



Regulation of Glucose Transporter 1 by IL-1 β Stimulation in Rat Articular Chondrocytes

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Background: The facilitative glucose transporters (GLUTs) are essential for chondrocyte survival and production of glycosaminoglycan. The objective of this study was to examine the glucose transporter 1 (GLUT1) expression in rat articular chondrocytes and its regulation in response to IL-1 β stimulation. **Methods:** Primary cultures of normal articular chondrocytes were isolated from rat knee joints. GLUT1 expression and regulation in the presence or absence of IL-1 β was evaluated by reverse transcription-polymerase chain reaction (RT-PCR), western blotting, and immunocytochemistry. SN50 peptide was used to inhibit nuclear factor-kappa B (NF-kB) activation. **Results:** IL-1 β stimulation upregulated GLUT1 mRNA and protein levels in a time dependent manner. The gene upregulation and protein expression of GLUT1 induced by IL-1 β were suppressed in the presence of SN50 ($P < 0.05$, in gene expression). **Conclusion:** GLUT1 upregulation may be associated with NF-kB activation, in rat articular chondrocytes following IL-1 β stimulation. Whether the expression of GLUT1 is related with the pathogenesis of osteoarthritis merits further investigation.

Key words: Glucose transporter, Chondrocyte, IL-1 β , Nuclear factor-kB.

INTRODUCTION

The extracellular matrix of articular cartilage, synthesized by chondrocytes, is comprised predominantly from collagen and aggrecan. Collagen fibers have the capacity to withstand tensile and shear forces. Aggrecan is a unique proteoglycan aggregate with glycosaminoglycan (GAG) chains. Glucose uptake by chondrocytes is essential not only for cellular energy but also for as the basis for glucosamine sulfate and other sulfated sugars required to biosynthesize of GAG¹⁻⁵. Chondrocytes have been shown to express several types of glucose transporters to maintain chondrocyte function⁶⁻⁸. Recent evidences have suggested that cartilage matrix degeneration, a characteristic feature of osteoarthritis, may be associated with metabolic glucose imbalance⁶.

Two major families of glucose transporters, the concentrative Na⁺/glucose transporters (SGLTs) and facilitative

glucose transporters (GLUTs), have been discovered^{9,10}. SGLTs are energized by H⁺ or Na⁺ gradients and are essential for cell survival. GLUTs facilitate passive diffusion of glucose across membranes by energy-independent stereo-specific mechanisms. The expression and distribution of glucose transporters is not constant in various tissues and throughout development as well^{6,11}. Fourteen members of the GLUT family have been cloned in humans¹²⁻¹³. The expression of GLUT isoforms is developmentally regulated in many tissues and organs. Normal mature articular chondrocytes express multiple GLUT isoforms (GLUT1, 3, 5, 6, 8, 9, 10, 11 and 12)¹⁴⁻¹⁷. GLUT4 is expressed in growth plate chondrocytes⁶. Although the reason for such GLUT isoform diversity in chondrocytes has not been fully examined, the presence of GLUT1 has been linked to the acute requirement of chondrocytes for glycolytic energy metabolism under the low oxygen tension conditions that are prevalent in avascular load-bearing articular cartilage¹⁴. GLUT1 has also been shown to be an IL-1 β inducible glucose transporter in cartilage¹⁸. However, the detailed signal pathway still needs to be determined. In this study we first examined the in vitro system and identified the increased expression of GLUT1 in rat articular chondrocytes following IL-1 β stimulation. The stimulator IL-1 β is a major cytokine which causes the cartilage matrix degradation. The signal transduction path-

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way of GLUT1 upregulation by IL-1 β in the normal rat articular chondrocytes was also investigated^{19,20}.

