CHAPTER 3

ER STRESS INHIBITS COLLAGEN SYNTHESIS INDEPENDENT OF COLLAGEN-MODIFYING ENZYMES IN DIFFERENT CHONDROCYTE POPULATIONS AND DERMAL FIBROBLASTS

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ABSTRACT

Chondrocytes respond to glucose deprivation with a decreased collagen synthesis due to disruption of a proper functioning of the endoplasmic reticulum: ER stress. Since the mechanisms involved in the decreased synthesis are unknown, we have investigated whether chaperones and collagen-modifying enzymes are affected by glucose deprivation. Chondrocytes obtained from nucleus pulposus, annulus fibrosus, articular cartilage, and meniscus and dermal fibroblasts were cultured under control conditions or exposed to the ER stress-inducing treatments of tunicamycin addition or glucose withdrawal. Both treatments resulted in an up-regulation of the gene expression of the ER stress markers in all cell types, but dermal fibroblasts showed a delayed response to glucose deprivation. Collagen gene expression was down-regulated and less collagen protein was present in the cells under both ER stress-inducing conditions. The expression levels of the prolyl 4-hydroxylases were either not affected (P4ha3) or increased (P4ha1 and P4ha2), the levels of the lysyl hydroxylases decreased, and the N-propeptidase Adamts2 decreased. Both treatments induced apoptosis. Chondrocytes respond more quickly to glucose deprivation, but it appears that chondrocytes can cope better with tunicamycin-induced ER stress than fibroblasts. Although collagen synthesis was inhibited by the treatments, some collagen-modifying enzymes and chaperones were up-regulated, suggesting that there is no causal relation between them.
INTRODUCTION

The chondrocyte is the single cell type in cartilage and is responsible for the maintenance of the extracellular matrix (ECM). Since cartilage is avascular, chondrocytes have to rely on diffusion for nutrient supply and waste exchange. In articular cartilage diffusion of solutes occurs to and from blood vessels of the underlying bone marrow spaces and through contact with the surrounding synovial fluid. Chondrocytes deep within the articular cartilage may be as much as 3 mm from a nutrient-supplying blood vessel\(^1\). In the intervertebral disc, only the outermost region of the annulus fibrosus is sparsely penetrated by capillaries and nerves. The other regions of the annulus fibrosus and the nucleus pulposus are supplied by a capillary network that arises from vertebral arteries penetrating the subchondral bone\(^2\). Chondrocytes in the centre of the nucleus pulposus in the lumbar intervertebral disc may be 7 to 8 mm from the nearest blood vessel\(^3\). The vascular portion of the fibrocartilaginous meniscus has the ability to repair itself. However, in adult tissue, only the outer part of the meniscus is vascular\(^4\).

Conditions that reduce the blood supply to cartilage, such as atherosclerosis or the occurrence of micro-emboli in subchondral vessels, can cause nutrients to drop to levels that no longer support cell activity or viability. Apart from vascular pathology, nutrients may not reach the chondrocytes if there is sclerosis of the subchondral bone or if the cartilaginous endplate calcifies. A fall in nutrient supply is associated with cartilage degeneration\(^3,5,6\). In particular glucose is a nutrient critical for maintaining chondrocyte viability since these cells obtain their energy primarily through glycolysis\(^1,7,8\). \textit{In vitro} studies have shown that glucose deprivation increased nucleus pulposus cell death\(^9\) and inhibited synthesis of type I and type II collagen by nucleus pulposus cells\(^10\). Glucose deprivation disrupts protein folding in the endoplasmic reticulum (ER), thereby inducing ER stress\(^11,12\). In collagen-producing cells like chondrocytes and fibroblasts, the ER stress response is accompanied by a strong decrease in collagen synthesis\(^13-15\).

ER stress is a condition in which the processes in the ER are disrupted, thereby causing accumulation of incorrectly folded proteins within the ER. The cell responds to ER stress by activating the unfolded protein response (UPR) and ER-associated degradation (ERAD). The UPR is the pathway in which the folding capacity of the ER is enhanced by up-regulation of ER-resident chaperones and foldases, expansion of the ER, and down-regulation of protein synthesis on transcriptional and translational level. The unfolded proteins present in the ER are ubiquitinated, retrotranslocated into the cytosol and targeted for proteosomal degradation, i.e. ERAD\(^16\). If cells are not able to remove incorrectly folded proteins from the ER, apoptosis is induced.

