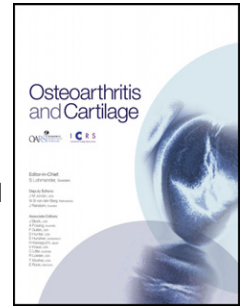


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Title: Expression and function of the insulin receptor in normal and osteoarthritic human chondrocytes: modulation of anabolic gene expression, glucose transport and GLUT-1 content by insulin

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**Title:** Expression and function of the insulin receptor in normal and osteoarthritic human chondrocytes: modulation of anabolic gene expression, glucose transport and GLUT-1 content by insulin

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**Running title:** Insulin receptor in chondrocytes

**Abstract:**

**Objective:** Chondrocytes respond to insulin, but the presence and role of the specific high affinity insulin receptor (InsR) has never been demonstrated. This study determined whether human chondrocytes express the InsR and compared its abundance and function in normal and OA human chondrocytes.

**Design:** Cartilage sections were immunostained for detection of the InsR. Non-proliferating chondrocyte cultures from normal and OA human cartilage were treated with 1 nM or 10 nM insulin for various periods. InsR, IGFR, aggrecan and collagen II mRNA levels were assessed by real time RT-PCR. InsR, GLUT-1, phospho-InsRbeta and phospho-Akt were evaluated by western blot and immunofluorescence. Glucose transport was measured as the uptake of [<sup>3</sup>H]-2-Deoxy-D-Glucose (2-DG).

**Results:** Chondrocytes staining positively for the InsR were scattered throughout the articular cartilage. The mRNA and protein levels of the InsR in OA chondrocytes were approximately 33% and 45%, respectively, of those found in normal chondrocytes. Insulin induced the phosphorylation of the InsRbeta subunit. Akt phosphorylation and 2-DG uptake increased more intensely in normal than OA chondrocytes. Collagen II mRNA expression increased similarly in normal and OA chondrocytes while aggrecan expression remained unchanged. The PI3K/Akt pathway was required for both basal and insulin-induced collagen II expression.

**Conclusions:** Human chondrocytes express functional InsR that respond to physiologic insulin concentrations. The InsR seems to be more abundant in normal than in OA chondrocytes, but these still respond to physiologic insulin concentrations, although some responses are impaired while others appear fully activated. Understanding the mechanisms that regulate the expression and function of the InsR in normal and OA chondrocytes can disclose new targets for the development of innovative therapies for OA.

**Keywords:** anabolic gene expression; glucose transport; human chondrocyte; insulin; insulin receptor; insulin-like growth factor receptor; osteoarthritis

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**Introduction:**

Insulin was shown to induce anabolic and inhibit catabolic responses in adult chondrocytes. In particular, insulin was shown to promote collagen II and proteoglycan synthesis<sup>1,2</sup> and to inhibit Interleukin-1 $\beta$  (IL-1)-induced catabolic effects in adult chondrocytes and cartilage explant cultures from various species<sup>3</sup>. Insulin also plays an important role in chondrogenesis as an inducer of chondrocyte differentiation<sup>4</sup> and was recently shown to reverse the cartilage loss associated with impaired fracture healing in diabetic mice<sup>5</sup>. Moreover, insulin was shown to ameliorate cartilage degeneration in a mouse model of osteoarthritis (OA) while its anabolic and anti-catabolic effects seemed to be independent of aging or disease state<sup>3</sup>.

Despite this, little attention has been devoted to the role of insulin in modulating adult articular chondrocyte functions other than the synthesis and degradation of proteoglycans and collagen II. In this regard, regulation of glucose transport may be especially important since it is a major function of insulin in target tissues. Besides its major effect on the facilitative glucose transporter (GLUT)-4, a member of the facilitative glucose/polyol or GLUT/SLC2A transporter family, expressed in skeletal muscle and adipose tissue cells, insulin was shown to increase glucose transport in other cell types, by promoting the expression and plasma membrane incorporation of GLUT-1<sup>6-9</sup>. Glucose is an essential nutrient for chondrocytes, being required for energy production, basically through the glycolytic pathway, and for the proper synthesis of cartilage matrix macromolecules, namely proteoglycans. Facilitated glucose transport represents the first rate-limiting step in glucose metabolism in chondrocytes and thus its regulation is a critical determinant of chondrocyte homeostasis<sup>10</sup>. Several members of the GLUT family were identified, at the protein level, in human chondrocytes, including GLUTs-1, -3, -6, -8, -9 and -10, but not GLUT-4<sup>10, 11</sup>. Among these, GLUT-1

seems to be especially important since it is regulated by anabolic, namely IGF-I, and catabolic stimuli<sup>10-13</sup>, as well as by the extracellular glucose concentration<sup>14</sup>.

It is unclear, however, whether physiologic nanomolar concentrations of insulin, which can reach chondrocytes by simple diffusion from the synovial fluid<sup>15</sup>, are able to promote anabolic gene expression and to modulate glucose transport in chondrocytes. This is especially important since many of the studies conducted so far used high supraphysiologic insulin concentrations<sup>1,2</sup>. In the few studies where insulin was used in the nanomolar range, the presence and role of the specific high affinity insulin receptor (InsR) in mediating insulin-induced responses were not demonstrated<sup>3</sup>. Since insulin also binds to the structural- and functionally-related insulin-like growth factor receptor (IGFR), although with lower affinity thus requiring higher concentrations<sup>16</sup>, the question remains as to the presence and role of the InsR in mediating insulin-induced responses in chondrocytes.

