

Recombinant tissue metalloproteinase inhibitor-3 protein induces apoptosis of murine osteoblast MC3T3-E1

L.-Q. Yuan^{1,*}, Y.-S. Liu^{2,*}, X.-H. Luo¹, L.-J. Guo¹, H. Xie¹, Y. Lu¹, X.-P. Wu¹, and E.-Y. Liao¹

¹ Institute of Metabolism and Endocrinology, The Second Xiangya Hospital, Central South University, Changsha, Hunan, China

² Geriatric Department of the Second Xiangya Hospital, Central South University, Changsha, Hunan, China

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Summary. Tissue inhibitor of metalloproteinases (TIMPs) plays an essential role in the regulation of bone metabolism. Here we report that recombinant tissue metalloproteinase inhibitor-3 (TIMP-3) protein induces the apoptosis of MC3T3-E1 osteoblasts. Cell apoptosis was detected by sandwich-enzyme-immunoassay. Fas and FasL protein levels were determined by Western blot analysis. The enzyme substrate was used to assess the activation of caspase-3 and caspase-8. The phosphorylation of JNK, p38 and ERK1/2 was examined by Western blot analysis. The ELISA suggested that TIMP-3 promoted MC3T3-E1 cell apoptosis. TIMP-3 treatment induced the expression of Fas and FasL proteins, and the activation of caspase-8 and caspase-3. TIMP-3 treatment induced p38 and ERK phosphorylation. SB203580 and PD98059, the inhibitor of p38 and ERK, respectively, abolished the TIMP-3 effect on osteoblast apoptosis. In conclusion, the signal pathway through which TIMP-3 induces MC3T3-E1 cell apoptosis, mediated by Fas and involves the p38 and ERK signal transduction pathways.

Keywords: Tissue metalloproteinase inhibitor-3 – Osteoblast – Apoptosis

Introduction

Apoptosis plays a critical role during embryonic limb development, skeletal maturation, adult bone turnover by modeling and remodeling processes, and during fracture healing and bone regeneration (Hock et al., 2001). Osteoblast apoptosis is considered to be an important determinant of bone formation and therefore of skeletal integrity (Manolagas, 2000; Weinstein et al., 2000), such disorders that promote the process are associated with increased bone fragility (Weinstein et al., 1998), and treatments that inhibit them are associated with antifracture efficacy (Jilka et al., 1999). Understanding the molecular mechanisms that regulate the survival of osteoblasts may promote the development of more effective therapeutics for disease states characterized by low bone mass.

Tissue inhibitor of metalloproteinases (TIMPs) is the inhibitor of matrix metalloproteinases (MMPs), which suppresses MMPs' activity to degrade extracellular matrix (ECM) and leads to the accumulation of ECM; therefore, TIMPs play an essential role in the regulation of bone metabolism (Nagase and Woessner, 1999; Liao and Luo, 2001). It has been found that the TIMP family includes four members, namely (in the order of discovery), TIMP-1, TIMP-2, TIMP-3 and TIMP-4. Different from the other three members, TIMP-3 only exists in ECM and is an insoluble protein bound to ECM. A previous study demonstrated TIMP-3 was expressed in osteoblasts and the TIMP-3 gene was an upregulated gene during the differentiation of MC3T3-E1 cells. Overexpression of TIMP-3 gene in osteoblast decreased the expression of alkaline phosphatase, osteopontin, osteocalcin, osteonectin, as well as the formation of mineralized nodules (Suzuki et al., 2003). These results support the role of TIMP-3 as an important mediator of osteoblast function. TIMP-3 has not previously been reported to promote osteoblast apoptosis; in fact, the available evidence suggests that it promotes apoptosis of prostate cancer cells (Deng et al., 2006) and fibroblasts (Drynda et al., 2005). In the current work we investigated the molecular mechanisms by which TIMP-3 promoted osteoblast apoptosis.