Newly synthesized proteins often undergo posttranslational modification before they can be correctly folded and subsequently translocated to their final destination. The synthesis of collagen involves a number of unique posttranslational modifications by collagen-modifying enzymes. Specific proline and lysine residues are hydroxylated in the rough ER by membrane-bound hydroxylases. Protein \(O\)-glycosylation of hydroxylsine
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(attachment of galactose and subsequently glucose) takes place in the rough ER, and a start is made with respect to the N-glycosylation of specific asparagine residues. The folding of the triple-helix also begins in the rough ER with the assembly of the C-propeptides and the formation of disulphide bonds. Completion of N-linked oligosaccharide chains takes place in the Golgi complex. During or following exocytosis the molecules undergo proteolytic cleavage to remove the propeptides. The resulting collagen molecule is capable of self-assembling into fibrils, and finally cross-links are formed. Glucose deprivation causes ER stress which is accompanied by a decrease in collagen protein synthesis. A disruption in posttranslational modification of collagen may lead to an accumulation of incorrectly folded collagen in the ER, thereby explaining the decrease in collagen protein synthesis. It is unknown how the collagen specific chaperone Serpinh1 and collagen-modifying enzymes react to ER stress. Therefore, the aim of this study was to investigate whether chaperones (e.g. Serpinh1) and enzymes (prolyl 4-hydroxylases, lysyl hydroxylases, and N-propeptidases) involved in collagen biosynthesis are affected by the pathophysiological situation of glucose deprivation. The response was studied in chondrocytes obtained from articular cartilage, nucleus pulposus, annulus fibrosus and meniscus. In addition, the effects were studied in dermal fibroblasts, cells that are less dependent on glucose for the production of collagen. The response to glucose depletion was compared to chemically induced ER stress using tunicamycin. To maintain the chondrocytic phenotype and the production of collagen and aggrecan by the chondrocytes, the chondrocytic cells were cultured in alginate beads.

MATERIALS AND METHODS

Harvesting of tissues
The tissues were obtained from skeletally mature female Dutch milk goats (n = 4 for cartilage samples, n = 3 for skin samples) that were used in other studies (the Animal care and use Committee of the VU University Amsterdam approved the use of goats in these studies). After sacrifice, the following cartilaginous tissues were collected: meniscus, intervertebral disc (from the thoracic part of the spine), and articular cartilage from the knee. The intervertebral discs were divided into the nucleus pulposus and annulus fibrosus. Skin samples were collected from the hip/thigh region.

Cell culture and treatments
Chondrocytes were isolated from the cartilaginous tissues. The tissues were dissected, minced and subjected to sequential treatments first with Dulbecco’s modified Eagle’s medium (DMEM, Gibco, Paisley, UK) supplemented with 1% foetal bovine serum (FBS, HyClone, Logan, UT), 100 U/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml amphotericin B (all from Gibco) and 2.5% (w/v) Pronase E (Sigma, St. Louis, MO) for 1 h,
then with DMEM supplemented with 25% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml amphotericin B and 0.125% (w/v) collagenase (CLS-2, Worthington, Lakewood, NJ) for 16 h at 37°C to release the chondrocytes from the tissues. After filtering the cell suspension through a 70 µm cell strainer (BD Biosciences, San Diego, CA), isolated cells were resuspended in an alginate solution (1.2% (w/v) Keltone LVCR sodium alginate (Monsanto, San Diego, CA) in physiological salt (0.9% NaCl, 0.2-µm sterile filtered), creating a cell suspension of 4 million cells/ml solution. This was mixed by slow pipetting and transferred to a sterile syringe. Alginate beads were formed by dripping the solution from the syringe needle (26 gauge) into calcium chloride (102 mM). The beads were allowed to polymerize for 10 min at room temperature. After washing twice in physiologic salt and twice in DMEM, the alginate beads were transferred to 24-well tissue culture dishes (Greiner Bio-one, Kremsmuenster, Austria).

Dermal fibroblasts were isolated from the skin samples. Care was taken that all adipose tissue was removed from the skin samples. The skin was cut into fragments (2 x 2 mm) and incubated in Hanks buffered salt solution (HBSS, Gibco) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml amphotericin B and 0.3% (w/v) Dispase II (Gibco) overnight at 4°C. The tissue was subsequently incubated at 37°C for 2 h and the dermis was separated from the epidermis. The dermis was incubated in DMEM supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml amphotericin B, 0.125% (w/v) collagenase, and 0.3% (w/v) dispase II for 4 h at 37°C. After filtering the cell suspension through a 70 µm cell strainer, isolated cells were cultured up to passage 2 to select for the dermal fibroblasts. The dermal fibroblasts were cultured both in monolayer and in alginate beads as described for the chondrocytic cells.

The cells were cultured in DMEM (DMEM contains 1 g/L D-Glucose) supplemented with 0.1% bovine serum albumin (BSA, fraction V, Sigma), 100 U/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml amphotericin B and with or without 1 µg/ml tunicamycin (Sigma), or in DMEM No Glucose (DMEM without D-Glucose, Gibco) supplemented with 0.1% bovine serum albumin (100 U/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml amphotericin B, and 50 µg/ml ascorbate-2-phosphate (Sigma) for 24 h and 48 h.

After culturing, the cells were first collected by dissolving the alginate beads in 10mM EDTA in physiologic salt and subsequently the cells were washed in physiologic salt.