Therefore, the first objective of this study was to determine whether adult human chondrocytes express the InsR and to compare its abundance in normal and OA chondrocytes. Then, we investigated whether the insulin receptors expressed in normal and OA chondrocytes were functional by examining the ability of insulin to induce its phosphorylation and the activation of the Phosphoinositol-3 Kinase (PI3K)/Akt (or protein kinase B) pathway, which is a major downstream effector of the InsR. To elucidate the role of the InsR in mediating the anabolic effects of insulin, we analyzed the mRNA expression of aggrecan and collagen II. Finally, we investigated the role of insulin and the InsR in modulating glucose transport and GLUT-1 content in normal and OA chondrocytes.

## Materials and methods

### CARTILAGE SAMPLES AND CHONDROCYTE CULTURES

Human knee cartilage was collected from the distal femoral condyles of 24 multi-organ donors (11 women and 13 men, 18-53 years old, mean=38,) or with informed consent from 29 patients (14 women, 15 men, 58-82 years old, mean=67) undergoing total knee replacement surgery at the Orthopedic Department of the University Hospital of Coimbra (HUC). The Ethics Committee of HUC approved all procedures. Normal cartilage had no macroscopic signs of degradation or osteophytosis. Cartilage from patients was severely damaged, with areas of extensive full thickness erosion. Chondrocytes were isolated by enzymatic digestion from non-pooled cartilage samples as described previously<sup>17</sup>. To avoid dedifferentiation, confluent non-proliferating monolayer cultures were established from each cartilage sample, allowed to recover in medium containing 5% fetal bovine serum for 24h, serum-starved overnight and maintained thereafter in serum-free culture medium. Serum-starved chondrocyte cultures were treated with 1 or 10 ng/ml recombinant human insulin (Sigma Chemical Co., St. Louis, MO), as indicated in the results section and figure legends.

### TOTAL RNA EXTRACTION, cDNA PREPARATION AND QUANTITATIVE REAL-TIME RT-PCR (qRT-PCR)

Total RNA was extracted with TRIzol (Invitrogen, Paisley, UK), analyzed using Experion RNA StdSens Chip (Bio-Rad) and quantified in a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE) at 260 nm. The cDNA was reverse transcribed from 1 µg of total RNA, using iScript™ Select cDNA Synthesis Kit (Bio-Rad), according to the manufacturer's instructions. The cDNA

obtained was stored at  $-20^{\circ}\text{C}$  until further analysis. Specific sets of primers were designed using Beacon Designer software (PREMIER Biosoft International, Palo Alto, CA). Details of the forward and reverse primers used are presented in table 1. The stability values for the expression of reference gene candidates were obtained by a Normfinder analysis using Genex<sup>®</sup> software (MultiD Analyses AB). The results obtained (Supplemental table) indicated that HPRT-1 is the best reference gene for comparisons between normal and OA chondrocytes, while RPL13A is the best to evaluate insulin-induced responses. qRT-PCR was performed with iTaq<sup>™</sup> DNA polymerase using iQ<sup>™</sup> SYBR Green Supermix (BioRad). Thermal cycling conditions included 3 minutes at  $95^{\circ}\text{C}$  to activate the iTaq<sup>™</sup> DNA polymerase, followed by 45 cycles, each consisting of a denaturation step at  $95^{\circ}\text{C}$  for 10 seconds, an annealing step at  $54^{\circ}\text{C}$  for 30 seconds and an elongation step at  $72^{\circ}\text{C}$  for 30 seconds. Fluorescence measurements were taken every cycle at the end of the annealing step and the specificity of the amplification products was checked by analysis of the melting curve. The efficiency of the amplification reaction for each gene was calculated by running a standard curve of serially diluted cDNA sample. In each assay, a control reaction without the cDNA was also subjected to PCR amplification.

Gene expression levels in normal and OA chondrocytes were normalized to HPRT-1 using the following formula  $E^{\Delta\text{Ct}}$ , where E is the qPCR efficiency of the genes evaluated and  $\Delta\text{Ct}$  is the difference between the cycle threshold (Ct) of HPRT-1 and that of each gene of interest. Gene expression changes relative to a control condition were analyzed using the built-in iQ5 Optical system software v2, which enables the analysis of the results with the Pfaffl method<sup>18</sup>, a variation of the  $\Delta\Delta\text{CT}$  method corrected for gene-specific efficiencies.

## WESTERN BLOT ANALYSIS



Total cell extracts were prepared as previously described<sup>14</sup>. The extracts and molecular weight markers (All blue, Precision Plus molecular weight markers, Bio-Rad Laboratories Inc., Hercules, CA) were subjected to SDS/PAGE, electroblotted onto PVDF membranes, probed with rabbit polyclonal antibodies against the  $\beta$  subunit of the InsR (InsRbeta) (1:1000 dilution; Santa Cruz Biotechnology, Inc, Santa Cruz, CA), the Ser 473-phosphorylated form of Akt (pAkt) (1:1000 dilution; Cell Signaling Technology, Danvers, MA) or the human GLUT-1 (1:4000 dilution; FabGennix Inc. International, Frisco, TX) and then with an alkaline phosphatase-conjugated secondary antibody (1:20,000 dilution; GE Healthcare). A mouse monoclonal antibody against the phosphorylated form of the InsRbeta subunit (1:200 dilution, Santa Cruz Biotechnology) was used to detect the activated form of the InsR (pInsRbeta). Immune complexes were detected with the Enhanced ChemiFluorescence reagent (GE Healthcare) in a Storm 840 scanner (GE Healthcare). A mouse anti-actin monoclonal antibody (1:10,000 dilution; Millipore Corporation, Billerica, MA) was used as a loading control. The intensity of the bands was analyzed using ImageQuant<sup>TM</sup> TL (GE Healthcare).

