

Arg⁹⁷² insulin receptor substrate-1 enhances tumor necrosis factor- α -induced apoptosis in osteoblasts

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Abstract. The presence of Arg⁹⁷² insulin receptor substrate-1 (IRS-1) is associated with impaired insulin/IRS-1 signaling to activate phosphatidylinositol-3 kinase (PI3K). Tumor necrosis factor- α (TNF- α), an inflammatory cytokine with a central role in the pathogenesis of rheumatoid arthritis (RA), induces apoptosis in osteoblasts, which are the principal cell type responsible for bone loss in RA. In our previous study, an association between Arg⁹⁷² IRS-1 and a high risk and severity of RA was identified. In the present study, the effects of Arg⁹⁷² IRS-1 and IRS-1 on TNF- α -induced apoptosis in human osteoblasts were examined. Normal and RA osteoblasts were stably transfected with Arg⁹⁷² IRS-1 and IRS-1. In addition, cells were stably transduced with IRS-1-shRNA to knock down IRS-1. Following stimulation with 10 nM insulin for 30 min, the stable overexpression of Arg⁹⁷² IRS-1 and knock down of IRS-1 significantly decreased IRS-1-associated PI3K activity and Akt activation/phosphorylation at serine 473 (ser473) and enhanced TNF- α -induced apoptosis in normal and in RA osteoblasts. By contrast, the stable overexpression of IRS-1 significantly increased the levels of IRS-1-associated PI3K activity and Akt phosphorylation (ser473) and inhibited TNF- α -induced apoptosis, which was eliminated by pretreatment with 50 μ M BJM120, a selective PI3K inhibitor, for 30 min. In conclusion, the present study provided the first evidence, to the best of our knowledge, that insulin stimulation of Arg⁹⁷² IRS-1 and IRS-1 enhanced and inhibited TNF- α -induced apoptosis, respectively in normal and RA osteoblasts by a PI3K-dependent mechanism. These findings suggest that insulin/IRS-1 signaling is important in the pathogenesis of RA.

Introduction

The effects of insulin are mediated by the activation of a signaling pathway involving insulin receptor substrate-1 (IRS-1) and phosphatidylinositol-3 kinase (PI3K) (1,2). Previous studies have reported a common polymorphism (rs1801278) in the IRS-1 gene, in which a Gly/Arg substitution occurs at codon 972 (Arg⁹⁷²) (3,4). The presence of Arg⁹⁷² IRS-1 is associated with impaired insulin/IRS-1 signaling to activate PI3K (3,4). In our previous study, an association between Arg⁹⁷² IRS-1 and increased risk and severity of rheumatoid arthritis (RA), a chronic inflammatory disease with progressive joint destruction, was identified (5,6). However, the role of Arg⁹⁷² IRS-1 in the pathogenesis of RA remains to be elucidated.

RA is characterized by an imbalance in bone remodeling and bone loss (7). It is well established that osteoclasts are the principal type of cell responsible for bone loss in RA (7). Tumor necrosis factor- α (TNF- α), an inflammatory cytokine has been elevated in the synovial fluid and the synovium of patients with RA (8) and has been demonstrated to have a central role in the pathogenesis of RA (9). Substantial *in vitro* and *in vivo* evidence has suggested that TNF- α induces apoptosis in osteoblasts (10-13). Insulin/IRS-1 signaling reportedly activates the PI3K/Akt pathway, which is important in cell survival against apoptotic stress (14). Thus, in the present study, the effects of Arg⁹⁷² IRS-1 and IRS-1 on TNF- α -induced apoptosis in normal and RA osteoblasts were examined.

Materials and methods

Plasmids and reagents. A fragment of human genomic DNA containing the entire coding sequence of IRS-1 was cloned and ligated into a pcDNA 3.1 expression vector and the Arg⁹⁷² IRS-1 expression vector (Invitrogen Life Technologies, Carlsbad, CA, USA) was constructed, as previously described (4,15). The cDNA construct containing the Arg⁹⁷² substitution was generated by site-directed mutagenesis using polymerase chain reaction (PCR) with the wild-type IRS-1 as a template. The PCR fragment containing the codon 972 variant of IRS-1 was digested with *Bam*HI and *Nhe*I restriction endonucleases and inserted into pcDNA3-WT-IRS-1, which was previously digested with the same enzymes. The presence of the substitution and the entire sequence of the fragment inserted was confirmed by sequencing. SuperFect transfection reagent was purchased from