**Real-time PCR**

Total RNA was isolated from cells with the RNeasy kit (Qiagen, Gaithersburg, MD), and DNase I treatment was performed as described by the manufacturer to remove any contaminating genomic DNA. Total RNA (750 ng) was reverse transcribed using 250 U/ml Transcriptor Reverse Transcriptase (Roche Diagnostics, Mannheim, Germany), 0.08 U random primers (Roche diagnostics), and 1 mM of each dNTP (Invitrogen, Carlsbad, CA) in Transcriptor RT reaction buffer at 42°C for 45 min followed by an inactivation of the
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enzyme at 80˚C for 5 min. Real-time PCR reactions were performed using the SYBRGreen reaction kit according to the manufacturer’s instructions (Roche Diagnostics) in a LightCycler 480 (Roche Diagnostics). The LightCycler reactions were prepared in 20 µl total volume with 7 µl PCR-H₂O, 0.5 µl forward primer (0.2 µM), 0.5 µl reverse primer (0.2 µM), 10 µl LightCycler Mastermix (LightCycler 480 SYBR Green I Master; Roche Diagnostics), to which 2 µl of 5 times diluted cDNA was added as PCR template. Primers (Invitrogen) used for real-time PCR are listed in Table 1. Specific primers were designed from sequences available in the data banks, based on homology in conserved domains between human, mouse, rat, dog and cow. The amplified PCR fragment extended over at least one exon-border. Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (Ywhaz) and hypoxanthine phosphoribosyltransferase 1 (Hprt1) were used as housekeeping genes and the gene expression levels were normalized for the normalization factor calculated with the equation \( \sqrt{(Ywhaz \times Hprt1)} \). With the Light Cycler software (version 4), the crossing points were assessed and plotted versus the serial dilution of known concentrations of the standards derived from each gene using Fit Points method. PCR efficiency was calculated by Light Cycler software and the data were used only if the calculated PCR efficiency was between 1.85 and 2.0.

Table 1. Primer sequences used for real time PCR

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**ER stress in chondrocytes**

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<td>5' TGCACCTGCTGCGGACTCA 3'</td>
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<td>5' TGCTGCAGAGGTGCACGTA 3'</td>
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<tr>
<td>Ywhaz</td>
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<td>5' CTATTTGTGGGACAGCATGGA 3'</td>
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Acan, aggrecan; Adamts2, and 3, a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 2, and 3; Col1a1, α1(I)procollagen; Col2a1, α1(II)procollagen; Hprt1, hypoxanthine phosphoribosyltransferase 1; Hspa5, heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa); P4ha1, 2, and 3, procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha polypeptide 1, 2, and 3; Plod1, 2, and 3, procollagen-lysine 2-oxoglutarate 5-dioxygenase 1, 2, and 3; Serpinh1, serpin peptidase inhibitor, clade H (heat shock protein 47); Xbp1t, total (spliced and unspliced forms) of X-box binding protein 1; Xbp1u, unspliced form of X-box binding protein 1; Ywhaz, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide.

**Immunoblotting**

At 0h, 24h, and 48h, cells were lysed in hot lysis buffer (1% SDS, 10 mM Tris, pH 7.4). After boiling for 5 min, the lysates were clarified by centrifugation. The protein concentration was determined using the BCA assay reagent kit (Pierce, Rockford, IL) with BSA as a standard. Lysate samples were denatured by heating at 95°C for 5 min in NuPage LDS sample buffer (Invitrogen, Carlsbad, CA) with NuPage reducing agent (Invitrogen). Equivalent amounts of protein were resolved on NuPage 10% Bis-Tris mini gels (Invitrogen) or on NuPage 3-8% Tris-Acetate mini gels (Invitrogen) for collagen detection. After SDS-PAGE, the proteins were transferred to Nitrocellulose membranes (Invitrogen) using the iBlot dry blotting system (Invitrogen) at program P3 for 7 min or for collagen transfer at program P3 for 8 min after a short wash of the gel in NuPage transfer buffer (Invitrogen).

The membranes were blocked in 2% (w/v) BSA 0.1% Tween in phosphate buffered saline (PBS) for 1 h and were then incubated with the primary antibody for 2 h. Rabbit polyclonal anti-78kDa glucose regulated protein (Hspa5, heat shock 70kDa protein 5 [glucose-regulated protein, 78kDa]) (sc-13968, Santa Cruz Biotechnology, Santa Cruz, CA) was used at 1:500 dilution; rabbit polyclonal anti-Heat shock protein 47 (Serpinh1, serpin peptidase inhibitor, clade H [heat shock protein 47], member 1) (AB3495,
Chemicon, Millipore, Billerica, MA) was used at 1:1000 dilution; mouse monoclonal anti-type II collagen (MAB1330, Chemicon) was used at 1:1000 dilution; mouse monoclonal anti-type I collagen (ab6308, Abcam, Cambridge, UK) was used at 1:1000 dilution; and mouse monoclonal anti-β-actin (AC-15, Sigma) was used at 1:5000 dilution.