#### IMMUNOFLUORESCENCE STAINING

Five mm diameter full thickness cartilage cylinders were collected from the distal femoral condyles of multi-organ donors, immersed in OCT embedding compound (TAAB Laboratories, UK) and immediately frozen at -80 °C. Cryostat sections, 10  $\mu$ m thick, were fixed in methanol at -20 °C for 10 min. Chondrocytes cultured on glass coverslips were fixed in 4% paraformaldehyde at 4 °C for 10 min and permeabilized with 0.1% Tween-20 in PBS for 10 min. For immunostaining of fixed cartilage sections and chondrocytes, a rabbit polyclonal antibody to the InsRbeta subunit (1:50 dilution; Santa Cruz Biotechnology) and Alexa fluor 488-conjugated goat anti-rabbit antibody (1:200 dilution; Molecular Probes, Eugene, OR) were used as primary and secondary

antibodies, respectively. Counterstaining was performed with DAPI (Sigma) to allow nucleus visualization. Specificity was assessed by omitting the first antibody (negative control). Images of cartilage sections were acquired in a PALM laser dissector fluorescence microscope (Zeiss Axiovert 200M) and images of cultured chondrocytes were acquired in a confocal laser scanning microscope (Zeiss LSM 510 Meta), using an excitation filter of 500 nm and an emission filter of 520 nm. The settings for contrast, brightness, acquisition mode and scanning time were maintained throughout the work in both microscopes.

#### 2-DEOXY-D-GLUCOSE UPTAKE ASSAY

Glucose transport was measured as the net uptake of 2-Deoxy-D-Glucose (2-DG) (Sigma), a non-metabolizable analogue of glucose, as previously described<sup>14</sup>.

#### STATISTICAL ANALYSIS

The two-tailed unpaired Student t-test was used to compare the mRNA and protein levels of the InsR and the IGFR mRNA levels in normal and OA chondrocytes. In all other figures and tables, the statistical analysis was performed using the one-tailed or two-tailed Student t-test for paired data. To assess normality for the observations themselves or for the observed differences, a graphical analysis based on normal quantile plots was used and showed no strong departure from normality thus supporting the use of the Student t-test. Each subject contributed only one cartilage sample. Single measurements of the various parameters being analyzed were obtained from each cartilage sample, except in qRT-PCR and 2-DG uptake where triplicates and duplicates, respectively, were analyzed. Nonetheless, multiple measurements contributed only once (as mean value) to the statistical analysis. Results are presented as mean values with 95% confidence intervals (95% CI), and were considered statistically significant for  $P < 0.05$ .

## Results

### *IN SITU* IMMUNOFLUORESCENCE STAINING OF THE InsR IN NORMAL HUMAN CARTILAGE.

Figure 1A shows chondrocytes staining positively for the InsRbeta subunit scattered throughout adult human cartilage, as indicated by the green fluorescence around nuclei stained in blue. Nonetheless, several chondrocytes are also present that show no staining. The absence of green staining in Fig. 1B (negative control) demonstrates the specificity of the staining in panel A. Therefore, these results demonstrate that the InsR is present at the protein level in adult human chondrocytes *in situ* in the articular cartilage.

### InsR AND IGFR mRNA EXPRESSION IN CHONDROCYTES FROM NORMAL AND OA HUMAN ARTICULAR CARTILAGE:

The results show that adult human chondrocytes, either normal or OA, express both the insulin and the IGF-I receptors at the mRNA level. However, the InsR mRNA level is 33% lower ( $P=0.0028$ ) in OA (mean=6.1, 95% CI=2.9-6.4, N=7) than in normal (mean=9.1, 95% CI=7.7-10.6, N=8) chondrocytes. Similarly, the abundance of the IGFR mRNA is 52.5% lower ( $P=0.0149$ ) in OA (mean= 2.2, 95% CI=1.9-2.6, N=7) than in normal (mean=4.6, 95% CI=2.8-5.4, N=8) chondrocytes.

### InsR PROTEIN LEVELS IN CHONDROCYTES FROM NORMAL AND OA HUMAN ARTICULAR CARTILAGE

The InsR expressed in chondrocytes was further characterized at the protein level by western blot and immunofluorescence staining. The antibody used in the western blot analysis is specific for the  $\beta$  subunit of the InsR, detecting both its precursor (PreInsR) and mature (InsRbeta) forms which have apparent molecular weights of 200 and 90 kDa, respectively. Accordingly, the two bands were detected both in a whole cell extract of 3T3-L1 adipocytes used as a positive control (PC) and in the whole cell extracts obtained from normal and OA chondrocytes (Fig. 2A). The levels of either form of the InsR were found to be significantly higher in normal than in OA chondrocytes, in which they represent 53.1% and 55.9%, respectively, of those found in normal chondrocytes (Fig. 2A). Immunofluorescence staining of normal and OA chondrocyte cultures (Fig. 2B) confirmed the results of the western blot analysis, showing a punctuated staining pattern consistent with the plasma membrane localization of the InsR that is more intense in normal than in OA chondrocytes.