METHODS

Chondrocyte isolation and culture

Male Sprague Dawley (SD) rats (8-week-old, 300 g) were obtained from BioLASCO Taiwan Co., Ltd (Taiwan). All experiments were approved by the local Institutional Review Board and performed in adherence to the National Institutes of Health Guidelines for the treatment of laboratory animals. The cartilage of knee joints was aseptically removed, cut into small fragments, incubated with antimicrobial solution (500 IU/mL penicillin/streptomycin, Gibco Invitrogen, Carlsbad, CA, USA) for 3 hours and washed with sterile phosphate-buffered saline (PBS). Cartilage tissue was then incubated with 0.25% trypsin (Gibco Invitrogen) at 37°C for 30 min, washed with PBS, and then digested with 3 mg/ml collagenase type H (Sigma, St Louis, MO, USA) at 37°C for 12 hours. The resultant cell suspension was centrifuged at 800 $\times$ g for 10 min, following which the supernatant was discarded and the pellet resuspended in PBS, and finally filtered with a 100 μ m cell strainer (BD Biosciences Falcon, Franklin Lakes, NJ, USA). After further centrifugation at 800 $\times$ g for 10 min, the cell pellet was resuspended and seeded in 10 ml of DMEM-F12 medium (Sigma) containing 10% fetal bovine serum (Sigma) and 100 μ l/ml penicillin/streptomycin (Gibco Invitrogen). The chondrocytes were then cultured in a humidified 5% CO₂ atmosphere at 37°C until confluent, detached with 0.05% trypsin-EDTA (Gibco Invitrogen) and seeded at a density of 5 $\times$ 10⁵ cells/dish in 60 mm tissue culture dishes (Orange Scientific, Braine-l'Alleud, Belgium) for further experimental procedures.

Chondrocyte stimulation

Dishes of chondrocytes were placed in serum-free media (3 ml) overnight and then treated with or without 10 ng/ml IL-1 β (R&D Systems, Minneapolis, MN, USA) in a 95% air/5% CO₂ incubator at 37°C for defined periods. SN50 (BIOMOL Research Laboratory Inc. Philadelphia, USA), a cell-permeable inhibitory peptide bearing the nuclear localization sequence (NLS) of nuclear factor kappaB (NF- κ B) p50 subunit was used as an NF- κ B inhibitor. The chondrocytes were incubated with SN50 at a concentration of 100 μ g/ml for 2 hours.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells using easy-BLUE™

Total RNA Extraction Kit (iNtRON Biotechnology, Gyeonggi-do, Korea). For first strand cDNA synthesis, 3 μ g of total RNA was used in a single-round RT reaction (total volume of 20 μ L), containing 0.75 μ g Oligo(dT)₁₄ primer, 1 mM dNTPs, 1 $\times$ first strand buffer, 0.4 mM DTT, 40 units RNaseOut recombinant ribonuclease inhibitor, and 200 units of superscript II reverse transcriptase (all from Gibco Invitrogen). RT reaction was performed at 42°C for 2 hours, followed by 95°C for 5 min. PCR was carried out using 0.9 μ l of the RT reaction mixture as template, 0.4 mM of gene specific primers, 1 $\times$ PCR buffer, 0.25 mM dNTPs, and 1.5 units of Taq DNA polymerase (BioMan, Taipei, Taiwan). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplification was carried out at 94°C for 2 min, then for 25 cycles at 94°C for 45 s, 56°C for 1 min, and 72°C for 45 s followed by a final extension at 72°C for 10 min. GLUT1 cDNA was amplified for 94°C for 2 min, then for 25 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1.5 min followed by a final extension at 72°C for 10 min. All PCR products were size-fractionated by a 1.5% agarose gel electrophoresis, and DNA bands were visualized by staining the gel with 0.1 μ g/ml ethidium bromide. The primers were as follows: GLUT1 5'-GCCTGAGACCAGTTGAAAGAAC-3' (sense) and 5'-CTGCTTAGGTAAAGTTACAG GAC-3' (antisense) (Gene bank accession number BC061873; PCR product 291 bp); GAPDH 5'-CCCATCACCATCTTCCAGGAG-3' (sense) and 5'-GTTGTCATGGATGACCTTGGC C-3' (antisense) (Gene bank accession number X02231; PCR product 284 bp).

Protein extraction and western blotting

Chondrocytes were washed with ice-cold PBS containing 100 μ M Na₃VO₄ (Sigma) and lysed in situ with ice-cold lysis buffer at 4°C for 15 min. Lysis buffer was prepared freshly and contained 1% Igepal (Sigma), 100 μ M Na₃VO₄, and a protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany). Whole cell lysates were collected after centrifugation at 10,000 $\times$ g for 15 min. Protein concentration was determined by the Lowry method. Equal amounts of protein (20 μ g) were electrophoresed on a 10% SDS-polyacrylamide gel and then were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore Immobilon-P, Sigma). The membranes were blocked overnight at 4°C with 2% BSA in TBST (12.5 mM Tris/HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20). After washing 3 times with TBST, PVDF membranes were incubated for 1 h at room temperature with primary antibody (1/1000 dilution) diluted with 2% BSA in TBST. After washing 6 times with TBST, the blots were then incubated with horseradish

peroxidase-labelled secondary antibody (1/2000 dilution) for 1 hour at room temperature. Membranes were rewashed extensively and binding was detected using Enhanced Chemiluminescence Western blotting detection system (Amersham-Pharmacia Biotech, Piscataway, NJ, USA), according to the manufacturer's instructions. Mouse monoclonal antibody tubulin Ab-4 (Lab Vision Thermo Fisher Scientific, Fremont, CA, USA) served as internal control.