After three washes with 0.1% Tween in PBS, the membranes were incubated with alkaline phosphatase-conjugated anti-rabbit or horseradish peroxidase-conjugated anti-mouse secondary antibody (DakoCytomation, Glostrup, Denmark) at a 1:5000 dilution for 1 h. Following three washes, immunoreactivity was visualized using CDP-Star (Roche Diagnostics, Mannheim, Germany) or Lumi-Lightplus (Roche Diagnostics).

Apoptosis

To measure apoptosis, an annexin V detection kit (Calbiochem, San Diego, CA) was used. The Annexin V binding and analysis by fluorescence microscopy were performed according to the manufacturer’s instructions. Briefly, the cells were washed with cold PBS and resuspended in cold 1x binding buffer. After the addition of fluorescein isothiocyanate (FITC) conjugated Annexin V, the suspension was incubated for 15 min at ambient temperature in the dark. The cells were resuspended in 1x binding buffer and propidium iodide was added. Numbers of viable and apoptotic cells were counted by using a Leica fluorescent microscope.

Statistical analysis

Data are expressed as mean ± SD. Gene expression data are expressed as mean ± SD of treated versus control (T/C) chondrocytes of target gene expression normalized for the equation √(Ywhaz x Hprt1). Differences between groups were tested with a two-tailed paired t-test and differences of T/C ratios were tested with a two-tailed t-test for single group mean and compared to 1 (T/C=1, no effect). Differences were considered significant if p<0.05.

RESULTS

Induction of ER stress

Since ER stress is characterized by an induction of heat shock 70kDa protein 5 [glucose-regulated protein, 78kDa] (Hspa5) and unconventional splicing of X-box binding protein 1 (Xbp1), the gene expression levels of these markers were measured by Real-time PCR to confirm an induction of ER stress by glucose deprivation and following addition of tunicamycin. Due to restrictions in primer design for goat mRNA, it was not possible to design primers for the spliced form of Xbp1 mRNA; instead the total amount of Xbp1 mRNA (Xbp1t) and the unspliced form of Xbp1 mRNA (Xbp1u) were measured.

We were unable to measure gene expression in dermal fibroblasts when they were cultured in alginate beads. Further analyses revealed that over 80% of these cells underwent
ER stress in chondrocytes

apoptosis, even under control culture conditions (data not shown). Therefore, subsequent experiments were performed with fibroblasts cultured on plastic.

Tunicamycin increased Hspa5 gene expression at 24 h in all cell types (Fig. 1A). Glucose deprivation resulted in an up-regulation of Hspa5 mRNA levels at 24 h in the different chondrocytes, but not in the dermal fibroblasts (Fig. 1A). In dermal fibroblasts, the gene expression level of Hspa5 was increased at the 48 h time point (Fig. 1D).

The gene expression levels of Xbp1t were increased after tunicamycin treatment at 24 h in all cell types (Fig. 1B). Glucose deprivation increased the Xbp1t gene expression in the chondrocytes at 24 h (Fig. 1B), whereas in the fibroblasts an effect was seen only after 48 h (Fig. 1D).

Tunicamycin decreased Xbp1u gene expression in all chondrocyte populations, but it had no effect on the expression of this gene in dermal fibroblasts (Fig. 1C). A similar effect was seen in the absence of glucose, down-regulation in chondrocytes and no effect in fibroblasts (Fig. 1C and Fig. 1D).

As ER stress was only apparent in the dermal fibroblasts after 48 h without glucose, this time point and condition was analyzed further for these cells. For the treatment with tunicamycin and both treatments in the chondrocytic cells, the 24 h time point was analyzed.

**ECM synthesis**

Gene expression levels of type I collagen could be quantified in nucleus pulposus, annulus fibrosus and meniscal chondrocytes, and dermal fibroblasts, whereas there were no quantifiable levels in chondrocytes derived from articular cartilage. Treatment with tunicamycin strongly decreased the type I collagen gene expression in all cell types (Fig. 2A). Glucose deprivation down-regulated gene expression of type I collagen by nucleus pulposus, annulus fibrosus and meniscal cells at 24 h and in dermal fibroblasts at 48 h.

In agreement with the gene expression levels, there was hardly any type I collagen protein detectable after glucose deprivation and after tunicamycin treatment in meniscal cells at 24 h (Fig. 2B). The protein expression of type I collagen in dermal fibroblasts was also in agreement with the mRNA levels; after treatment with tunicamycin hardly any type I collagen was detectable at 24 h and there was a decrease after 48 h without glucose.

