NO donor or NOS inhibitor. We found that NO regulated JNK phosphorylation without changing the total JNK protein level.
levels. SNP significantly decreased the phosphorylation of JNK while L-NMMA up-regulated JNK phosphorylation (Fig. 6A). To further investigate the effect of JNK on NO-mediated autophagy regulation, SP600125 (20 mM, Sigma-Aldrich, St Louis, MO, USA), a JNK inhibitor, was used. In L-NMMA-treated meniscal cells, we noted that increasing levels of LC3-II, Beclin-1 and autophagy incidence were prevented in the presence of SP600125 (Fig. 6B and C). The incidence of apoptosis also showed that SP600125 suppressed the protective effect of L-NMMA (Fig. 6D).

Discussion
NO plays an important role in the degeneration of meniscal tissue. The pro-apoptotic effect of NO on meniscal
cells has been well demonstrated. Also, increasing the NO level can enhance the expression of aggrecanases, which could accelerate the degradation of ECM [26]. As a potential protective mechanism, autophagy is involved in many degenerative diseases [9, 10, 27–29]. However, there is evidence showing that extensive autophagy is commonly associated with cell death, suggesting that controversy still exists in addressing the role of autophagy in both cell survival and death [30]. Recently, up-regulation of autophagy resulted in reducing the severity of experimental OA in an animal model, indicating that autophagy might function as a survival mechanism in chondrocytes [31]. Nevertheless, the interplay between autophagy and NO has never been documented in meniscal cells.

In this study we demonstrated that NO enhanced meniscal cell apoptosis by suppressing autophagy, as NO treatment resulted in a significant decrease in autophagy. Conversely, NOS inhibitors increased autophagy as well as the cell survival rate. Furthermore, when autophagy was suppressed upon LC3 knockdown, NOS inhibitors failed to rescue meniscal cells from apoptosis. We also detected that NOS inhibitors reduced the mRNA expressions of MMPs and aggrecanases in serum-deprived meniscal cells. The results indicated that increasing the NO level might aggravate meniscus degeneration by inhibiting autophagy.

Our results also demonstrated the protective role of autophagy in human meniscal cells. Increasing autophagy levels augmented the survival rate of meniscal cells incubated in serum deprivation medium. Conversely, inhibition of autophagy resulted in the augmentation of apoptosis in meniscal cells. Up-regulation of autophagy also reduced the production of intracellular NO. In addition, up-regulation of autophagy suppressed the mRNA expression of MMPs and aggrecanases in the meniscal cells. However, the protective effect of l-NMMA was blocked by 3-MA, which is known as an inhibitor of autophagy [32]. Thus we thought that autophagy played a protective role in human meniscal cells. Our results were consistent with those of several studies reporting that the autophagy inducer delayed the pathological process of degenerative diseases [31, 33–35].

The repair of meniscus damage is closely associated with the progression of knee OA. Up-regulation of MMPs and aggrecanases, especially MMP-1, -3 and -13 and ADAMTS (a disintegrin and metalloprotease with thrombospondin motifs)-4 and -5, could enhance the degradation of meniscal tissue, which is stimulated by inflammatory cytokines [14, 18]. Therefore we investigated the potential relationship between these catabolic markers and autophagy. Serum deprivation significantly increased the mRNA level of MMPs and aggrecanases in human meniscal cells. l-NMMA inhibited the mRNA expression of MMPs as well as aggrecanases in serum deprivation media, suggesting that the up-regulation of MMPs and aggrecanases in meniscal cells might occur through an NO-mediated signal pathway. Furthermore, this protective effect of l-NMMA was significantly suppressed by 3-MA, indicating that l-NMMA suppressed MMP and aggrecanase expression through up-regulation of autophagy. This protective effect of autophagy might be due to

**Fig. 3** NO regulated lysosome activity and apoptosis in meniscal cells

Meniscal cells were treated with SNP or l-NMMA under serum deprivation medium for 24 h. The lysosome activity was detected by the density of lysosomes with Lyso-Tracker. The density of mitochondria was detected by Mito-Tracker. The LC3 expression was measured by immunofluorescence. The apoptotic cells were stained with Hoechst 33258. l-NMMA increased lysosome density as well as LC3 expression, while SNP increased Hoechst-positive cells. Magnification: 200×.
Meniscal cells were treated with rapamycin or 3-MA under serum deprivation medium for 24 h. (A and B) Autophagy inducer increased the cell viability of meniscal cells while autophagy inhibitor augmented serum deprivation-induced apoptosis ($n = 6$). (C) Meniscal cells transfected with LC3 siRNA (+) or control siRNA (−) were tested by western blot analysis of LC3 expression ($n = 3$). (D and E) Knockdown of LC3 resulted in an increase in apoptotic meniscal cells and a decrease in cell viability ($n = 6$). (F and G) L-NMMA failed to rescue cell viability of LC3 knockdown meniscal cells from serum deprivation-induced apoptosis ($n = 6$). Values are given as mean (s.d.). *$P < 0.05$, **$P < 0.01$.