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Qiagen (Valencia, CA, USA). Anti- β -actin (cat. no. 8H10D10, 3700) antibody was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). IRS-1 (cat. no. sc-29376-V) short hairpin (sh)RNA lentiviral particles, control shRNA lentiviral particles-A (cat. no. sc-108080), selective PI3K inhibitor BKM120 (cat. no. sc-364437A) and rabbit anti-human polyclonal IRS-1 (C-20; cat. no. sc-559; 1:1,000 dilution), mouse anti-human monoclonal Akt (5C10) (cat. no. sc-81434; 1:1,000 dilution) and rabbit anti-human polyclonal phosphorlyated (phospho)-Akt [Serine 473 (ser473) cat. no. sc-101629; 1:1,000 dilution] antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The DeadEndTM Fluorometric terminal deoxynucleotidyl transferase mediated nick-end labeling (TUNEL) system was purchased from Promega (Madison, WI, USA). Recombinant human TNF- α , G418, puromycin and insulin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) was purchased from Life Technologies (Beijing, China).

Cell culture. Adult human osteoblasts, isolated from normal individuals (cat. no. 406-05a) and patients with RA (cat. no. 406RA-05a) were purchased from Cell Applications (San Diego, CA, USA). The cells were grown in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Sigma-Aldrich) at 37°C in a humidified, 5% CO₂ atmosphere. The normal and the RA osteoblasts were genotyped by sequencing and found to be wild type IRS-1 homozygotes.

Transfection and lentiviral transduction. The IRS-1 and Arg⁹⁷² IRS-1 expression constructs were transfected into the osteoblast cells using SuperfectTM transfection reagent (Qiagen) according to the manufacturer's instructions. Pools of stable transductants were generated via selection using G418 (600 μ g/ml) according to the manufacturer's instructions. Lentiviral transduction was performed and pools of stable transductants were generated via selection with puromycin (4 μ g/ml).

Western blot analysis. The osteoblasts were lysed in 250 μ l 2X SDS loading buffer containing 62.5 mM TrisHCl, (pH 6.8), 2% SDS, 25% glycerol, 0.01% bromophenol blue, 5% 2-mercaptoethanol (Sigma-Aldrich), and incubated at 95°C for 10 min. Equal quantities of the proteins (100 μ g) of each sample were separated by 8-15% SDS-polyacrylamide gel electrophoresis and blotted onto a polyvinylidene difluoride microporous membrane (Millipore, Billerica, MA, USA). The membranes were incubated for 1 h with a 1:1,000 dilution of primary antibody, washed three times with phosphate-buffered saline for 5 min and incubated with secondary antibodies with horseradish peroxidase conjugate (1:5,000, 1 h). The peroxidase was visualized using a GE Healthcare electrochemiluminescence kit (Shanghai, China).

IRS-1-associated PI3K activity assay. The IRS-1-associated PI3K activities were determined, as previously described (16). Briefly, 700 μ g total protein was immunoprecipitated with anti-IRS-1 antibody (Santa Cruz Biotechnology, Inc.). PI3K activity was measured in a reaction mixture containing phosphatidylinositol (Sigma-Aldrich) and [γ -³²P]ATP