Type II collagen gene expression levels could be quantified in nucleus pulposus, annulus fibrosus and articular cartilage chondrocytes, whereas there were no detectable levels in meniscal chondrocytes and dermal fibroblasts. Both treatments, with tunicamycin and glucose deprivation, resulted in strongly decreased type II collagen expression levels in nucleus pulposus, annulus fibrosus and articular cartilage cells (Fig. 3A). The protein levels of type II collagen were decreased at 24 h with tunicamycin treatment and with glucose deprivation (Fig. 3B).
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Fig. 1

A

B

C

D

- Glu

- Glu

- Glu
Figure 2: Type I collagen expression is down-regulated under ER stress-inducing conditions. Chondrocytes isolated from nucleus pulposus (NP), annulus fibrosus (AF), articular cartilage (AC), and meniscus (M), and dermal fibroblasts (DF) were cultured under control conditions (C) or exposed to the ER stress-inducing treatments of tunicamycin (Tm) or glucose withdrawal (-G). A, Real-time PCR was performed on reverse-transcribed RNA isolated from each condition. The results are presented as relative gene expression of type I collagen (Col1a1) in treated versus control cells (T/C). Data are shown as mean ± SD. *: p<0.05; **: p<0.01; ***: p<0.001. B, Type I collagen protein expression was detected by Western blot analysis. The expression of β-actin was used as a loading control.

Figure 1: Induction of ER stress markers. Chondrocytes isolated from nucleus pulposus (NP), annulus fibrosus (AF), articular cartilage (AC), and meniscus (M), and dermal fibroblasts (DF) were cultured under control conditions or exposed to the ER stress-inducing treatments of tunicamycin (left panel) or glucose withdrawal (right panel) for 24 h. Real-time PCR was performed on reverse-transcribed RNA isolated from each condition. The results are presented as relative gene expression of heat shock 70kDa protein 5 [glucose-regulated protein, 78kDa] (Hspa5, A), total X-box binding protein 1 (Xbp1t, B), or unspliced form of X-box binding protein 1 (Xbp1u, C) in treated versus control cells (T/C). As no effects were seen in dermal fibroblasts after glucose deprivation for 24 h, these cells were subjected to glucose deprivation for 48 h (D). Data are shown as mean ± SD. *: p<0.05; **: p<0.01; ***: p<0.001.
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Figure 3: Type II collagen expression is down-regulated under ER stress-inducing conditions. Chondrocytes isolated from nucleus pulposus (NP), annulus fibrosus (AF), articular cartilage (AC), and meniscus (M), and dermal fibroblasts (DF) were cultured under control conditions (C) or exposed to the ER stress-inducing treatments of tunicamycin (Tm) or glucose withdrawal (-G). A, Real-time PCR was performed on reverse-transcribed RNA isolated from each condition. The results are presented as relative gene expression of type II collagen (Col2a1) in treated versus control cells (T/C). Data are shown as mean ± SD. *: p<0.05; **: p<0.01; ***: p<0.001. B, Type II collagen protein expression was detected by Western blot analysis. The expression of β-actin was used as a loading control.

Aggrecan is another important component of the ECM in cartilage and its gene expression levels could be quantified in the chondrocytic cells. Both stress-inducing treatments down-regulated gene expression of aggrecan and in meniscal cells the gene expression levels dropped to levels no longer quantifiable (Fig. 4).

Taken together, these results suggest a substantial decreased ECM synthesis under ER stress conditions.
Figure 4: Aggrecan gene expression is decreased under ER stress-inducing conditions. Chondrocytes isolated from nucleus pulposus (NP), annulus fibrosus (AF), articular cartilage (AC), and meniscus (M), and dermal fibroblasts (DF) were cultured under control conditions or exposed to the ER stress-inducing treatments of tunicamycin (left panel) or glucose withdrawal (right panel). Real-time PCR was performed on reverse-transcribed RNA isolated from each condition. The results are presented as relative gene expression of aggrecan (Acan) in treated versus control cells (T/C). Data are shown as mean ± SD. **: p<0.01; ***: p<0.001

Collagen chaperones and collagen-modifying enzymes

Molecular chaperones are necessary for correct protein folding and especially in situations where protein folding is affected; they are up-regulated to prevent aggregation. Serpinh1 is a chaperone protein specific for collagen. It is unknown how Serpinh1 reacts to ER stress, especially since the synthesis of its target protein collagen is decreased. Both treatment with tunicamycin and glucose deprivation increased Serpinh1 gene expression (Fig. 5A). On protein level, Serpinh1 could be detected in all the samples (Fig. 5B). Thus, although collagen expression is down-regulated under ER stress, there is an up-regulation of the collagen-specific chaperone Serpinh1.