Materials and methods

Antibodies and reagents

Recombinant human TIMP-3, PD098059 (specific inhibitor of the ERK/MAPK kinase), SB203580 (specific inhibitor of p38 kinase), and SP600125 (JNK inhibitor II) were purchased from Calbiochem Biotechnology Inc (San Diego, CA, U.S.A.); fetal bovine serum (FBS)

* These authors contributed equally to this work.

was purchased from GIBCO BRL (Grand Island, NY); α -MEM and antimouse β -actin monoclonal antibody were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.); Anti-Bcl-2, Bax, caspase-3, phospho-c-Jun N-terminal kinase (p-JNK), JNK, phospho-p38, p38, phosphorylated extracellular signal-regulated kinase (p-ERK)1/2, ERK1/2 antibody, anti-mouse and anti-rabbit IgG peroxidase conjugate antibodies were purchased from Santa Cruz Biotechnology (Waltham, MA).

Cell culture

MC3T3-E1, a mouse osteoblastic cell line, was obtained from American Type Culture Collection (ATCC). MC3T3-E1 cells were maintained as previously described (Yuan et al., 2007). Briefly, the cells were cultured in α -minimum essential medium (α -MEM) containing 10% FBS, 20 mM HEPES, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 50 μ g/ml ascorbic acid at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. The medium was changed twice a week and the cells were subcultured using 0.05% trypsin with 0.01% EDTA.

Cell apoptosis measurement

For apoptosis assay, cells were plated at a density of 1×10^4 cells/well in 24-well plates and incubated until they were approximately 75–85% confluent, incubated for 2 days in culture medium supplemented with 0.5% FBS and then changed to assay medium. For most experiments, cells were incubated with the absence or presence of 100–600 ng/ml TIMP-3 for 48 h (for dosage study) and 600 ng/ml TIMP-3 for 4–48 h (for time course study). Apoptosis was assessed directly by the measurement of cytoplasmic nucleosomes (i.e., DNA complexed with histone in the cytoplasm) using a Cell Death Detection kit (Roche Diagnostics GmbH, Roche Molecular Biochemicals, Mannheim, Germany), according to the kit protocol. The cell layers were rinsed with PBS and extracted with 0.5 ml of lysis buffer after 30-min incubation at 4 °C. The cell lysates were then centrifuged for 10 min at 15,000 rpm, and the aliquots of aqueous supernatant were tested for apoptosis using the Cell Death Detection kit. In some experiments, cells were preincubated for 2 h with 10 μ M PD098059 (ERK inhibitor), 20 μ M SB203580 (p38 inhibitors), 20 μ M SP600125 (JNK-1 inhibitors) or vehicle (DMSO) which were also incubated in assay medium, and then analyzed by Cell Death Detection kit. The concentration of inhibitors was selected based on published studies.

Western blot analysis

MC3T3-E1 cells were incubated with 100–600 ng/ml TIMP-3 for 48 h as described above and the cell layers were homogenated with Triton lysis buffer (50 mM Tris-HCl, pH 8.0 containing 150 mM NaCl, 1% Triton X-100, 0.02% NaN₃, 10 mM EDTA, 10 μ g/ml aprotinin, and 1 μ g/ml aminoethylbenzenesulfonyl fluoride. The lysates were centrifuged for 15 min at 12,000 g to remove debris. Protein concentrations were determined using the Bradford protein assay. Forty micrograms protein of each cell layer homogenate was loaded onto a 10% polyacrylamide gel, and transferred to a PVDF membrane. After blocking with 5% nonfat milk, membranes were incubated with anti-mouse Bcl-2 monoclonal antibody, anti-mouse Bax monoclonal antibody, or anti-rabbit caspase-3 antibody. The membrane was re-probed with peroxidase-conjugated secondary antibodies. The same membrane was stripped and then re-probed with anti-mouse β -actin monoclonal antibody as a loading control. Blots were processed using an ECL kit (Santa Cruz) and exposed to film, then analyzed by densitometry.

Phosphorylation levels of p38, ERK and JNK were examined by Western blot to evaluate the role of the MAPK survival signal pathway. First, MC3T3-E1 cells were treated with 600 ng/ml TIMP-3 for a desired time. Then, the cells were washed quickly with cold PBS containing 5 mM of EDTA and 0.1 mM of Na₃VO₄, and lysed with a lysis buffer consisting of 20 mM of Tris-HCl (pH 7.5), 150 mM of NaCl, 1% Triton X-100, 10 mM of NaH₂PO₄, 10% glycerol, 2 mM of Na₃VO₄, 10 mM of NaF,

1 mM of aminoethylbenzenesulfonyl fluoride, 10 μ g/ml leupeptin, and 10 μ g/ml aprotinin. The protein concentrations were determined using Bradford protein assay. Western blots were performed with anti-p-JNK, JNK, p-p38, p38, p-ERK, and ERK antibodies.