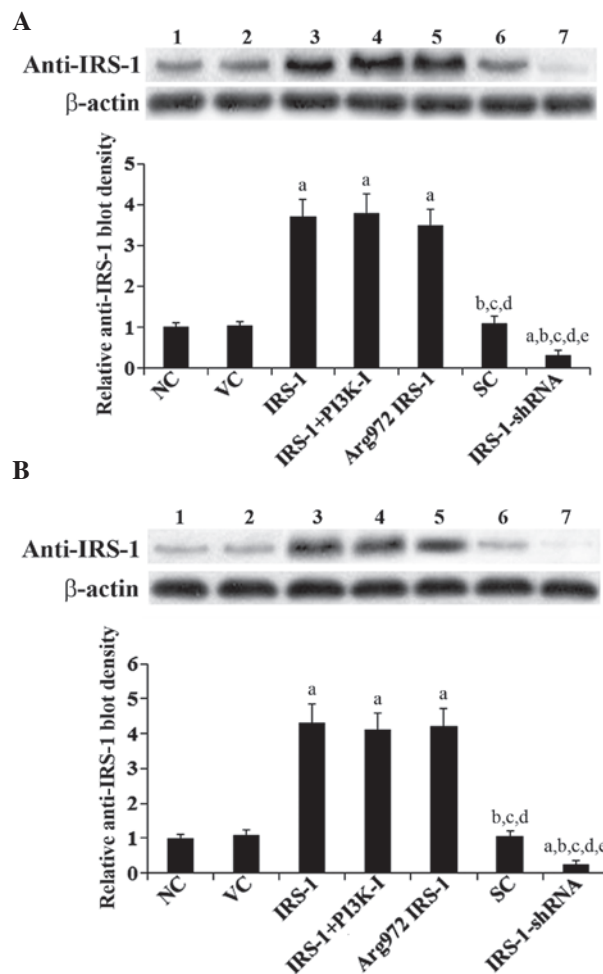


Figure 1. Stable overexpression of IRS-1 or Arg⁹⁷² IRS-1 or knock down of IRS-1 in normal human osteoblasts and RA osteoblasts. Western blot analyses were performed with an anti-IRS-1 antibody in (A) normal human osteoblasts and (B) RA osteoblasts. Lane 1, control cells (NC); lane 2, cells stably transfected with empty pcDNA3 vector (VC); lane 3, cells stably transfected with IRS-1; lane 4, cells stably transfected with IRS-1 plus pre-treatment with selective PI3K inhibitor (50 μ M BKM120) for 30 min; lane 5, cells stably transfected with Arg⁹⁷² IRS-1; lane 6, cells stably transfected with scramble control shRNA (SC); lane 7, cells stably transfected with IRS-1-shRNA. β -Actin blotting was used as a loading control. The density of the anti-IRS-1 blot was normalized against that of β -actin to obtain a relative blot density, which is expressed as the fold change to the relative anti-IRS-1 blot density of NC (designated as 1). ^aP<0.05, vs. NC or VC; ^bP<0.05, vs. IRS-1; ^cP<0.05, vs. IRS-1+PI3K-1; ^dP<0.05, vs. Arg⁹⁷² IRS-1; ^eP<0.05, vs. SC. IRS-1, insulin receptor substrate-1; PI3K, phosphatidylinositol-3 kinase; RA, rheumatoid arthritis.

(Sigma-Aldrich). After 5 min, the reaction was stopped by the addition of HCl and chloroform:methanol and analyzed by thin-layer chromatography. PI3K activity was detected by the appearance of a specific radioactive spot corresponding to ³²P-labeled phosphatidylinositol 3-phosphate [³²P]PI-3-P (17). The PI3K activity was normalized against 10⁶ cells. The autoradiographic signals were quantified using the National Institutes of Health Image J software, version 1.63 (National Institutes of Health, Bethesda, MO, USA).

Measurement of apoptosis using a TUNEL assay. The TUNEL assay was performed using the DeadEndTM Fluorometric TUNEL system according to the manufacturer's instructions

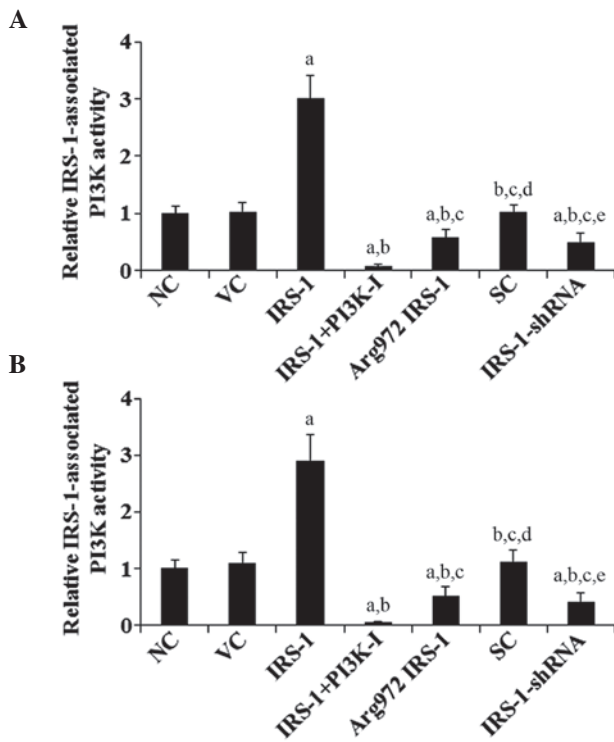


Figure 2. IRS-1-associated PI3K activity in normal human osteoblasts and RA osteoblasts with overexpression of IRS-1 or Arg⁹⁷² IRS-1 or the knock down of IRS-1. IRS-1-associated PI3K activity was measured in (A) normal human osteoblasts and (B) RA osteoblasts stimulated by 10 nM of insulin for 30 min. The IRS-1-associated PI3K activities in Lane 1, control cells (NC); lane 2, cells stably transfected with empty pcDNA3 vector (VC); lane 3, cells stably transfected with IRS-1; lane 4, cells stably transfected with IRS-1 plus pre-treatment with selective PI3K inhibitor (50 μ M BKM120) for 30 min; lane 5, cells stably transfected with Arg⁹⁷² IRS-1; lane 6, cells stably transfected with scramble control shRNA (SC); lane 7, cells stably transfected with IRS-1-shRNA. Blot density is expressed as fold changes to that of NC (designated as 1). ^aP<0.05, vs. NC or VC; ^bP<0.05, vs. IRS-1; ^cP<0.05, vs. IRS-1+PI3K-I; ^dP<0.05, vs. Arg⁹⁷² IRS-1; ^eP<0.05, vs. SC. IRS-1, insulin receptor substrate-1; RA, rheumatoid arthritis; PI3K, phosphatidylinositol-3 kinase.