#### EVALUATION OF THE FUNCTIONAL STATE OF THE InsR EXPRESSED IN NORMAL AND OA HUMAN CHONDROCYTES

To determine whether the InsR expressed in normal and OA human chondrocytes is functional and mediates insulin-induced responses, we evaluated the ability of nanomolar insulin concentrations to induce the phosphorylation of Tyrosine residues in positions 1150 and 1151 of the beta subunit of the InsR and the phosphorylation of the Serine 473 residue of Akt.

Treatment of normal and OA chondrocytes with 10 nM insulin induced the phosphorylation of the InsRbeta subunit, in a time-dependent manner (Fig. 3A). Normal and OA chondrocytes have a similar basal Akt phosphorylation, which was markedly increased by treatment with 1 nM and 10 nM insulin both in normal (Fig. 3B) and OA

chondrocytes (Fig. 3C). However, in OA chondrocytes, Akt phosphorylation induced by either insulin concentration was lower than that observed in normal ones.

#### ROLE OF INSULIN AND ITS SPECIFIC RECEPTOR IN MODULATING AGGREGAN AND TYPE II COLLAGEN GENE EXPRESSION:

To further characterize the functions of the InsR, insulin-induced anabolic gene expression, namely aggrecan and type II collagen, was evaluated by qRT-PCR. Treatment of normal human chondrocytes with 10 nM insulin for 12 h (mean=1.002, 95% CI=0.72-1.28, P=0.5089, N=5) did not alter aggrecan mRNA levels relative to untreated cells, whereas it induced a 2.4 fold increase in type II collagen mRNA level (Table 2).

To further elucidate the mechanisms by which the InsR mediates insulin-induced collagen II expression, specific inhibitors of PI3K (wortmannin) and MEK1/MEK2 (U0126) were added to the chondrocyte cultures 30 min before addition of 10 nM insulin for 12 h. Treatment with wortmannin alone for the same period significantly decreased basal collagen II mRNA levels, while in insulin-treated chondrocytes, it returned collagen II mRNA levels to those found in untreated cells (Table 2). The MEK1/MEK2 inhibitor slightly increased collagen II mRNA levels relative to untreated cells, but had no effect on insulin-induced collagen II expression.

To determine whether the InsR is still functional in OA chondrocytes, we measured collagen II mRNA levels in response to insulin. Basal mRNA levels did not differ significantly between normal and OA chondrocytes (data not shown). Treatment of OA chondrocytes with 10 nM insulin for 12 h increased collagen II mRNA levels to 2.0 (95% CI=0.8-3.3, P=0.0455, N=4) of those found in untreated cells, which, although

barely reaching statistical significance, is a response of magnitude similar to that observed in normal chondrocytes.

#### REGULATION OF GLUCOSE TRANSPORT AND GLUT-1 CONTENT BY INSULIN IN NORMAL AND OA CHONDROCYTES

To determine the role of insulin and its specific receptor in the regulation of glucose transport, we measured 2-DG uptake in normal and OA chondrocytes. Treatment of normal chondrocytes with 1 nM and 10 nM insulin for 48 h increased 2-DG uptake in a concentration-dependent manner relative to untreated cells (Table 3). In OA chondrocytes, however, the increase in 2-DG uptake induced by insulin was lower than in normal ones and the difference between the responses induced by the two insulin concentrations tested did not reach statistical significance.

To elucidate the mechanism by which insulin increased 2-DG uptake, total GLUT-1 protein content was analyzed by western blot in total cell extracts from normal and OA chondrocytes. Treatment with 10 nM insulin increased total GLUT-1 protein content in normal (Fig. 4A), but not in OA chondrocytes (Fig. 4B), whereas 1 nM insulin had no effect, either in normal or OA chondrocytes.

## Discussion

The results obtained in this study demonstrate for the first time that adult human chondrocytes express the InsR, both *in situ* in the articular cartilage (Fig. 1) and *in vitro* after isolation and culture (Fig. 2). In agreement with this, a physiologic nanomolar insulin concentration was effective in directly activating the InsR both in normal and OA chondrocytes, thus indicating that the InsR expressed in human chondrocytes is functional. Consistent with this, insulin also induced Akt phosphorylation in normal and, to a lesser extent, in OA chondrocytes (Fig. 3). This finding, together with the low insulin concentrations tested that are unlikely to be sufficient to activate the IGFR<sup>16</sup>, further support the involvement of the specific InsR in mediating Akt activation induced by physiologic insulin concentrations. On the other hand, the lower response of OA chondrocytes to the same insulin concentrations can directly result from reduced expression of the InsR (Fig. 2), as well as from defective downstream signaling.

