**Protein profiles of Akt, STAT-3, NF-κB, and TLR4 in human chondrosarcoma cells: Potential therapeutic targets of insulin signaling pathway**

Sumeyya Akyol1,2, Sevda Yuksel3, Mehmet Akif Gulec4, Semsettin Sahin2, Kadir Demircan1, Omer Akyol5, Huseyin Ozyurt2

**ABSTRACT**

**Objectives:** Currently there is no effective chemotherapy for chondrosarcoma. Recent studies report that mesenchymal chondrosarcoma is relatively resistant to radiotherapy but sensitive to chemotherapy in some extend. It is unknown whether receptor tyrosine kinase is activated in chondrosarcoma. Potential new systemic treatment targets have been widely investigated. This study aimed to determine insulin-induced phosphorylation rate of signal proteins Akt, STAT-3, NF-κB and TLR4 by using Western Blot technique to find out a possible therapeutic target for human chondrosarcoma.

**Methods:** Human chondrosarcoma cells (OUMS-27) were induced by 10 μmol/mL insulin for 60 min. The first group was untreated control group, while the others were insulin-induced groups for 10, 30, and 60 min by applying same amount of insulin. After the induction periods, cells were harvested and protein extractions were performed. Phosphorylated and unphosphorylated individual protein levels were detected.

**Results:** Both pSTAT-3/STAT-3 and pNF-κB/NF-κB ratios were found to be remarkably increased (almost 3-fold) in 60 min group (almost 3-fold) compared those of controls.

**Conclusions:** The mechanism of insulin action in OUMS-27 chondrosarcoma cell lines and other human chondrosarcomas has not yet been illuminated. According to our findings, STAT-3 and/or NF-κB could be intracellular molecules that transmit the message of insulin to inside of OUMS-27 cells. Especially the fact that the phosphorylated forms of these proteins increase 3 times after 60 minutes of the insulin induction supports our perspective. These signaling molecules might be considered as potential targets of effective chemotherapy alternatives.

**KEY WORDS:** OUMS-27 cells; chondrosarcoma; insulin; Akt; STAT-3; NF-κB; TLR4

---

**INTRODUCTION**

Insulin has effects on both anabolic and catabolic pathways of adult chondrocytes, in this regard, it induces anabolic responses and inhibits catabolic responses. It also play significant roles in chondrogenesis by inducing chondrocyte differentiation [1]. Furthermore, insulin was demonstrated to ameliorate cartilage degeneration in osteoarthritis which shows its anabolic properties [2]. Rat chondrosarcoma chondrocytes are insulin- and IGF-1-dependent tumors. This tumor, which can be transplanted in vivo, is shrunken significantly in hypophysectomized rats and also shows a distinct reduction in streptozotocin-induced diabetic rats [3]. When insulin is introduced into Swarm rat chondrosarcoma (SRC) chondrocyte cells, the insulin receptors has been increased and this is not interpreted as an increase in the de novo receptor synthesis or a translocation of other intracellular receptors located in the cell compartments to the cell surface but most likely as a decrease in the rate of a falling receptor [4]. Chondrocytes has an ability to form an extracellular matrix (ECM) by synthesizing and secreting huge amount of macromolecules. SRC chondrocytes respond in a very special way to physiological concentrations of porcine insulin at in vitro conditions and produce plenty of secretaur proteins, collagen (type 2), hyaluronate, and cartilage-like proteoglycans [5-8].

Although clinical and experimental evidence support the growth-promoting properties of insulin [9,10], it is not yet known whether insulin might affect glucose transportation to and from chondrocytes because the mission of the very well-known function for insulin in target tissues [11]. In this regard, insulin was shown to increase glucose transport in most of the cells by promoting the expression and plasma membrane incorporation of GLUT proteins [12,15]. The effect of insulin on the proliferation of chondrocytes in the growth plate and the differentiation is investigated by targeted silencing of the expression of insulin and IGF-1 receptors. Consequently, it is found in the mice feeding of high-fat diet that insulin resistance and accelerated skeleton growth are related. During in vitro experiments it was shown that insulin, itself, directly modulates the skeleton growth in the growth plate by activating the insulin receptors. However, due to the fact that insulin-like growth factor receptor resembles insulin receptor functionally and structurally, insulin can also binds to this receptor and induce some effects [14]. Lately, the effect of insulin on
the enzymes that are very active on extracellular matrix structure was widely investigated [15-19].