Statistical analyses

Data are presented as means $\pm$ standard deviation (SD). Comparisons were made with one-way analysis of variance. All experiments were repeated at least twice, and representative experiments are shown.

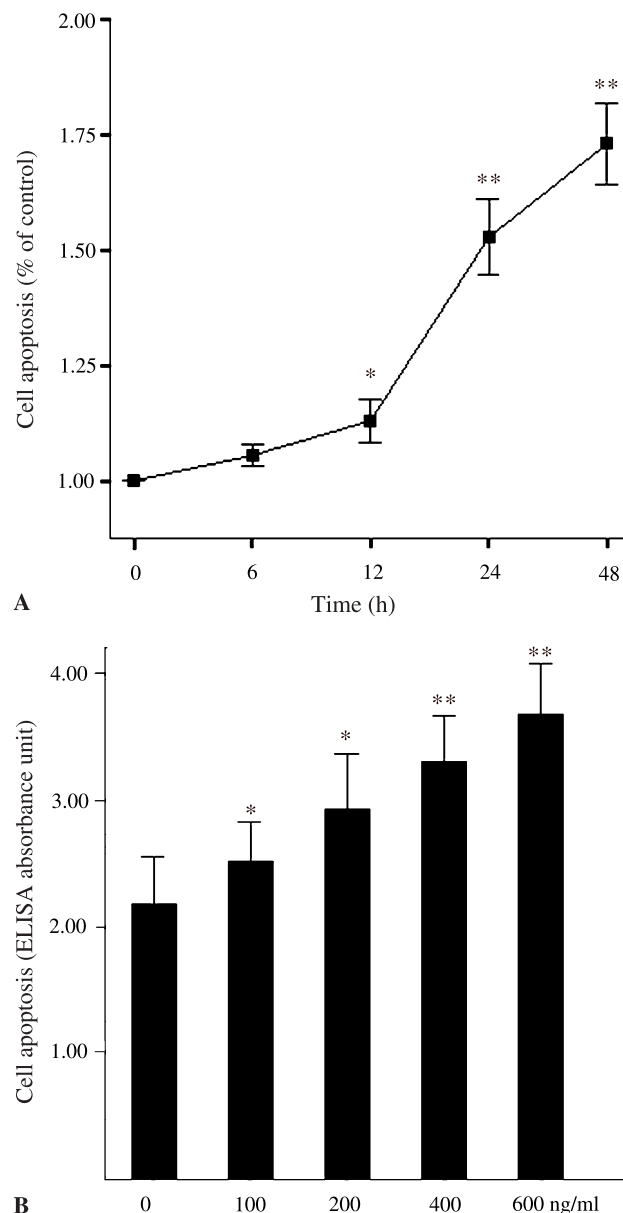


Fig. 1. Cells were exposed to 600 ng/ml TIMP-3 in serum-free medium for 48 h. Apoptosis was assessed using a Cell Death ELISA Detection kit and expressed as ELISA absorbance units. (A) Time-course effects of TIMP-3 on cell apoptosis. Dots represent the percentage viability at various time points. * $P < 0.05$ and ** $P < 0.001$ compared with control. (B) Dose-response effects of TIMP-3 on cell apoptosis. Bars represent means $\pm$ SD ($n = 4$). * $P < 0.05$ and ** $P < 0.001$ compared with control.

Results

TIMP-3 promoted MC3T3-E1 cells apoptosis induced by serum deprived

The Cell Death Detection ELISA results indicated that TIMP-3 stimulated MC3T3-E1 cell apoptosis in a dose-dependent manner (Fig. 1A). At 48 h of culture, apoptotic cells at 100 ng/ml TIMP-3 (2.73 ± 0.25 ELISA absorbance units) were higher than in controls (2.20 ± 0.24 , $p < 0.05$), and the maximal apoptotic effect was 600 ng/ml (3.74 ± 0.46 , $p < 0.001$) after 48 h of incubation. There were also statistically significant differences between the 100, 200, 400, and 600 ng/ml TIMP-3 treatment groups. Figure 1B showed 600 ng/ml TIMP-3 promoted MC3T3-E1 cell apoptosis in a time-dependent manner.