Several processes can contribute to the reduced expression of the InsR in OA chondrocytes. The InsR is regulated both at the transcriptional and post-transcriptional levels by glucocorticoids<sup>19</sup>, insulin<sup>19,20</sup> and nutrient availability<sup>20-22</sup>. Additionally, inflammatory mediators, like IL-1, TNF- $\alpha$  and reactive oxygen species (ROS) which play an important role in OA<sup>23,24</sup>, may also contribute to downregulate the InsR and/or its downstream signaling pathways. Indeed, studies in endothelial cells demonstrated that TNF- $\alpha$  decreases the InsR content<sup>25</sup>. Moreover, TNF- $\alpha$ , IL-1 and ROS have all been shown to inhibit the generation of downstream signaling intermediates<sup>26-29</sup>, leading to insulin resistance. Accordingly, the lower InsR content and the reduced Akt activation in OA chondrocytes relative to normal ones may be another consequence of the accumulation and actions of those inflammatory and catabolic mediators in osteoarthritic joints. On the other hand, given the different mean age of the normal and OA groups analyzed, the influence of aging on the decreased InsR expression and

function in OA chondrocytes cannot be excluded. Nonetheless, since aging is the major risk factor for OA, elucidation of the mechanisms that regulate the InsR expression and function in human chondrocytes and how these are affected by aging and OA deserve further investigation.

We also found the IGFR expression to be decreased in OA relative to normal chondrocytes. This is likely to contribute to the reported resistance of OA chondrocytes to IGF-I, along with other mechanisms already identified<sup>30,31</sup>. Similarly, the lower InsR expression and activity in OA chondrocytes suggest that these may also have some degree of insulin resistance. However, although less effectively, OA chondrocytes still activated Akt in response to physiologic insulin concentrations (Fig. 3B), indicating that they are partially sensitive to insulin which can contribute to sustain chondrocyte functions, since Akt is upstream of key survival and metabolic pathways<sup>31</sup>.

To elucidate this question, we determined whether insulin signaling through its specific receptor can promote anabolic responses, namely aggrecan and collagen II expression, both in normal and OA chondrocytes. Under the experimental conditions used, the mRNA expression of aggrecan was not affected, while collagen II mRNA levels were increased by treatment of human chondrocytes with a physiologic insulin concentration (Table 2). In addition, collagen II expression involved a mechanism partly dependent on the PI3K, but not on the MEK1/MEK2 pathway (Table 2). This demonstrates that, at least, some anabolic responses in human chondrocytes are physiologically regulated by activation of the InsR. Although expressing lower levels of the InsR, the ability of OA chondrocytes to express collagen II in response to insulin was found to be similar to that observed in normal chondrocytes. This suggests that the remaining InsR molecules are sufficient to ensure full or nearly full induction of collagen II expression. Nonetheless, this finding is somewhat puzzling since OA chondrocytes showed defective Akt activation (Fig. 3B) and the InsR-mediated collagen II expression



was found to involve the PI3K (Table 2), which taken together would predict a lower ability of OA chondrocytes to express collagen II mRNA in response to insulin. In agreement with this, the difference in collagen II mRNA levels between insulin-treated and untreated OA chondrocytes barely reached statistical significance, likely reflecting a significant variability between individuals. This suggests that some individuals with OA will respond less effectively to physiologic insulin concentrations in terms of collagen II expression, which in the current study may have been underestimated due to the small sample size available. Further studies with larger cohorts of OA patients stratified by age, sex, comorbidities and possibly other individual variables may help elucidate which patients will more likely respond to physiologic insulin concentrations. Moreover, identification of the signaling pathways involved in InsR-mediated collagen II expression and evaluation of their activity in normal and OA chondrocytes deserves further investigation as it may disclose novel mechanisms to promote the chondrocyte anabolic responses.

The finding that, unlike collagen II, aggrecan expression was not increased by low insulin concentrations is somewhat unexpected, especially as other studies showed increased aggrecan and proteoglycan production<sup>1,2</sup>. As these studies used high supraphysiologic insulin concentrations, it is possible that the effects of insulin on aggrecan and proteoglycan synthesis were mediated solely by the IGFR. Nonetheless, it is also possible that insulin, even in low physiologic concentrations, can increase the production of proteoglycans acting by mechanisms independent of gene transcription. Such studies are beyond the scope of the current study, but are worthwhile being undertaken as new targets to increase the chondrocyte anabolic activity may be disclosed.

To further characterize the role of physiologic insulin concentrations in regulating normal and OA chondrocyte functions, we evaluated its ability to modulate glucose

transport and observed that it significantly increased in normal and to a lesser extent in OA chondrocytes (Table 3). The highest insulin concentration tested also augmented total GLUT-1 protein content in normal chondrocytes (Fig. 4A), suggesting that this contributed, at least in part, to the observed increase in 2-DG uptake. In OA chondrocytes, however, total GLUT-1 content remained unaffected with both insulin concentrations (Fig. 4B). In other cells, insulin was shown to increase glucose uptake by promoting GLUT-1 incorporation in the plasma membrane<sup>32</sup>. Other studies demonstrated that GLUT-1 exists in a dynamic equilibrium that involves the plasma membrane, intracellular storage in the trans Golgi network and the lysosomes where it is degraded<sup>14,33,34</sup>. Accordingly, the results presented suggest that, even in concentrations as low as 1 nM acting in OA chondrocytes, insulin can promote the recruitment of GLUT-1 from intracellular stores to the plasma membrane. Nonetheless, we also cannot exclude the possibility that other isoforms of the GLUT family are involved in insulin-stimulated glucose uptake in chondrocytes. Indeed, insulin has been shown to regulate other GLUT isoforms, namely GLUTs-8 and -12<sup>35,36</sup> which are insulin sensitive and expressed by human chondrocytes, at least at the mRNA level<sup>10,11</sup>. GLUT-3, which is constitutively expressed in chondrocytes<sup>10</sup> and in other cells was shown to be regulated by insulin<sup>37</sup>, also deserves to be investigated as a possible insulin target in human chondrocytes.