(Promega). The cells were treated with 20 ng/ml TNF- α for 6 and 12 h in the presence of 10 nM insulin. Subsequently, 50 μ l of TdT reaction mix was added to the cells on an area no larger than 5 cm² and slides were covered with plastic coverslips to ensure even distribution of the mix. The slides were incubated for 60 min at 37°C in a humidified chamber. Apoptotic cells exhibit a marked nuclear green fluorescence, which can be detected using a standard fluorescein filter. Cells stained with 4',6-diamidino-2-phenylindole (Sigma-Aldrich) exhibit a marked blue nuclear fluorescence. The slides were visualized using fluorescence microscopy (IX83; Olympus, Beijing, China) and the relative quantity of apoptotic cells were determined by counting the number of TUNEL-positive cells in five randomly selected fields (magnification, x100) for each sample.

Statistical analysis. Statistical analyses were performed using SPSS 15.0 software (SPSS, Inc., Chicago, IL, USA). All continuous variable values are expressed as the mean \pm standard deviation. Comparisons of the means among multiple groups were performed with one-way analysis of variance, followed by post hoc pairwise comparisons using Tukey's

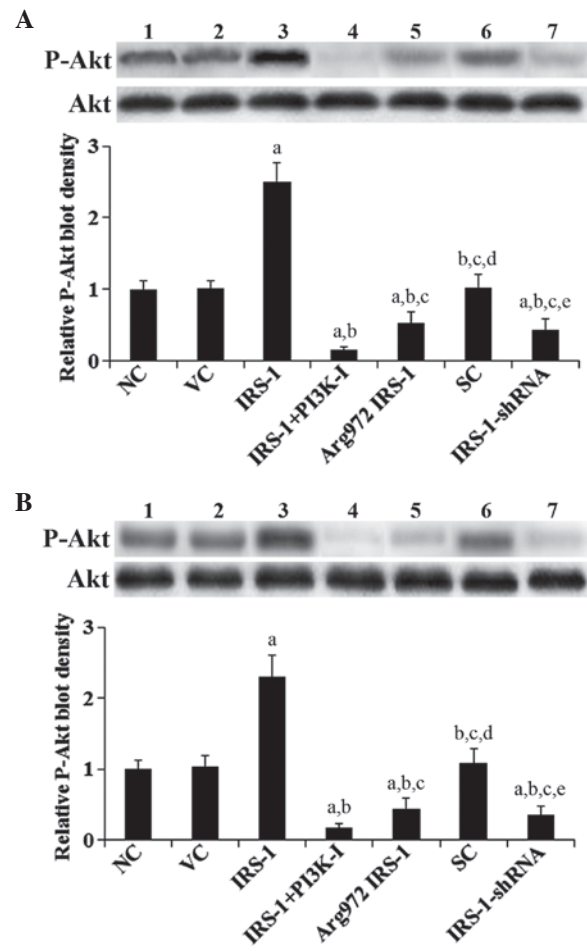


Figure 3. P-Akt level in normal human osteoblasts and RA osteoblasts with overexpression of IRS-1 or Arg⁹⁷² IRS-1 or knock down of IRS-1. Western blot analyses were performed to determine total Akt and P-Akt (at serine 473) levels in (A) normal human osteoblasts and (B) RA osteoblasts stimulated by 10 nM insulin for 30 min. Lane 1, control cells (NC); lane 2, cells stably transfected with empty pcDNA3 vector (VC); lane 3, cells stably transfected with IRS-1; lane 4, cells stably transfected with IRS-1 and pretreated with selective PI3K inhibitor (50 μ M BKM120) for 30 min (IRS-1+PI3K-I); lane 5, cells stably transfected with Arg⁹⁷² IRS-1; lane 6, cells stably transfected with scramble control shRNA (SC); lane 7, cells stably transfected with IRS-1-shRNA. The density of the P-Akt blot was normalized against that of total Akt to obtain a relative blot density, which is expressed as fold changes to the relative P-Akt blot density of NC (designated as 1). ^aP<0.05 vs. NC or VC; ^bP<0.05 vs. IRS-1; ^cP<0.05 vs. IRS-1+PI3K-I; ^dP<0.05 vs. Arg⁹⁷² IRS-1; ^eP<0.05 vs. SC. P-Akt, phosphorylated Akt; IRS-1, insulin receptor substrate-1; RA, rheumatoid arthritis; PI3K, phosphatidylinositol-3 kinase.

test. P<0.05 was considered to indicate a statistically significant difference for the two-tailed analysis.