TIMP-3 activated ERK and p38 phosphorylation in MC3T3-E1 cells

MAPK were the important regulators of apoptosis, and the influence of blockers of the MAPK signal transduction path-

way on TIMP-3's action was observed. Western blot analysis revealed that TIMP-3 treatment induced ERK and p38 phosphorylation; and p-ERK was activated at 10 min after TIMP-3 treatment and its phosphorylation reached a peak at 30 min; p-p38 was activated at 5 min after TIMP-3 treatment and its phosphorylation reached a peak at 30 min. However, TIMP-3 had no effect on JNK kinase phosphorylation (Fig. 2). These data demonstrated that TIMP-3 activated ERK and p38 signaling pathway in MC3T3-E1 cells.

The apoptosis ELISA assay indicated that the ERK signal transduction blocker PD098059 and the p38 signal transduction blocker SB203580 suppressed the effect of TIMP-3 on cell apoptosis, that is, they abolished the promotion of cell apoptosis by TIMP-3; nevertheless, the JNK blocker SP600125 did not significantly affect TIMP-3 action, that is, it has no effect on TIMP-3-induced osteoblast apoptosis (Fig. 3, the DMSO group as control).

Effect of TIMP-3 on the expression of apoptosis-related protein in MC3T3-E1 cells

The effect of TIMP-3 on the expression of Fas and FasL, and activities of caspase-8, caspase-3 was assessed by

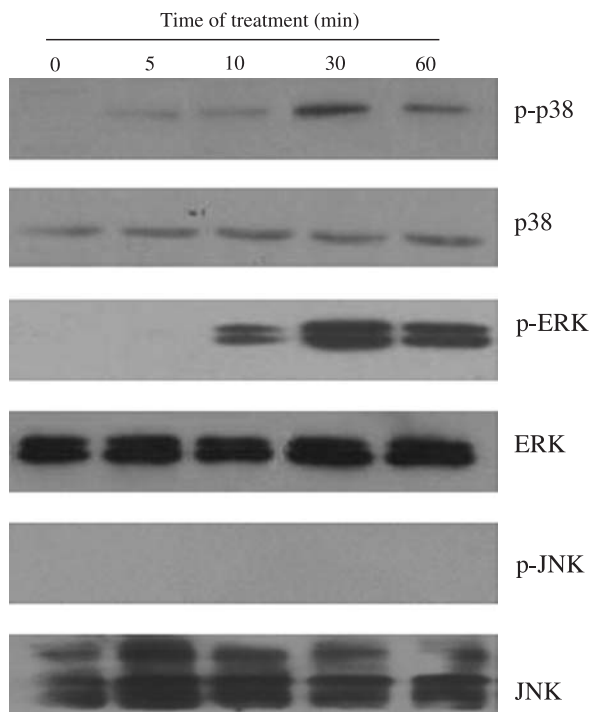


Fig. 2. MC3T3-E1 cells were treated with or without 600 ng/ml recombinant TIMP-3 proteins for 0, 5, 10, 30, and 60 min. Cell lysates were subjected to Western blot with anti-active ERK, p38 and JNK1/2 and anti-ERK, -p38, and -JNK1/2 antibodies. TIMP-3 treatment induced p38 phosphorylation, which was activated at 5 min and reached a peak at 30 min ($P < 0.05$); TIMP-3 treatment induced ERK phosphorylation, which was activated at 10 min and reached a peak at 30 min ($P < 0.05$); TIMP-3 treatment did not affect JNK kinase phosphorylation