Inhibition of nuclear factor κB (NF-κB) activation or reactive oxygen species (ROS) production [36]. Impairment of autophagy induction has been reported to increase the level of ROS or activate the NF-κB signal pathway [6]. Increased ROS activity or activation of the NF-κB signal pathway could enhance the production of MMPs [37, 38]. Inhibition of multiple MMPs could enhance the repair of meniscus in vitro [18]. Thus we thought autophagy might have a potential protective effect in meniscus repair. In accordance with our results, Carames et al. [31] also reported that up-regulation of autophagy could maintained cartilage cellularity and decreased ADAMTS-5 and IL-1β expression in chondrocytes.

Previous studies demonstrated that NO could induce apoptosis of chondrocytes via mitochondrial dysfunction [39, 40]. However, controversy exists over the effect of mitochondrial dysfunction on autophagy [41, 42]. This led us determine whether autophagy might be another mechanism that contributes to meniscal cell apoptosis observed with increasing NO level. We found that NO
The meniscal cells were treated in the presence or absence of 3-MA in serum-free media for 24 h. The meniscal cells incubated in 10% FBS were used as controls. (A-F) Results of real-time PCR for MMPs and aggrecanases after stimulation of L-NMMA and 3-MA (n = 6). The results showed that up-regulation of autophagy by L-NMMA suppressed the mRNA expression of matrix catabolic markers. The protective effect of L-NMMA was blocked by 3-MA, a selective autophagy inhibitor. Values are given as mean (S.D.). *P < 0.05, **P < 0.01.
impaired autophagy activity of human meniscal cells. Our results suggested that inhibition of autophagy was one of the potential mechanisms in NO-induced meniscal cell apoptosis.

Our data also showed that NO reduced autophagy via inactivation of JNK. JNK regulation contributes to cell death in various cell types [43]. One of the major mechanisms by which JNK mediates autophagy is to phosphorylate Bcl-2 and dissociate it from Beclin-1 [44, 45]. It has also been shown that the activity of JNK could be regulated by NO [46, 47]. In the present study, our observations suggested that phosphorylation of JNK is one of the potential mechanisms by which NO contributes to the inhibition of autophagy. Interestingly, we noted that the increase in autophagy was not fully blocked by SP600125, indicating that there might be other mechanisms independent of JNK in NO-mediated autophagy regulation.

Our study has several limitations. Although it is considered better to culture the meniscal cells within the three-dimensional (3D) microenvironment for ECM production [48], we only obtained non-degenerative meniscal cells. However, our data also showed that NO reduced autophagy via inactivation of JNK. Dimethyl sulphoxide (DMSO) is the vehicle of SP600125 for 24 h in serum-free medium. (A) Western blot results show that SNP inhibited the phosphorylation of JNK while L-NMMA increased the phosphorylation of JNK. (B and C) SP600125 reduced the up-regulated effect of L-NMMA on LC3-II, Beclin-1 (n = 3) and the incidence of autophagy (n = 6). (D) SP600125 suppressed the anti-apoptotic effect of L-NMMA in apoptosis induced by serum deprivation medium in meniscal cells (n = 6). Values are given as mean (s.d.). *P < 0.05, **P < 0.01.
cells from inner meniscus tissue in monolayer culture. Monolayer culture of meniscus might influence meniscal cell ECM production and potentially change cell signal events and proliferation. The second limitation is that normal meniscal tissues were harvested from young cases. In order to avoid contamination of other types of cells, only the inner one third of non-degenerative meniscus tissues were used for meniscal cell isolation. It has been reported that autophagy might decrease during OA and the aging process [9,10], therefore different mechanisms could underlie impairment of autophagy in aging or OA development. Further studies are needed that include arthritis cases and age-matched normal cases.

In summary, we have shown, for the first time, that NO regulates autophagy in normal human meniscal cells. Furthermore, we found that NO increased apoptosis via inhibition of autophagy in meniscal cells. Up-regulation of autophagy maintains both cell viability and ECM homeostasis. These results indicate that a potential treatment of meniscus degeneration in OA or RA is via up-regulation of autophagy achieved by selectively targeting NO.

Rheumatology key messages

- Nitric oxide (NO) suppresses autophagy in human meniscus-derived cells by inhibiting Jun-N-terminal kinase activation.
- Up-regulation of autophagy might provide potential treatment of meniscus degeneration in OA or RA.

Funding: This study was supported by the National Natural Science Foundation of China (81101379, 81171705, 81000793) and the Natural Science Fund of the Shanghai Jiao Tong University School of Medicine (11XJZ21022).

Disclosure statement: The authors have declared no conflicts of interest.

Supplementary data

Supplementary data are available at Rheumatology Online.

References

19. Weinberg JB, Femor B, Guilkaf F. Nitric oxide synthase and cyclooxygenase interactions in cartilage and
NO inhibits autophagy via JNK suppression