Results

The normal and RA osteoblasts were stably transfected with Arg⁹⁷² IRS-1 and IRS-1. By contrast, the cells were stably transfected with IRS-1-shRNA to knock down IRS-1. As shown in Fig. 1, compared with the controls, use of the anti-IRS-1 antibody revealed that IRS-1 and Arg⁹⁷² IRS-1 were overexpressed >3.5-fold, while the level of endogenous IRS-1 was knocked down by ~70% in the normal and the RA osteoblasts. Insulin stimulation (10 nM) had no significant effects on the overexpression of Arg⁹⁷² IRS-1 and IRS-1 or on the knock down of IRS-1 in the cells (data not shown).

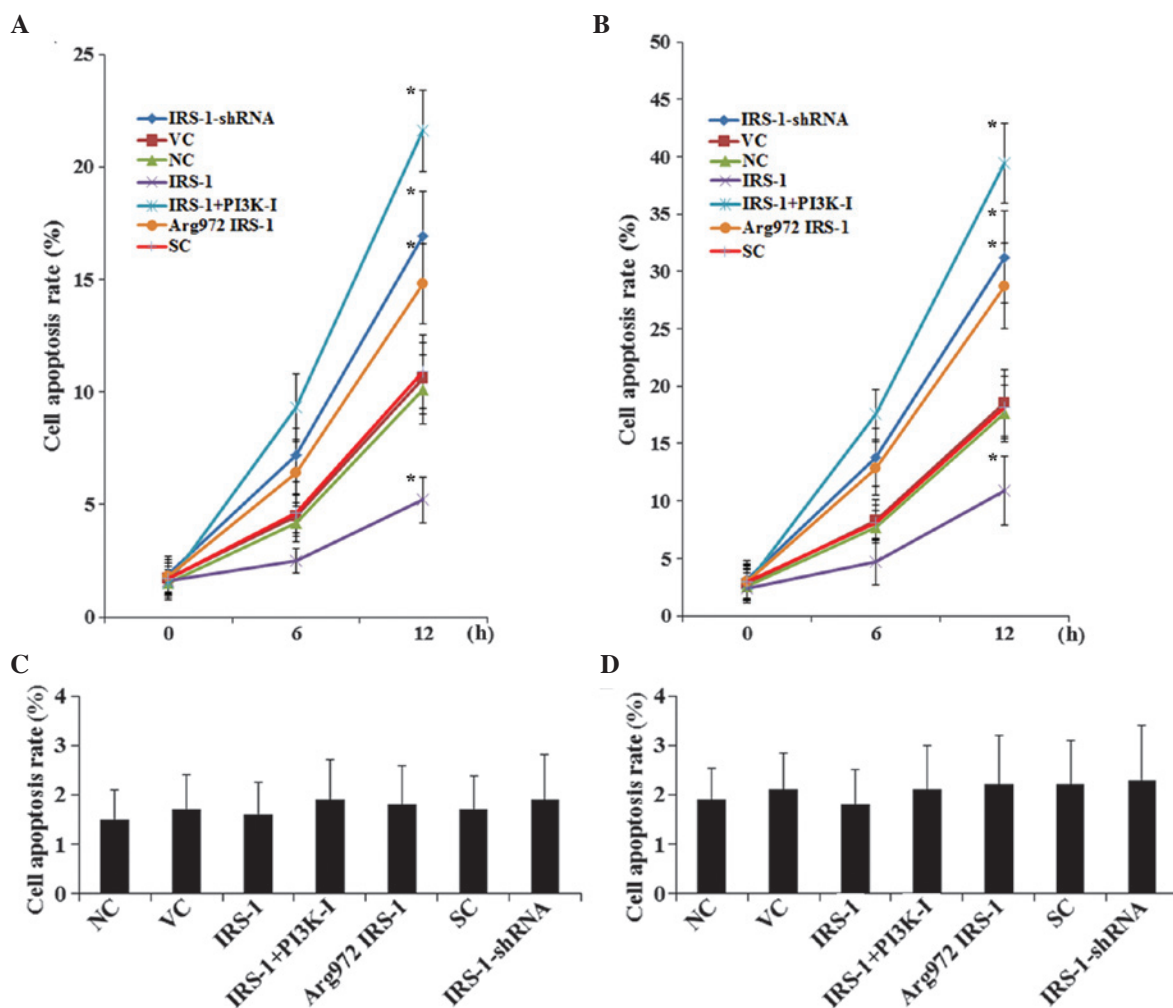


Figure 4. TNF- α -induced apoptosis in normal human osteoblasts and RA osteoblasts with overexpression of IRS-1 or Arg⁹⁷² IRS-1 or knock down of IRS-1. (A) Normal human osteoblasts and (B) RA osteoblasts were stimulated by 10 nM insulin for 30 min and then treated with 20 ng/ml TNF- α for 6 and 12 h. TUNEL assays were performed in the control cells (NC), cells stably transfected with empty pcDNA3 vector (VC), cells stably transfected with IRS-1, cells stably transfected with IRS-1 plus pre-treatment with selective PI3K inhibitor BKM120 (50 μ M) for 30 min (IRS-1+PI3K-I), cells stably transfected with Arg⁹⁷² IRS-1, cells stably transfected with scramble control shRNA (SC) and cells stably transfected with IRS-1-shRNA. The rates of cell apoptosis at 6 and 12 h are presented as the percentage of TUNEL positive cells in the total cells. TUNEL assays were also performed at 12 h in (C) normal human osteoblasts and (D) RA osteoblasts stimulated by 10 nM insulin for 30 min, but without TNF- α treatment. * P <0.05, compared with NC, VC or SC at 12 h. IRS-1, insulin receptor substrate-1; RA, rheumatoid arthritis; PI3K, phosphatidylinositol-3 kinase; TNF- α , tumor necrosis factor- α ; TUNEL, terminal deoxynucleotidyl transferase mediated nick-end labeling.