Hydroxylation of proline residues by prolyl hydroxylases plays a central role in the synthesis of collagen. There are three isoenzymes of the ER transmembrane prolyl 4-hydroxylases (P4ha). The type 1 isoenzyme, P4ha1, was up-regulated under both ER stress-inducing conditions in the chondrocyte populations (Fig. 6A). Treatment with tunicamycin for 24 h did not significantly increase P4ha1 gene expression in dermal fibroblasts, but there was a trend towards up-regulation (Fig. 6A). Glucose deprivation did significantly increase P4ha1 gene expression at 48 h by the fibroblasts. The type 2 isoenzyme, P4ha2, was not detectable in dermal fibroblasts. Both tunicamycin treatment and glucose deprivation resulted in an increase of P4ha2 gene expression at both time-points by all chondrocyte populations (Fig. 6B). The type 3 isoenzyme, P4ha3, was not affected by ER stress-inducing conditions in all the cells (Fig. 6C). Thus, P4ha gene expression levels were either not affected or they were up-regulated under ER stress conditions.
Figure 5: ER stress results in an up-regulation of Serpinh1. Chondrocytes isolated from nucleus pulposus (NP), annulus fibrosus (AF), articular cartilage (AC), and meniscus (M), and dermal fibroblasts (DF) were cultured under control conditions (C) or exposed to the ER stress-inducing treatments of tunicamycin (Tm) or glucose withdrawal (−G). A, Real-time PCR was performed on reverse-transcribed RNA isolated from each condition. The results are presented as relative gene expression of Serpinh1 [serpin peptidase inhibitor, clade H (heat shock protein 47), member 1] in treated versus control cells (T/C). Data are shown as mean ± SD. *: p<0.05; **: p<0.01; ***: p<0.001 B, Serpinh1 protein expression was detected by Western blot analysis. The same expression was detected in AF and M (not shown). The expression of β-actin was used as a loading control.

Figure 6: ER stress either up-regulates or does not affect prolyl 4-hydroxylases. Chondrocytes isolated from nucleus pulposus (NP), annulus fibrosus (AF), articular cartilage (AC), and meniscus (M), and dermal fibroblasts (DF) were cultured under control conditions or exposed to the ER stress-inducing treatments of tunicamycin (left panel) or glucose withdrawal (right panel). Real-time PCR was performed on reverse-transcribed RNA isolated from each condition. The results are presented as relative gene expression of prolyl 4-hydroxylase type 1 isoenzyme (P4ha1, A), type 2 isoenzyme (P4ha2, B), or type 3 isoenzyme (P4ha3, C) in treated versus control cells (T/C). Data are shown as mean ± SD. *: p<0.05; **: p<0.01
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Fig. 6

A

Tunicamycin

Glucose deprivation

Relative P4ha1 gene expression (T/C)

NP AF AC M DF

Relative P4ha1 gene expression (T/C)

NP AF AC M DF

B

Tunicamycin

Glucose deprivation

Relative P4ha2 gene expression (T/C)

NP AF AC M

Relative P4ha2 gene expression (T/C)

NP AF AC M

C

Tunicamycin

Glucose deprivation

Relative P4ha3 gene expression (T/C)

NP AF AC M DF

Relative P4ha3 gene expression (T/C)

NP AF AC M DF
Hydroxylation of specific lysine residues is also a key event in collagen posttranslational modification. Hydroxylysine residues are important for intermolecular cross-linking and they are the attachment sites for protein O-glycosylation. There are three isoenzymes of procollagen-lysine 2-oxoglutarate 5-dioxygenase (Plod), Plod1-3. This enzyme is responsible for the hydroxylation of lysine residues. Both treatments, with tunicamycin and glucose deprivation, resulted in Plod1 gene expression levels that were detectable, but not quantifiable (because they were close to the detection limit) in the chondrocyte populations. In the fibroblasts, there was a decrease in Plod1 gene expression after both treatments (Fig. 7A). The second isoenzyme, Plod2, was down-regulated under ER stress-inducing conditions in the chondrocytes (Fig. 7B). Treatment with tunicamycin did not significantly decrease Plod2 gene expression by dermal fibroblasts (Fig. 7B). Glucose deprivation decreased Plod2 gene expression at 48 h in dermal fibroblasts. The third isoenzyme, Plod3, was detectable in all the cells, but the values were under the quantification limit of the assay.

**Figure 7:** Lysyl hydroxylases are down-regulated under ER stress-inducing conditions. Chondrocytes isolated from nucleus pulposus (NP), annulus fibrosus (AF), articular cartilage (AC), and meniscus (M), and dermal fibroblasts (DF) were cultured under control conditions or exposed to the ER stress-inducing treatments of tunicamycin (left panel) or glucose withdrawal (right panel). Real-time PCR was performed on reverse-transcribed RNA isolated from each condition. The results are presented as relative gene expression of procollagen-lysine 2-oxoglutarate 5-dioxygenase isoenzyme type 1 (Plod1, A) or procollagen-lysine 2-oxoglutarate 5-dioxygenase isoenzyme type 2 (Plod2, B) in treated versus control cells (T/C). Data are shown as mean ± SD. *: p<0.05, **: p<0.01
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During or following exocytosis, the collagen triple helices undergo proteolytic cleavage to remove the propeptides. The N-propeptides of type II collagen are removed by a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 3 (Adamts3). Adamts2 is capable to remove the N-propeptides from type I, II and V collagen. After the cleavage of the propeptides, the collagen is capable of self-assembling into fibrils. The gene expression levels of Adamts3 were not affected by tunicamycin or glucose deprivation (Fig. 8). Both conditions resulted in Adamts2 gene expression levels that were detectable, but not anymore quantifiable because it was close to the detection limit of the assay (data not shown).