How insulin affects the cartilage formation and resorption, which is one of the important stages of cell proliferation in in vitro and in vivo conditions, is an unsearched and untouched area. This topic is important because if the signaling pathway in chondrocytes is very well-known for insulin, some new chemotherapeutic approaches might be possible for chondrosarcomas as well. The main purpose of this cell culture study on OUMS-27 cell line is to examine signaling pathways that insulin activates and to try to find molecular targeted therapy for the treatment of chondrosarcoma. This topic is not investigated until today and so we investigated insulin-induced phosphorylation rate of signal proteins, Akt, STAT-3, NF-κB and TLR4 by Western Blot technique.

MATERIALS AND METHODS

OUMS-27 cell culture: Dulbecco’s modified Eagle’s medium (DMEM) (added fetal bovine serum 10% plus penicillin/streptomycin) was used to culture OUMS-27 cells in physical conditions adjusted to 37 °C and humidified air containing within 5% CO2. Every 7-10 days, OUMS-27 cells were subcultured by using trypsin and EDTA (split ratio was 1:2-1:4). All experiments were performed with cells at passages from 7 to 14. The medium of control and study groups was changed every second days. All the cells were attached to the bottom and expanded up to the edge of the dishes. It took 5 days to get this result to start insulin induction.

Commercial lyophilised insulin was dissolved within 0.01N HCl to prepare stock solution in a concentration of 2 mg/dl. Working insulin solution was prepared from this stock solution to get 10 μg/mL in culture medium. In the experiments, the incubation time of cells with insulin was 10 min to 60 min. Four groups of cells (contains 6 of 20-mm wells in each plate) were subjected to insulin except control cells: First group of cells (control) subjected to insulin and insulin was removed after 10 minutes. Second group of cells was exposed to insulin for 10 min and then insulin was removed. Third and fourth groups were exposed to same amount of insulin for 30 and 60 min, respectively. All the groups were harvested at the end of whole procedure and protein isolations were performed.

Protein isolation and Western blot: Anti-Akt, Anti-pAkt, Anti-STAT-3, Anti-pSTAT-3, Anti-NF-κB, Anti-pNF-κB, Anti-TLR4, and Anti-GAPDH primary antibodies were purchased from Santa Cruz (SC Biotechnology, Inc., CA, USA). The dilutions of antibodies were given in Table 1. Phosphorylated antibody for TLR4 was not commercially available. OUMS-27 cells were scraped from the plates following one-wash step with phosphate-buffered saline (PBS). Then CellLytic M solution (Sigma Aldrich, St. Louis, MO) including protease and phosphatase inhibitor mixture was used to lyze cells. Supernatant was obtained by sequential incubation (4°C for 15 min) and centrifugation (12,000g). This supernatant was used to determine total protein concentration by a detection kit (Bradford Assay Kit, Thermo Fisher Scientific, Boston, MA) with a protein standard (bovine serum albumin, BSA). The binding of protein molecules to Coomassie dye under acidic condition results in a color change in the principle of the assay. The presence of basic amino acid residues such as arginine, lysine and histidine contributes to formation of protein-dye complex. Afterwards, the samples were subjected to Laemmli sample buffer (BioRad, Hercules, CA) and β-mercaptoethanol within boiling water at 95°C for 5 min in order to get easy-reactant proteins to antibodies. Total of 10 μL samples and the same amount of protein marker (Precision Plus Protein Western C, BioRad, Hercules, CA) were loaded to the ready-to-use gels (BioRad Mini-PROTEAN TGX Stain-Free Gels, 4-15%, Herkules, CA) in a running buffer (1XTris/Glycerine/SDS, BioRad, Hercules, CA) within Western box adjusted to 250 V for 20 min. BioRad Trans-Blot Turbo Transer System (Singapore) was used to transfer proteins onto polyvinylidene-difluoride-membrane (BioRad Trans-Blot Turbo Transfer Pack, 0.22 μM PVDF, Herkules, CA) right after electrophoresis. Blocking of membranes was achieved within nonfat dried skim milk (2.5%) prepared within TBS containing Tween 20 (0.05%). Primary antibodies Anti-Akt, Anti-pAkt, Anti-STAT-3, Anti-pSTAT-3, Anti-NF-κB, Anti-pNF-κB, Anti-TLR4, and Anti-GAPDH (Table 1) (diluted within blocking buffer) were applied to the membranes overnight. Membranes were incubated with corresponding secondary antibodies (Table 1) at room temperature for 1 h after they were washed three times (8 min each) with TBS with Tween-20. Immunoactive bands onto membranes were visualized by an enhanced chemiluminescence system (BioRad Immun-Star Western Kit, Herkules, CA) for 90 sec follow-up period just after washing membranes with