Arg⁹⁷² IRS-1 is reportedly associated with impaired insulin/IRS-1 signaling to activate the PI3K/Akt pathway (3,4), which is important in cell survival against apoptotic stress (14). The present study also examined the IRS-1-associated PI3K activity and Akt activation/phosphorylation in osteoblasts. In the absence of insulin, the overexpression of Arg⁹⁷² IRS-1 and IRS-1 and the knock down of IRS-1 exhibited no significant effects on IRS-1-associated PI3K activity, Akt activation/phosphorylation or TNF- α -induced apoptosis in the osteoblasts. (figs. 2-4). Different techniques used to stimulate insulin were assessed, which revealed that treatment with 10 nM insulin for 30 min had the most marked stimulatory effects on IRS-1-associated PI3K activity in the osteoblasts (data not shown). Thus, in all the subsequent experiments, osteoblasts were pre-stimulated with 10 nM insulin for 30 min. As shown in Fig. 2, overexpression of IRS-1 increased IRS-1-associated PI3K activity by \sim 3 fold in the normal and RA osteoblasts, compared with the controls, which was

eliminated by pretreatment with 50 μ M BKM120, a selective PI3K inhibitor, for 30 min. By contrast, the overexpression of Arg⁹⁷² IRS-1 and the knock down of IRS-1, decreased IRS-1-associated PI3K activity by 40 and 60%, respectively in the normal and RA osteoblasts. Similar trends were observed in the phosphorylation of Akt at ser473, which is required for full activation of Akt by PI3K (Fig. 3) (14).

Subsequently, the effects of IRS-1 and Arg⁹⁷² IRS-1 on TNF- α -induced apoptosis in osteoblasts were examined. As shown in Fig. 4A and B, osteoblasts treated with 20 ng/ml TNF- α for 12 h in the presence of insulin (10 nM), exhibited significant differences in apoptosis. Compared with the controls at 12 h, overexpression of IRS-1 decreased cell apoptosis by \sim 5% in the normal osteoblasts and by \sim 7% in the RA osteoblasts and this was eliminated completely by pretreatment with 50 μ M BKM120, a selective PI3K inhibitor, for 30 min. By contrast, overexpression of Arg⁹⁷² IRS-1 increased cell apoptosis by \sim 4.5% in the normal osteoblasts