In conclusion, our results show for the first time that adult human chondrocytes express functional InsR. Even though expressing lower InsR levels and showing defective activation of Akt than normal ones, OA chondrocytes still seem capable of responding to physiologic insulin concentrations. Even though the relatively small number of cartilage samples available precludes definitive conclusions to be taken, some responses, namely glucose transport, seem to be impaired while others, like collagen II mRNA expression, appear fully activated, at least in some patients. The reduced ability of OA chondrocytes to increase glucose transport in response to physiologic insulin

concentrations may compromise energy production and plastic functions, namely glucosaminoglycan synthesis, what can contribute to chondrocyte damage and OA progression. On the other hand, taking into account the structural and functional homology between the InsR and the IGFR, modulation of the InsR and of its downstream signaling pathways may be an effective strategy to overcome the resistance of OA chondrocytes to IGF-1, thus representing a novel pharmacological target for the development of innovative anti-osteoarthritic therapies.

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#### **Authors' Contributions**

SCR (susrosa@gmail.com) carried out chondrocyte cultures, 2-DG uptake assays, western blots, immunofluorescence and real time RT-PCR experiments, participated in the study design and drafted the manuscript; ATR isolated and set up chondrocyte cultures and performed western blots; FJ collected normal and OA cartilage and participated in the study design; CT performed the statistical analysis; MCL participated in the study design; AFM (afmendes@ff.uc.pt) conceived, designed and coordinated the study, set up some chondrocyte cultures and drafted the manuscript. All authors made intellectual contributions to the project, revised critically and approved the final manuscript.

**Conflict of Interest Statement:**

None to declare.

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**Figure legends:**

**Figure 1: *In situ* Immunofluorescence staining of the InsR in normal human cartilage.** (A) representative image of the InsRbeta subunit (green) in normal human cartilage sections (N=3) counterstained with DAPI (blue) to allow nuclei visualization, viewed with a 200x magnification (inset: 400x magnification). (B) representative image of the negative control (N=3) viewed with a 200x magnification (inset: 400x magnification).

**Figure 2: InsR protein levels in chondrocytes from normal and OA human articular cartilage.** (A) preInsR and InsRbeta protein content in total cell extracts of chondrocyte cultures established from normal (N=6) and OA (N=6) non-pooled cartilage samples. The image shown is representative of the results obtained in normal and OA chondrocytes. **MW**= molecular weight marker; **PC**=positive control; **N**=normal chondrocytes; **OA**=osteoarthritic chondrocytes. (B) Immunofluorescence staining of the InsRbeta subunit (green) expressed in normal and OA chondrocytes counterstained with DAPI (blue) to show the cell nuclei, as described in section 2.6. The images shown are representative of the results obtained in 3 normal and 3 OA independent chondrocyte cultures; **NC**= Negative control.

**Figure 3: Evaluation of the functional state of the InsR expressed in normal and OA human chondrocytes.** (A) InsRbeta phosphorylation in chondrocyte extracts from 1 normal and 2 OA cartilage samples untreated (Control) or treated with insulin 10 nM for the indicated periods. Akt phosphorylation in total cell extracts from 4 normal (B) and 4 OA (C) non-pooled independent chondrocyte cultures treated with 1 or 10 nM insulin for 30 min relative to untreated cells.

**Figure 4: Regulation of GLUT-1 content by insulin in normal and OA chondrocytes.** Total GLUT-1 protein levels in **(A)** normal (N=6) and **(B)** OA (N=6) chondrocytes treated with 1 or 10 nM insulin for 48 h relative to untreated cells.

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**Table 1:** Oligonucleotide primer pairs used for qRT-PCR

Gene	Primer sequences (5'-3')	RefSeq ID
$\beta$ -Actin (ACTB)	<b>F:</b> AACTACCTTCAACTCCAT <b>R:</b> TGATCTTGATCTTCATTGTG	NM_001101
Beta-2-microglobulin (B2M)	<b>F:</b> CTCCAAAGATTTCAGGTTTACTCAC <b>R:</b> AGTCAACTTCAATGTCGGATGG	NM_004048
Peptidylprolyl isomerase A (Cyclophilin A)	<b>F:</b> CAGTCCCAGGAAGTGTCAATG <b>R:</b> CAGCGTCTCACTATGTTGCC	NM_021130
Hypoxanthine phosphoribosyltransferase- 1 (HPRT-1)	<b>F:</b> TGACACTGGCAAACAAT <b>R:</b> GGCTTATATCCAACACTTCG	NM_000194
Glyceraldehyde 3- phosphate dehydrogenase (GAPDH)	<b>F:</b> ACAGTCAGCCGCATCTTC <b>R:</b> GCCCAATACGACCAAATCC	NM_002046
Ribosomal protein S18 (18S rRNA)	<b>F:</b> GAAGATATGCTCATGTGGTGTG <b>R:</b> CTTGTACTGGCGTGGATTCTG	NM_022551
Ribosomal protein L13a (RPL13A)	<b>F:</b> GGAAGAGCAACCAGTTACTATGAG <b>R:</b> CAGAGTATATGACCAGGTGGAAAG	NM_012423
TATA box binding protein (TBP)	<b>F:</b> TTCCACTCACAGACTCTC <b>R:</b> ACAATCCCAGAACTCTCC	NM_003194
Insulin receptor (INSR)	<b>F:</b> GCCTCTACAACCTGATGAAC <b>R:</b> ACAGATGTCTCCCACTCC	NM__000208
Insulin like growth factor receptor (IGFR)	<b>F:</b> GAAGTGAGTGCTCCTTGATG <b>R:</b> CCACGGATGACTGCTGAG	NM_000875
Collagen type II (COL2A1)	<b>F:</b> GGC AGA GGT ATA ATG ATA AGG <b>R:</b> ATT ATG TCG TCG CAG AGG	NM_001844
Aggrecan (ACAN)	<b>F:</b> .CCT GGT GTG GCT GCT GTC <b>R:</b> CTG GCT CGG TGG TGA ACT C	NM_001135