Table 1. The primary and secondary antibodies used in Western blotting tests for signaling pathways and their technical details (Ab: Antibody, kDa: kilo dalton)

<table>
<thead>
<tr>
<th>No</th>
<th>Primary Ab</th>
<th>Concentration</th>
<th>Secondary Ab</th>
<th>Concentration</th>
<th>Reaction</th>
<th>kDa</th>
<th>Catalogue no</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Akt</td>
<td>1/1000</td>
<td>Rabbit</td>
<td>1/4000</td>
<td>Mouse, Rat, Human</td>
<td>62</td>
<td>sc-5298</td>
</tr>
<tr>
<td>2</td>
<td>pAkt</td>
<td>1/1000</td>
<td>Rabbit</td>
<td>1/4000</td>
<td>Mouse, Rat, Human</td>
<td>62</td>
<td>sc-135650</td>
</tr>
<tr>
<td>3</td>
<td>STAT-3</td>
<td>1/1000</td>
<td>Rabbit</td>
<td>1/4000</td>
<td>Mouse, Rat, Human</td>
<td>86</td>
<td>sc-482</td>
</tr>
<tr>
<td>4</td>
<td>pSTAT-3</td>
<td>1/1000</td>
<td>Rabbit</td>
<td>1/4000</td>
<td>Mouse, Rat, Human</td>
<td>86</td>
<td>sc-8059</td>
</tr>
<tr>
<td>5</td>
<td>NF-κB</td>
<td>1/1000</td>
<td>Rabbit</td>
<td>1/4000</td>
<td>Mouse, Rat, Human</td>
<td>65</td>
<td>sc-372</td>
</tr>
<tr>
<td>6</td>
<td>pNF-κB</td>
<td>1/1000</td>
<td>Rabbit</td>
<td>1/4000</td>
<td>Mouse, Rat, Human</td>
<td>65</td>
<td>sc-33020</td>
</tr>
<tr>
<td>7</td>
<td>TLR4</td>
<td>1/1000</td>
<td>Rabbit</td>
<td>1/4000</td>
<td>Mouse, Rat, Human</td>
<td>95</td>
<td>sc-10741</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Internal control</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 GAPDH</td>
</tr>
<tr>
<td>1/10000-1/500000</td>
</tr>
<tr>
<td>1/4000</td>
</tr>
<tr>
<td>36</td>
</tr>
</tbody>
</table>
TBS with Tween-20.

Signals were detected with BioRad ChemiDoc MP Imaging System (Singapore), and the densitometry was performed with Image J software (W. Rasband, Research Services Branch, NIMH, NIH) and normalized to the signal intensity of GAPDH for equal protein loading control of each sample in each experiment. This quantification was performed with the linear range of the standard curve defined by the standard sample, GAPDH, for all densitometry analysis. The expression ratio of proteins was calculated by dividing phosphorylated antibody results to normal antibody results to get an arbitrary unit.

RESULTS

This study has been planned to understand the mechanism of insulin signaling pathway(s) in cultured OUMS-27 cells in vitro. Western blot analysis of signaling proteins using antibodies which recognize specific proteins of several signaling pathways was performed. We basically examined four different signaling pathways in order to find out the possible signaling pathway that is used by insulin in OUMS-27 cells. Because we are unable to find phosphorylated TLR4 antibody, only unphosphorylated protein form was investigated. As usual, GAPDH was used to compare all phosphorylated and unphosphorylated proteins.