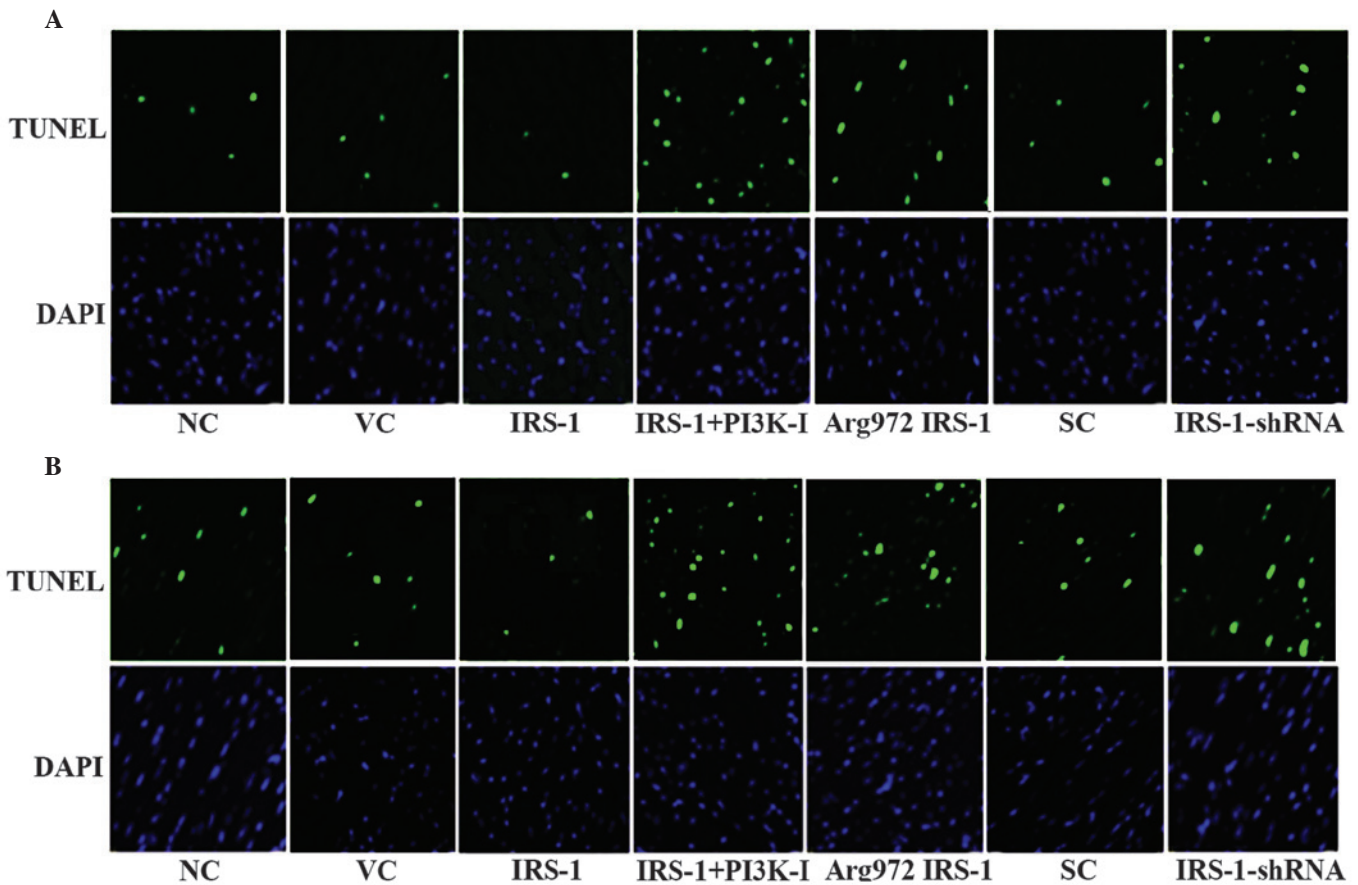


Figure 5. Fluorescent TUNEL assay in normal human osteoblasts and RA osteoblasts with overexpression of IRS-1 or Arg⁹⁷² IRS-1 or knock down of IRS-1. (A) Normal human osteoblasts and (B) RA osteoblasts were stimulated by 10 nM insulin for 30 min and then treated with 20 ng/ml TNF- α for 6 and 12 h. Fluorescent TUNEL assays were performed in control cells (NC), cells stably transfected with empty pcDNA3 vector (VC), cells stably transfected with IRS-1, cells stably transfected with IRS-1 plus pre-treatment with selective PI3K inhibitor BKM120 (50 μ M) for 30 min (IRS-1+PI3K-I), cells stably transfected with Arg⁹⁷² IRS-1, cells stably transfected with scramble control shRNA (SC) and cells stably transfected with IRS-1-shRNA. Apoptotic cells exhibited marked nuclear green fluorescence, which was detected using a standard fluorescein filter. All cells stained with DAPI exhibited marked blue nuclear fluorescence. TUNEL, terminal deoxynucleotidyl transferase mediated nick-end labeling; DAPI, 4',6-diamidino-2-phenylindole; IRS-1, insulin receptor substrate-1; RA, rheumatoid arthritis PI3K, phosphatidylinositol-3 kinase; TNF- α , tumor necrosis factor- α .

and by ~10.5% in the RA osteoblasts, and knock down of IRS-1 increased cell apoptosis by ~6.5% in the normal osteoblasts and ~13% by in the RA osteoblasts (Fig. 4A and B). In the absence of TNF- α treatment, no significant differences were observed in the apoptotic rate at 12 h in the normal or the RA osteoblasts (Fig. 4C and D). Representative fluorescent TUNEL assay images at 12 h are shown in Fig. 5. DAPI staining of the cell nuclei indicated a similar cell number/density in the experiment groups (Fig. 5).