**F:** Forward sequence; **R:** Reverse sequence

**Table 2:** Role of signaling pathway inhibitors on collagen type II gene expression relative to untreated cells

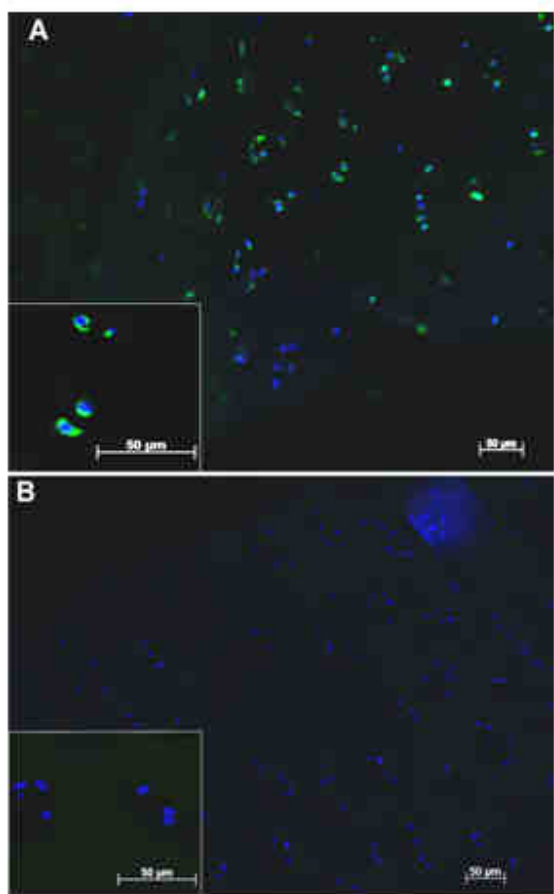
Insulin, 10 nM, 12h	Inhibitor	Mean (95% CI)	P value	N
+	–	2.48 (1.24-3.72)	0.0138 <sup>a</sup>	6
–	Wortmannin, 200 nM	0.41 (0.31-0.50)	0.0014 <sup>a</sup>	3
+	Wortmannin, 200 nM	0.80 (0.73-0.88)	0.9962 <sup>a</sup> /0.0021 <sup>b</sup>	3
–	U0126, 10 $\mu$ M	1.21 (1.17-1.25)	0.0022 <sup>a</sup>	3
+	U0126, 10 $\mu$ M	2.14 (1.18-3.10)	0.0180 <sup>a</sup> /0.8556 <sup>b</sup>	3

<sup>a</sup> vs untreated cells; <sup>b</sup> vs insulin, 10nM

**Table 3:** Regulation of 2-DG uptake by insulin in normal and OA chondrocytes

Relative 2-DG uptake (% of untreated cells)						
	Normal			OA		
Insulin, nM	Mean (95% CI)	P	N	Mean (95% CI)	P	N
1	125 (118-132)	1.16e-05 <sup>a</sup>	16	112 (107-117)	0.0049 <sup>a</sup>	8
10	141 (134-147)	5.78e-10 <sup>a</sup> /0.0084 <sup>b</sup>	18/12	123 (118-129)	3.8e-07 <sup>a</sup> /0.0511 <sup>b</sup>	16/4