The protein was expressed equally in all situations. Signaling pathway Akt gave a band on 62 kDa after treated with related antibody (Figure 1). While the control and 60 min bands are hardly seen in Western membranes, 10 min and 30 min bands were quite distinctive. In case of pAkt, control and 60 min bands were getting more evident. 60 min band was wilted. 10 and 30 min bands became apparent in the membrane. After the band densities were normalized by GAPDH band densities, pAkt/Akt ratio was found to be decreased in 10, 30, and 60 min experiments compared control (Figure 5). It was apparent that insulin led Akt protein to be unphosphorylated.

The bands were seen at about 86 kDa for both phosphorylated and unphosphorylated proteins of STAT-3 (Figure 2) as anticipated. It was obvious that STAT-3 protein was expressed in all groups of conditions (10, 30, and 60). The bands were rather pale in control and 60 min groups, being more pale in 60 min group compared to control group. Regarding pSTAT-3 bands, it was noticed that 60 min bands were more prominent. 10 and 30 min bands were decreased in phosphorylated proteins compared to unphosphorylated ones. After the density results were normalized by GAPDH results, pSTAT-3/STAT-3 ratio were found to be decreased in 30 min group but remarkably increased in 60 min group (almost 3-fold) (Figure 5). The increased phosphorylation of protein STAT-3 induced by insulin was observed around 60 min, not 10 min or 30 min.

Figure 1. The Western blot results of the phosphorylated and unphosphorylated forms of signaling pathway Akt protein in OUMS-27 cells induced by insulin (WB: Western blot, kDa: kilo dalton, M: marker).

Figure 2. The Western blot results of the phosphorylated and unphosphorylated forms of signaling pathway STAT-3 protein in OUMS-27 cells induced by insulin (WB: Western blot, kDa: kilo dalton, M: marker).

Figure 3. The Western blot results of the phosphorylated and unphosphorylated forms of signaling pathway NF-kappaB protein in OUMS-27 cells induced by insulin (WB: Western blot, kDa: kilo dalton, M: marker).
The bands were detected around 65 kDa for both phosphorylated and unphosphorylated proteins of NF-κB (Figure 3), which was already expected based on commercial instruction. NF-κB was expressed in all groups. The bands were found to be pale in control and 60 min groups. Regarding pNF-κB bands at 60 min were distinctive but control and other bands were considerably pale. After the density results were normalized by GAPDH results (Figure 5), pNF-κB/NF-κB ratios were found to be similar and depressed compared to control in 10 and 30 min groups (50% of control values) whereas the ratio was increased almost 3-fold compared to control group. The increased phosphorylation of protein NF-κB induced by insulin was seen around 60 min, not 10 min or 30 min groups.

The bands were detected around 95 kDa for unphosphorylated proteins of TLR4 (Figure 4) as it was expected from commercial prospectus. When looking at TLR4 bands, it was realized that 60 min bands were more prominent. After the density results were normalized by GAPDH results (Figure 5), unphosphorylated protein amount was found to be increased almost two-fold at 60 min compared to control group. Because we were unable to obtain commercial pTLR4 antibodies for our experiment, phosphorylated and unphosphorylated proteins of TLR4 could not be compared.

DISCUSSION

Cartilage and bone can grow abnormally in chondrosarcoma, which is known as a malignant cancer. Currently there is no effective chemotherapy for chondrosarcoma. Optimal therapy regimens for low-grade chondrosarcoma remain a dilemma. In case of higher-grade tumors, adjuvant therapies might be considered. Recent studies report that chondrosarcoma is relatively radiotherapy resistant. However, chemotherapy has some beneficial effects in mesenchymal chondrosarcoma. It has been suggested that
this type of chondrosarcoma may give a good answer to the aggressive surgical removal in addition to chemoradiotherapy and reduce recurrence risk [20]. It is unknown whether receptor tyrosine kinase is activated in chondrosarcoma. Potential new systemic treatment targets are very important to find effective chemotherapy alternatives. When insulin has been applied into chondrocyte cells as in our study, the levels/activity of these proteins have been decreasing and the removal of the proteoglycan groups have been slowing down. Diabetes increases the number of apoptotic cells and insulin therapy provides these apoptotic cells to return to a normal level. A loss of chondrocytes is considered to be physiologically significant because the death of chondrocyte cells triggers the production of several signals, which stimulates cartilage resorption.