Immunocytochemistry

Cells on dishes were washed with ice-cold PBS and fixed with 1:1 methanol/acetone for the immunodetection of GLUT1 protein using a standard avidin-biotin-peroxidase complex detection kits (Dakocytomation, Glostrup, Denmark). Endogenous peroxidase activity and non-specific binding were blocked by incubation with 3% hydrogen peroxide and non-immune goat serum, respectively. The dishes were then incubated sequentially with primary antibody (1:100) for 60 min, biotinylated secondary antibody (1:200) for 30 min, and peroxidase-conjugated streptavidin for 20 min. The chromogen 3-amino-9-ethylcarbazole (AEC) was used to localize positive staining by light microscopy. Cells were counterstained with hematoxylin.

Scanning system and data analysis

The bands on the agarose gel were analyzed with a gel documentation system (DOC-008, UVItect, Cambridge, UK) and calculated as the ratio of the intensity of the target gene band to that of the control GAPDH gene. The control condition was normalized to 1. The "fold" was defined as GLUT1/GAPDH and then the ratio value was compared with each other group. Variance and p values were analyzed by AlphaImage 1220 V5.5 (Alpha Innotech Corporation, San Leandro, CA, USA). A Student t-test was used for statistical comparison between groups. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Upregulation of GLUT1 by IL-1 β stimulation in articular chondrocytes

To ascertain whether GLUT1 expression was associated with chondrocyte insult during disease progression, normal articular chondrocytes were isolated and cultured from rat knee joint cartilage. Cells without IL-1 β stimulation for 3, 6, 12, and 24 hours showed no changes in

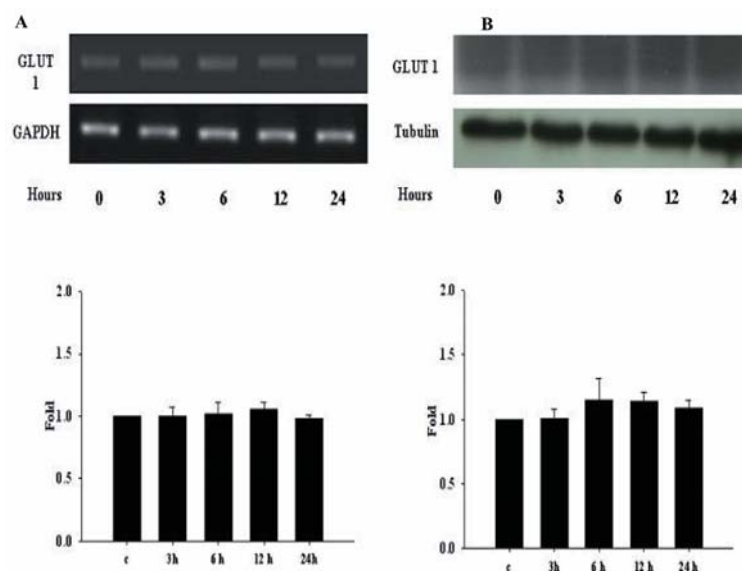


Fig. 1 GLUT1 gene alteration without IL-1 β stimulation. (A) A representative gel shows no increase of GLUT1 gene expression. (B) A representative blot shows no increase of GLUT1 protein. The semi-quantitative data showed no significant change. (n=3, *P* > 0.05)

mRNA and protein levels of GLUT1 (Fig. 1). Cells were then incubated with IL-1 β for 2, 4, 6, 12, and 24 hours to investigate the GLUT1 gene alteration. IL-1 β caused GLUT1 gene upregulation in a time course dependent manner. A significant increase in GLUT1 gene expression over basal conditions was seen at 4 hours (1.8 ± 0.6 -fold increase), 6 hours (2.58 ± 1.26 -fold increase), 12 hours (2.88 ± 1.4 -fold increase), and 24 hours (3.22 ± 1.78 -fold increase) incubation (*P* < 0.05) (Fig. 2). The expression of GLUT1 protein by western blotting showed a pattern similar to that of mRNA expression (Fig. 3A). Cells were incubated with IL-1 β for 24 hours and then subjected to fixation and immunocytochemistry. Increased staining intensity in cells with IL-1 β stimulation was identified (Fig. 3B). Cells without IL-1 β stimulation showed no increase of the staining intensity of GLUT1 protein over 24 hours (data not shown), consistent with the data from western blotting.