<sup>a</sup> vs untreated cells; <sup>b</sup> vs insulin, 1 nM



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Figure 2

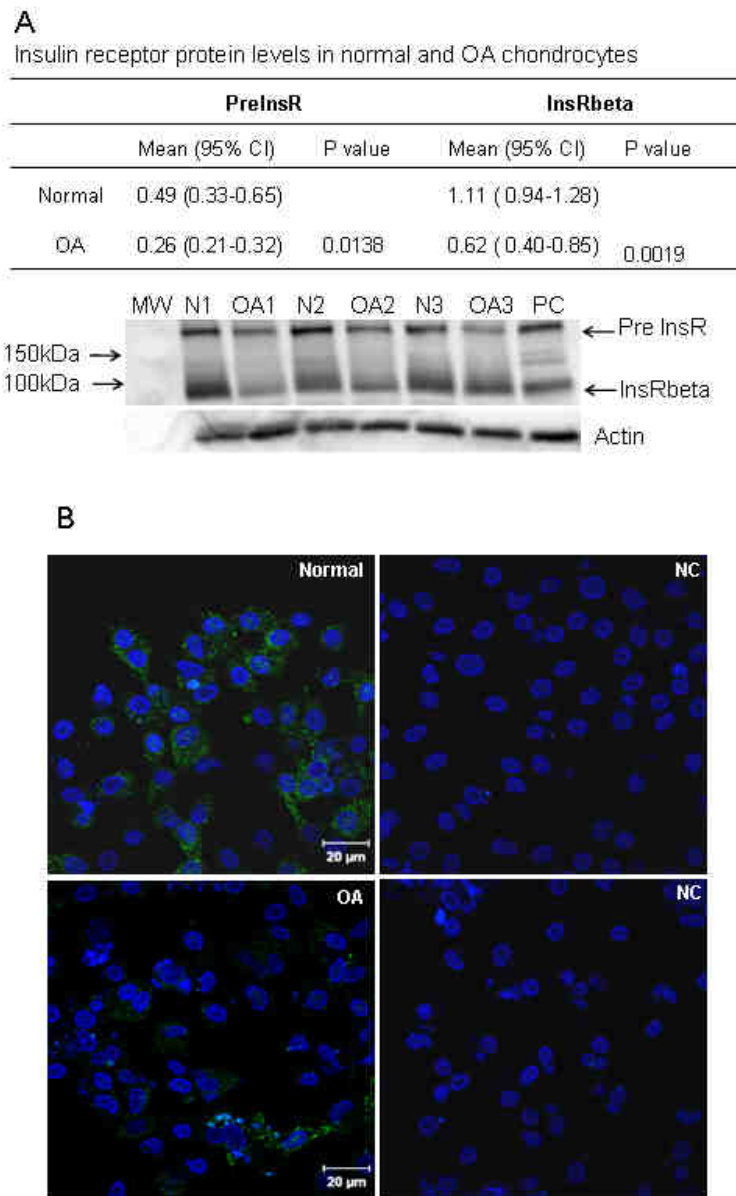




Figure 3

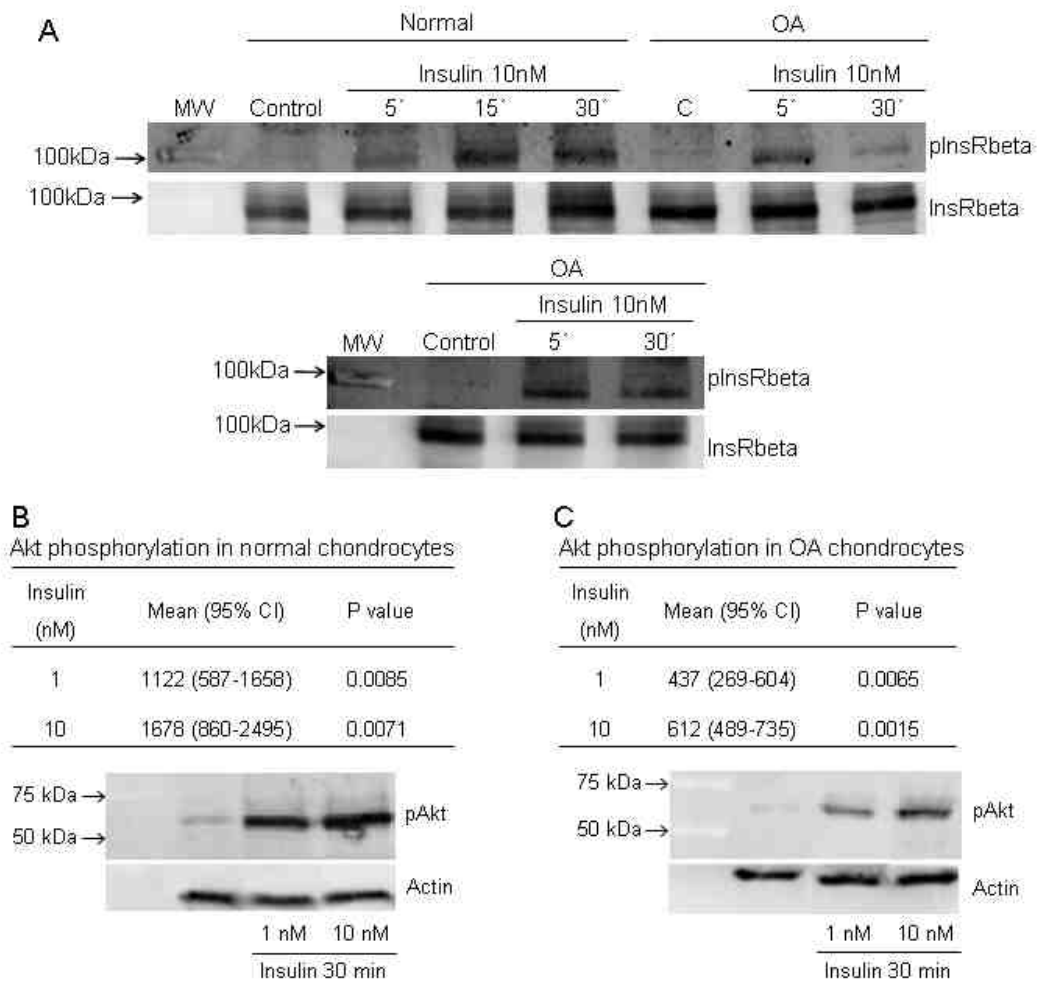


Figure 4