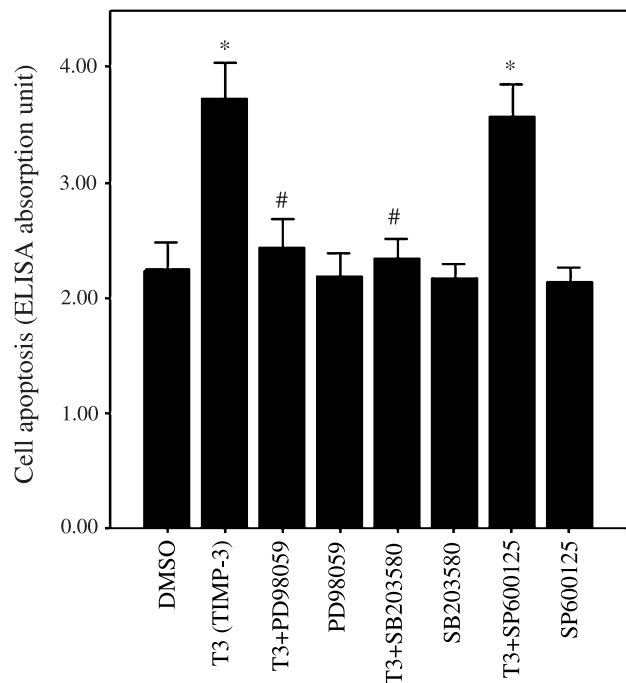


Fig. 3. MC3T3-E1 cells were cultured in a serum-free medium as control (DMSO) or with 600 ng/ml recombinant TIMP-3 (T3), T3 + PD98059, PD98059, T3 + SB203580, SB203580, T3 + SP600125, or SP600125. After 48 h of culture, cell apoptosis was determined by Cell Death Detection kit. Bars represent means $\pm$ SD. $p < 0.05$ versus the DMSO group, # $p < 0.05$ versus the T3 group

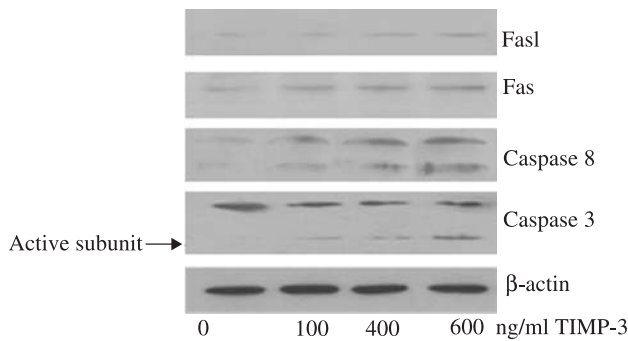


Fig. 4. Cells were exposed to 100–600 ng/ml TIMP-3 for 48 h. Western blot was performed using anti-Fas, FasL, caspase-8, and caspase-3 antibodies. The bottom panel shows the β -actin levels of the same blot were re-probed with an anti- β -actin monoclonal antibody. Compared to the control group, in the TIMP-3 treatment group FasL and Fas protein expression increased; and caspase-8 and -3 activation was enhanced; A representative western blot from three independent experiments is shown

Western blot analysis. The results demonstrated that the Fas and FasL expression and the bands of cleaved caspase-3 and caspase-8 were increasing obviously with increasing concentrations of TIMP-3.

Discussion

Osteocyte apoptosis is a mode of bone metabolism, for instance, osteoblast apoptosis may initiate bone resorption. Postmenopausal abnormal apoptosis of osteoblasts and osteoclasts may cause uncoupling of bone remodeling, which is an important mechanism for postmenopausal osteoporosis. Osteocyte apoptosis increases bone fragility, resulting in higher risks of bone fracture (Brixen et al., 2004). Bone metabolism is modulated by both systemic hormones and various local cytokines such as TGF- β , IL-1 and IL-6, and local cytokines are even more important than systemic hormones in this regard. In the present study, recombinant TIMP-3 protein was used to treat MC3T3-E1 cells directly, and then the effect of TIMP-3 on apoptosis of MC3T3-E1 cells and the mechanism involved were investigated.

TIMP-3 contains 39 amino acid residuals (12 cysteine residuals and 27 other amino acid residuals), which are the same in the sequence of all members of the TIMP family. However, TIMP-3 has properties different from those of the other three members, and as an insoluble protein bound to ECM, it exists only in ECM. Many studies have confirmed that TIMP-3 induces apoptosis in different cells (Ahonen et al., 2003; Majid et al., 2002; Span et al., 2004). As a local cytokine of bone, TIMP-3 suppressed osteoblasts differentiation (Suzuki et al., 2003) regulating bone metabolism. Nevertheless, the effect of

TIMP-3 on osteoblast apoptosis has not been reported yet. In the present study, recombinant TIMP-3 protein was used to treat MC3T3-E1 cells cultured in serum-free medium. Detection of apoptosis by ELISA demonstrated that TIMP-3 induced MC3T3-E1 cell apoptosis in a dose and time-dependent manner. These results demonstrated that TIMP-3 induced apoptosis in MC3T3-E1 cells.