Apoptosis
Treatment with tunicamycin induced apoptosis in all the cells after 24 h and 48 h (Fig. 9). After 48 h with tunicamycin, the percentage of apoptotic cells was higher in the dermal fibroblasts compared to the chondrocyte populations. Glucose deprivation induced apoptosis at 24 h in the chondrocyte populations. At 48 h, glucose deprivation also increased apoptosis in dermal fibroblasts, but there were less apoptotic dermal fibroblasts compared to the chondrocyte populations.

Figure 8: Adams3 gene expression is not affected by ER stress. Chondrocytes isolated from nucleus pulposus (NP), annulus fibrosus (AF), articular cartilage (AC), and meniscus (M), and dermal fibroblasts (DF) were cultured under control conditions or exposed to the ER stress-inducing treatments of tunicamycin (left panel) or glucose withdrawal (right panel). Real-time PCR was performed on reverse-transcribed RNA isolated from each condition. The results are presented as relative gene expression of Adams3 [a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 3] in treated versus control cells (T/C). Data are shown as mean ± SD.
DISCUSSION

It is well known that nutrient deprivation may cause ER stress in cells\textsuperscript{11,12}. Due to the avascularity of cartilage, chondrocytes are more prone to nutrient deprivation than cells in most other types of tissue. In this study we have shown that chondrocytes respond to glucose deprivation by initiating an ER stress response at a relatively early time point, 24 h. Dermal fibroblasts proved to be less glucose dependent; they showed a delayed response and ER stress was noticeable after 48 h. All the cells did respond to treatment with tunicamycin after 24 h. Not only is glucose important for the synthesis of glycosaminoglycans, it is also necessary for the energy metabolism. The energy metabolism by chondrocytes differs from most other eukaryotic cells, including fibroblasts. Chondrocytes derive most of their energy from substrate level phosphorylation during glycolysis in stead of oxidative phosphorylation, even in an aerobic environment\textsuperscript{20-22}. Since substrate level phosphorylation generates less adenosine triphosphate than oxidative phosphorylation, more glucose is needed to obtain a certain amount of energy. It is likely that this energy metabolism makes chondrocytes more sensitive to glucose deprivation than fibroblasts.
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The splicing of Xbp1 mRNA was suggested by increased gene expression levels of Xbp1t and similar or decreased gene expression of Xbp1u. In chondrocytes derived from nucleus pulposus, annulus fibrosus, articular cartilage and meniscus there was, besides an up-regulation of Xbp1t gene expression, a down-regulation of Xbp1u gene expression, which was not seen in fibroblasts. This might be a fine-tuning system of the ER stress-response such as seen in yeast with the splicing of the Xbp1 homolog HAC23.

We found a decrease in collagen synthesis under ER stress conditions, which is consistent with earlier observations13-15. Although there was ER stress in fibroblasts, the effect on collagen synthesis was stronger in chondrocytes. An explanation might be that chondrocytes are preconditioned to survive a variety of stresses. They are destined to survive osmotic stress, oxidative stress, and mechanical stress and it is likely that they have already encountered ER stress in their harsh in vivo environment. Cells can only survive ER stress when they are able to remove incorrectly folded proteins from the ER. In order to do so, cells enhance the folding capacity of the ER, the unfolded proteins in the ER are targeted for proteasomal degradation and further protein synthesis is down-regulated11,12,16. The more decreased collagen synthesis by the chondrocytes suggests that chondrocytes are better in down-regulating protein synthesis during ER stress and in consequence that they can manage ER stress better than fibroblasts. Although cells are not able to survive the severe ER stress-inducing conditions we have used, we did observe more apoptotic fibroblasts after treatment with tunicamycin than apoptotic chondrocytic cells, indicating that chondrocytes can indeed cope better with ER stress. One may even speculate that there is a difference in sensitivity to ER stress between the different chondrocyte populations, but this remains to be investigated.

Diseases that are associated with ER stress in chondrocytes do have severe phenotypes. Pseudoachondrodysplasia (PSACH) and multiple epiphyseal dysplasia (MED) result from mutations in cartilage oligomeric protein (COMP) which accumulates in the ER24,25. Both PSACH and MED are characterized by disproportional short stature, joint laxity, and early onset of osteoarthritis. Mutations in the COL10A1gene result in Schmid metaphyseal dysplasia (SMCD) that is characterized by irregularities of the metaphyseal ends of bones of the extremities, bowlegs and coxa vara. Many different mutations are known that lead to the same phenotype in SMCD, but they have in common that the UPR is up-regulated26,27. Nanomelia is a lethal autosomal recessive defect in chickens. It is caused by mutant aggrecan that is retained in the ER and as a consequence normal chondrocyte metabolism is disrupted28,29.