Discussion

Arg⁹⁷² IRS-1 has been previously reported an independent risk factor for RA and a case-control study demonstrated it is significantly associated with the severity of RA (5). In the present study, a mechanistic explanation for this was obtained by revealing that Arg⁹⁷² IRS-1 enhanced TNF- α -induced apoptosis in normal and RA osteoblasts.

The presence of Arg⁹⁷² IRS-1 is associated with impaired insulin/IRS-1 signaling to activate the PI3K/Akt pathway (3,4). The results of the present study were in agreement with this, which demonstrated that, in the presence of

insulin, stable overexpression of Arg⁹⁷² IRS-1 and knock down of IRS-1 significantly decreased IRS-1-associated PI3K activity and Akt activation/phosphorylation (ser473) in osteoblasts. By contrast, the stable overexpression of IRS-1 significantly increased IRS-1-associated PI3K activity and Akt activation/phosphorylation (ser473), which was completely eliminated by a selective PI3K inhibitor. For insulin stimulation, the cells were pretreated with 10 nM insulin, which had been used in a previous study to stimulate osteoblast-lineage cells (18).

The present study demonstrated that treatment with 20 ng/ml TNF- α for 12 h induced significant apoptosis in the normal and RA osteoblasts, concordant with a previous study, which observed peaks in the activities of caspase-3, caspase-8 and caspase-9 in human bone marrow stromal cells after 12 h of treatment with 20 ng/ml TNF- α (19). In agreement with their inhibitory effects on the PI3K/Akt pathway, which is important in promoting cell survival against apoptotic stress (14), overexpression of Arg⁹⁷² IRS-1 and knock down of IRS-1 in the present study significantly enhanced TNF- α -induced apoptosis in the normal and RA osteoblasts. This was corroborated by the finding that the overexpression of IRS-1 significantly

increased IRS-1-associated PI3K activity/Akt phosphorylation and reduced TNF- α -induced apoptosis in the osteoblasts. The overexpression of Arg⁹⁷² IRS-1 and knock down of IRS-1 in the RA osteoblasts exhibited a more pronounced inhibitory effect on TNF- α -induced apoptosis compared with the normal osteoblasts. This suggested that, Arg⁹⁷² IRS-1, or the impairment of insulin/IRS-1 signaling, was important in the pathogenesis of RA and that other signaling pathways, besides insulin/IRS-1 signaling, are involved in the pathogenesis of RA.

The normal and the RA osteoblasts used in the present study were genotyped and found to be wildtype IRS-1 homozygotes. Thus, the overexpression of Arg⁹⁷² IRS-1 in the osteoblasts resulted in the expression of a mixture of Arg⁹⁷² IRS-1 and wild type IRS-1. This resembles an Arg⁹⁷² IRS-1 heterozygote, which is the major source of Arg⁹⁷² IRS-1 carriers and the frequency of Arg⁹⁷² IRS-1 heterozygote, wild-type IRS-1 homozygote and Arg⁹⁷² IRS-1 homozygote are 12.5, 87 and 0.6%, respectively, in Exome Sequencing Project cohort populations (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=rs1801278). By demonstrating that the overexpression of Arg⁹⁷² IRS-1 and IRS-1 enhanced and inhibited TNF- α -induced apoptosis in normal and RA osteoblasts, respectively, the results of the present study suggested that Arg⁹⁷² IRS-1 carriers may develop RA more easily and that insulin/IRS-1 signaling is important in the pathogenesis of RA. It also suggested that insulin/IRS-1 signaling may be a new target for treating RA.

TNF- α has significant effects on other cell types in the synovial membrane, including synoviocytes, macrophages, chondrocytes and osteoblasts (20). Thus, it may be useful to examine how Arg⁹⁷² IRS-1 and IRS-1 affect the regulatory effects of TNF- α on cell types other than osteoblasts in future studies. In addition, inflammatory cytokines, including interleukin-1 β and interleukin-6 have also been found to have important roles in the pathogenesis of RA (9). Therefore, identifying whether insulin/IRS-1 signaling can regulate the effects of interleukins on cells involved in the pathogenesis of RA may be of interest.

In conclusion, the present study provided the first evidence, to the best of our knowledge, that under insulin stimulation, Arg⁹⁷² IRS-1 and IRS-1 enhanced and inhibited TNF- α -induced apoptosis in normal and RA osteoblasts, respectively, by a PI3K-dependent mechanism. These findings suggest an important role for insulin/IRS-1 signaling in the pathogenesis of RA.

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