There are two fundamental apoptosis pathways of mammal cells, either endogenous (caspase-9 dependent) or exogenous (caspase-8 dependent). The common endogenous signal-inducing apoptosis is DNA damage. Exogenous signals trigger apoptosis through cell surface death receptors such as Fas (CD95 or TNF receptor-1). Bond et al. found that the Fas pathway mediated TIMP-3-induced apoptosis of human embryonic kidney 293 cells (Bond et al., 2002). Fas, a member of the tumor necrosis factor (TNF) receptor/growth factor receptor superfamily, is a 45 kD type I membrane protein, and its extracellular segment has a reserved sequence comprising 60–70 amino acid residuals. This reserved sequence is a death domain (DD) and is associated with apoptosis. Fas ligand (FasL), the natural ligand of Fas, is a member of the TNF family and a type II membrane protein, which can bind to Fas and induce apoptosis. The Fas/FasL system is a major pathway through which exogenous stimuli trigger apoptosis. In the present study, Western blot analysis revealed upregulation of Fas and FasL protein expression, suggesting that TIMP-3 regulates MC3T3-E1 cell apoptosis by activating the Fas/FasL system.

Extracellular pro-apoptotic signals can trigger the intrinsic apoptotic cascade only through intracellular signal transduction. After binding to FasL, Fas may form trimers that transmit signals, and then the trimers bind to Fas associated protein with death domain (FADD) and transmit apoptotic signals to caspase-8 through the death effector domain (DED or MORTI domain) at FADD's N-terminal, resulting in cascade reactions of the caspase family. Activated caspases react with various substrates and result in various effects, finally leading to apoptosis of Fas-expressing cells. Both endogenous and exogenous apoptotic pathways converge at caspase-3, a protease that executes apoptosis, and caspase-3 activation ultimately causes apoptosis (Ding et al., 2004). In the present study, the effect of TIMP-3 treatment on caspase-8 and -3 activation was assessed by Western blot analysis. The results demonstrated that the bands of cleaved caspase-8 and -3 were observed both in the control group and in the TIMP-3 treatment group, and the bands were increasing obviously with increasing concentrations of TIMP-3. The findings suggested that TIMP-3 treatment activated the exog-

enous (caspase-8 dependent) apoptosis pathway and induced MC3T3-E1 cell apoptosis.

In Fas-mediated apoptosis, apoptotic signals might be transduced by MAPK. MAPK were serine/threonine kinases which resided in cytoplasm and could phosphorylate both serine and tyrosine. The MAPK signal transduction pathway was one of the pathways through which extracellular signals were given rise to nuclear responses, and the pathway contributed to cell morphogenesis, kinesis, apoptosis, proliferation, differentiation, growth and other physiological processes (Tanaka et al., 2002). It was demonstrated that the MAPK signal transduction pathway contributed to osteoblast differentiation, proliferation and apoptosis, and that glucocorticosteroid-induced, Fas-mediated osteocyte apoptosis was related to ERK phosphorylation. In the present study, TIMP-3-induced MC3T3-E1 cell apoptosis, which was accompanied by changes in ERK and p38 phosphorylation, but not in JNK phosphorylation; the ERK signal transduction blocker PD098059 and the p38 signal transduction blocker SB203580 might abolish TIMP-3's effect of suppressing cell viability, while the JNK signal transduction blocker SP600125 did not affect TIMP-3's action. These findings demonstrated that ERK and p38 contribute to signal transduction in TIMP-3 induced MC3T3-E1 cell apoptosis.

In the present study, it was found that TIMP-3 may promote MC3T3-E1 cell apoptosis, the signal pathway involved was mediated by Fas, and the p38 and ERK signal transduction pathways. TIMP-3 may regulate bone metabolism by promoting osteoblasts apoptosis.

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Authors' address: Er-Yuan Liao, Institute of Metabolism and Endocrinology, The Second Xiang-Ya Hospital, Central South University, Changsha, Hunan 410011, P.R. China,
Fax: +86-0731-5361472, E-mail: allenylq@hotmail.com