NF- κ B activation is associated with GLUT1 upregulation

To assess whether increased expression of GLUT1 was associated with NF- κ B activation, NF- κ B was inhibited with SN50. Cells on dishes were pre-treated with SN50 at a concentration of 100 μ g/ml for 2 hours. The gene upregulation and protein expression of GLUT1 induced by IL-1 β were suppressed in the presence of SN50 (Fig. 4A, *P* < 0.05; and Fig. 4B).

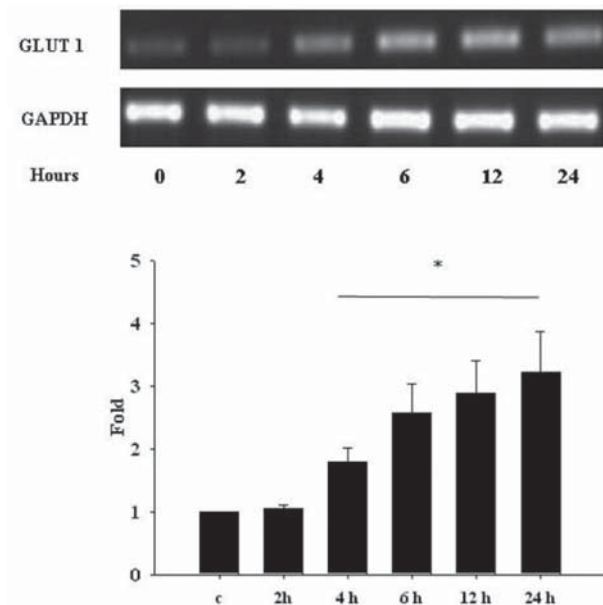


Fig. 2 GLUT1 gene upregulation following IL-1 β stimulation. GLUT1 gene expression showed significant upregulation at 4, 6, 12, and 24 hours. (n=6, * P < 0.05)

DISCUSSION

The present study has demonstrated that IL-1 β stimulation induces GLUT1 gene and protein upregulation in normal rat articular chondrocytes. The signal transduction pathway may be associated with the activation of NF- κ B.

GLUT proteins are expressed in specific cells and tissues. They expressed their distinct kinetic and regulatory properties, involved in the basal glucose requirement and the lymphocyte functions and survival^{21,22}. Activation of T lymphocytes causes a large increase of GLUT1 expression and cell membrane localization via signal transducer and activator of transcription 5 (STAT5)-mediated activation of Akt⁵. GLUT1 gene expression, protein level and glucose transport activity by human adipocytes are markedly increased under hypoxic condition²³. Anaerobic glycolysis is the only pathway for a vertebrate cell to produce energy under anoxic conditions; this requires a large amount of glucose and glucose transport. Glucose transport becomes the rate-limiting step for glucose metabolism. GLUT1 is critical to neuronal survival during anoxic exposure²⁴. Cancer cells are also known to have accelerated metabolism with high glucose requirement. High levels of GLUT1 expression in breast and prostate cancer patients have been associated with poor survival²⁵.