One of the first responses of cells to ER stress is the up-regulation of chaperones and foldases to prevent aggregation of unfolded proteins. Hspa5 is such a chaperone that is immediately up-regulated after sensing ER stress and is even used as a marker to characterize ER stress. Serpinh1 is a chaperone that is believed to be specific for collagen30,31. We also observed an up-regulation of Serpinh1 when ER stress was induced. This is in agreement with a mouse model of osteogenesis imperfecta in which a Col1a1 mutation causes ER stress and an up-regulation of Serpinh132. However, it is remarkable
that the expression of Serpinh1 is elevated even though there was in our case no type I and/or type II collagen synthesis. An explanation might be that under the condition of ER stress no causal relationship exists between collagen and Serpinh1. It is possible, however, that other types of collagen, like types III, VI, IX, and XI, are still being synthesized.

P4ha2 was not detectable in dermal fibroblasts. This is in agreement with previous observations. In chondrocytes, P4ha2 is responsible for about 80% of the prolyl 4-hydroxylase activity. ER stress induced P4ha1 and P4ha2 by the different chondrocyte populations, while P4ha3 was not affected. P4ha seems to play an important role in the ER retention of procollagen next to Hspa5. It is striking that just like Serpinh1, P4ha1 and P4ha2 gene expression levels were up-regulated while type I and/or type II collagen synthesis was inhibited. This supports our conclusion formulated in the previous paragraph that under the condition of ER stress no causal relationship exists between synthesis of collagen types I and II and P4ha1 and P4ha2. A general response by cells to ER stress is a down-regulation of expression of secreted molecules and an up-regulation of chaperones and foldases. This seems to happen here; the expression of type I and type II collagen is down-regulated, while Serpinh1, P4ha1 and P4ha2 are up-regulated to help with any misfolded or aggregated proteins in the ER.

The mRNA levels Plod1 and Plod2 were down-regulated by ER stress. The behaviour of the third isoenzyme, Plod3, remains unclear; gene expression was detectable, but the values were below the quantifiable limit of the assay. The hydroxylation of lysine residues is a posttranslational modification which is essential for certain properties of mature collagen molecules, such as the stability and formation of intermolecular cross-links. Several mutations in PLOD1 are responsible for Ehlers-Danlos Syndrome Type VI and mutations in PLOD2 are responsible for Bruck syndrome leading to underhydroxylated collagen, but the secretion of collagen is normal. This indicates that it is unlikely that the lysyl hydroxylases are necessary for the intracellular folding of collagen and this may help to explain the down-regulation of these genes during ER stress.

An interesting observation was that in the different chondrocyte populations the gene expression of the N-propeptidase Adamts3 was not affected by ER stress, whereas the gene expression levels of Adamts2 were decreased. This implies different regulatory pathways for those N-propeptidases. The regulation of the expression of Adamts genes is still poorly understood, but they are expressed by a wide range of tissues under normal conditions. In line with our results, it was found that Adamts3 is preferentially expressed in cartilage, an enzyme that cleaves the N-propeptide of type II procollagen.

This study demonstrates that chondrocytes obtained from a variety of cartilaginous tissues respond more severely to glucose deprivation by ER stress than fibroblasts derived from skin. Under these conditions collagen synthesis is strongly decreased. This is primarily due to a drop in mRNA levels, as mRNA expression of enzymes involved in the processing of collagen molecules is far less or not affected. Interestingly some collagen-modifying enzymes and chaperones were up-regulated in cells exposed to ER stress, thus suggesting an attempt by the cell to compensate for the reduced level of collagen synthesis.
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by up-regulating expression of these proteins. This study supports the view that chondrocytes are sensitive to glucose deprivation and that a fall in nutrient supply is associated with impaired ECM production.

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REFERENCES

8. Heywood HK, Bader DL, Lee DA. Rate of oxygen consumption by isolated articular chondrocytes is sensitive to medium glucose concentration. J Cell Physiol 2006;206:402-410
ER stress in chondrocytes

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35. Chessler SD, Byers PH. BiP binds type I procollagen pro alpha chains with mutations in the carboxyl-terminal propeptide synthesized by cells from patients with osteogenesis imperfecta. J Biol Chem 1993;268:18226-18233


37. Walmsley AR, Batten MR, Lad U, Bulleid NJ. Intracellular retention of procollagen within the endoplasmic reticulum is mediated by prolyl 4-hydroxylase. J Biol Chem 1999;274:14884-14892


39. Yeowell HN, Walker LC. Mutations in the lysyl hydroxylase 1 gene that result in enzyme deficiency and the clinical phenotype of Ehlers-Danlos syndrome type VI. Mol Genet Metab 2000;71:212-224