Despite the fact that there are some studies that focused on the effect of insulin on chondrocytes, [21] only few studies have been found about insulin application in chondrosarcoma cell lines in mice besides human, where some signaling pathways and aggrecan degradation has been compared. Mouse embryonal carcinoma obtained from ATDC5 cells needs insulin or IGF-1 for differentiation [22,23]. If the cultural environment is supplemented with insulin or IGF-1, it leads to a typical cell condensation and then to chondrogenesis. It is claimed that PI3K/PKB (Akt) plays an important role in IGF-1/insulin-induced chondrogenesis [24]. Because insulin/IGF-1 stimulation causes PI3K production in several types of cells [25]. Again, it was demonstrated in previous studies that when vanadate is added to the cultural environment as an tyrosine phosphatase inhibitor, it has become the reason for chondrocyte differentiation in the rabbit rib cage [26]. Tyrosin residues of insulin needs to be phosphorylated for its stimulation which in turn leads activation of PI3K followed by PI3 production and then PI3 production [27]. It has been investigated whether insulin activates the stimulation of PI3K/PKB pathway of chondrocytes in ATDC5 cells [24]. In the same study, it has also been investigated whether the conversion of ATDC5 cells to chondrocytes after the insulin induction takes place only after the activation of PKB pathway [24]. It has been found out that PI3K/PKB signaling pathway takes an active role in chondrogenic differentiation as an answer to insulin/IGF-1 in ATDC5 cells. When we compare the present study and other studies, we can say that our findings about signaling pathways can be characterized as original. The mechanism of insulin action in OUMS-27 chondrosarcoma cell lines and other human chondrosarcomas has not yet been investigated. According to our findings, STAT-3 and/or NF-kB could be intracellular molecules that transmit the message of insulin inside the cell. Especially the fact that the phosphorylated forms of these proteins increase 3 times after 60 minutes of the insulin induction supports our perspective. The signaling pathway activation seen after 60 minutes of insulin induction to the OUMS-27 cells and the predicted mechanisms that take place starting at the cell membrane is representatively shown in Figure 6.

When insulin is induced into SRC chondrocyte cells, the insulin receptors has increased and this is not interpreted as an increase in the de novo receptor synthesis or a translocation of other intracellular receptors located in the cell compartments to the cell surface but most likely as a decrease in the rate of receptor degradation [4]. Experiments with human recombinant insulin by ruling and all possibilities have shown that increased proteoglycan synthesis is entirely caused by insulin itself [4]. The most important extracellular cartilage compound that chondrocyte cells produce and secrete is aggrecan which is a proteoglycan and gives the cartilage features such as strength against the pressure force, dynamic weight-bearing function.
and an osmotic feature [19]. It has extremely hydrophilic properties that consists of glycosaminoglycan chains (100 chondroitin sulfate and 30 keratin sulfate residues) [28]. The breaking down of the aggrecan is one of the important indicators of osteoarthritis. In the analysis of synovial fluid of arthritis patients, it has been shown that the aggrecan is pathologically cut from the aggrecanase cutting field [29]. This extracellular environment is also important for treatment of chondrosarcoma in cartilage tissue. Therefore, molecular targeted therapy modalities for insulin signaling pathway in OUMS-27 cells might lead to shutdown this kind of anabolic pathways and give an opportunity to stop/slow down the metastasis and tumor growth.

As a conclusion, our results show for the first time that insulin affects chondrosarcoma cells most probably through STAT-3 and NF-κB signal proteins. Analyses regarding determination of signal pathway triggered by insulin in human chondrosarcoma cells revealed a high rate of phosphorylation of STAT-3 and NF-κB proteins for 60 min, which might indicate one or both of these as possible pathways. These molecular signal pathways might be considered as potential targets for effective chemotherapy alternatives in chondrosarcoma. New studies with new cell lines and alternative signaling pathways are needed to illuminate the subject in all aspects.

ACKNOWLEDGEMENT
Authors would like to thank to Dr. T. Kunisada from Graduate School of Medicine and Dentistry of Okayama University, Okayama, Japan who provided human chondrosarcoma (OUMS-27) as a gift.

CONFLICT OF INTEREST STATEMENT
There is no conflict of interest of authors.

REFERENCES