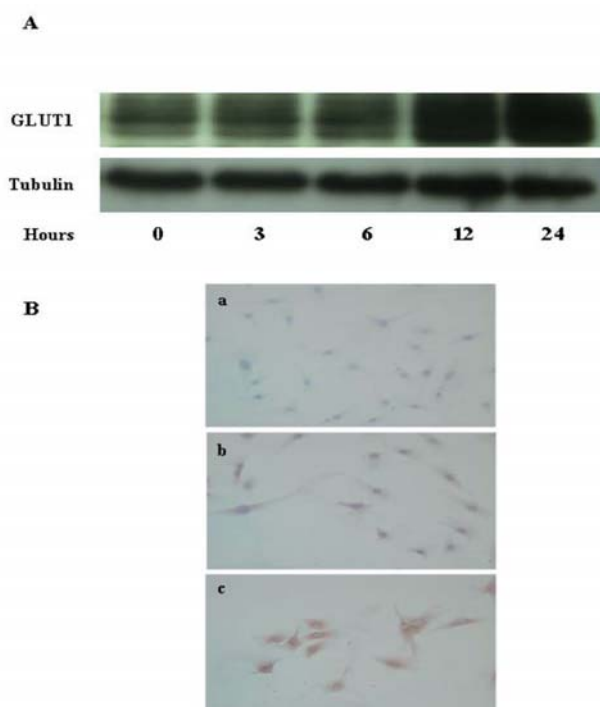


Fig. 3 Increased GLUT1 protein expression following IL-1 β stimulation. (A) A representative blotting shows increased GLUT1 protein following IL-1 β stimulation for 12 and 24 hours. (B) A representative immunocytochemistry image shows increased signal of GLUT1 protein expression. a. As a staining control, cells were reacted only with secondary antibody. b. Cells without IL-1 β stimulation were immunostained with anti-GLUT1 antibody. c. Cells with IL-1 β stimulation were immunostained with anti-GLUT1 antibody. (n=3)

By immunohistochemistry, it has been observed that osteoarthritic chondrocytes have increased GLUT1 protein levels which may be associated with the energy demand for cartilage repair. Our results demonstrated that the expression of GLUT1 could be induced by IL-1 β , consistent with the previous study⁶.

SN50, a synthetic peptide, contains a cell membrane-permeation motif and a nuclear localization sequence (NLS). It could inhibit the translocation of the NF- κ B p50 subunit containing an NLS. It could inhibit IL-1 β -stimulated increases in MMP-2 and -9 expression and activity in adult rat cardiac fibroblasts²⁶. SN50 may prevent the TNF- α -induced increase in cytochrome p450 enzyme 7b (Cyp7b) activity in the fibroblast-like synoviocytes which are isolated from rheumatoid arthritis patients²⁷. It may also inhibit TNF- α -induced increases of RelA and tenascin-C expression in human osteoarthritic chondrocytes²⁸.

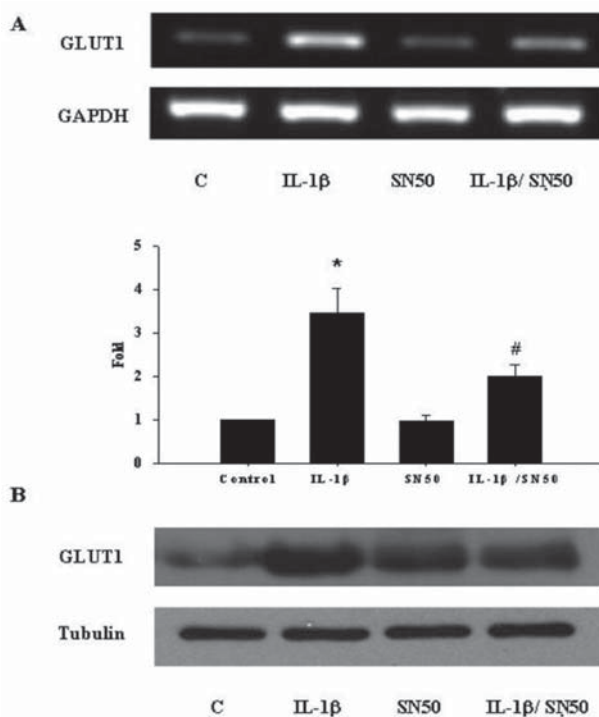


Fig. 4 GLUT1 upregulation associated with NF- κ B activation. (A) A representative agarose gel is shown. Treating with only SN50 showed no effect on GLUT1 gene expression. Significant suppression by SN50 was identified by the semi-quantitative analysis (* $P < 0.05$ for control vs. IL-1 β ; # $P < 0.05$ for IL-1 β vs. SN50+IL-1 β). (B) IL-1 β -induced GLUT1 protein upregulation was partially suppressed by SN50. A representative western blot is shown. (n=3)

Herein, we showed that SN50 could suppress the upregulation of GLUT1 in rat normal articular chondrocytes following IL-1 β stimulation. The promoter of GLUT1 gene has been demonstrated to contain a NF- κ B binding site²⁹. These evidences suggest that GLUT1 expression may be mediated by NF- κ B.

In conclusion, the upregulation of GLUT1 in articular chondrocytes, which was induced by IL-1 β , was partly suppressed by SN50. The results indicated that the IL-1 β -induced expression of GLUT1 may be associated with NF- κ B activation. Whether the expression of GLUT1 is related with the pathogenesis of osteoarthritis merits further investigation.

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