Decrease of GSK3β Ser-9 Phosphorylation Induced Osteoblast Apoptosis in Rat Osteoarthritis Model*

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Summary: Nowadays, the cumulative intake of glucocorticoids has become the most common pathogenic factor for non-traumatic osteonecrosis of the femoral head (ONFH). Apoptosis of osteoblasts is considered as the main reason of ONFH at the molecular level. Glycogen synthase kinase 3β (GSK3β) is an important regulator of cellular differentiation and apoptosis pathway, which can modulate the balance between osteoblasts and osteoclasts. Several studies have reported about its function in osteoporosis, but little is known about it in osteonecrosis. In our study, lipopolysaccharide and methylprednisolone were utilized to establish a rat ONFH model. The phosphorylation of GSK3β Ser-9 was decreased in the model. Western blotting examination of β-catenin, Bcl-2, Bax and caspase-3 revealed that the osteoblasts were apoptotic. In dexamethasone (Dex)-incubated primary osteoblasts, the expression profile of GSK3β phosphorylation and apoptotic factors were consistent with those in the rat ONFH model. To further investigate the regulation of osteonecrosis caused by GSK3β, the expression and function of GSK3β were inhibited in Dex-incubated primary osteoblasts. The knockdown of GSK3β by siRNA decreased the expression of Bax and cleaved caspase-3, but increased Bcl-2 and β-catenin. On the other hand, selective inhibition of GSK3β function by LiCl counteracted the activation of caspase-3 induced by Dex. Our work is the first study about the GSK3β phosphorylation in ONFH, and provides evidence for further therapeutic methods.
Key words: osteonecrosis of the femoral head; GSK3β; phosphorylation; apoptosis; dexamethasone

Every year, tens of millions of patients suffer from pain of osteonecrosis of the femoral head (ONFH). Glucocorticoid is prescribed to patients for treatment of inflammatory or autoimmune diseases[1], but the overload of glucocorticoids has become one of the most common pathogenic factors of ONFH. Nowadays, several hypotheses have been proposed to explain the pathologic changes at the molecular level. Osteocyte apoptosis and oxidative stress are thought to be the vital cause of ONFH[2–5].

Glycogen synthase kinase 3β (GSK3β) is a pivotal regulator of cell differentiation process. It distributes in cytosol, mitochondria and nucleus[6]. In different cell types, GSK3β may modulate pro- or anti-apoptosis function[7–9]. The paradoxical apoptosis-regulating actions of GSK3β depend on the phosphorylation on serine-9 (Ser-9) and tyrosine 216 (Tyr-216). Indeed, when cells undergo apoptosis, the phosphorylation level of Ser-9 is decreased, while Tyr-216 is phosphorylated to activate GSK3β[10]. This observation has been validated by studies containing various inhibitors of GSK3β including the first-known and the most-used inhibitor, lithium[11–13]. Kinases in PI3K/Akt, Wnt/β-catenin and p38-MAPK signaling pathways could regulate the phosphorylation of Ser-9 and Tyr-216[14–17].

The functions of GSK3β in orthopedic diseases are discussed in several reports. Yun *et al* had demonstrated that activation of GSK3β facilitated the dexamethasone (Dex)-mediated apoptosis of osteoblasts in osteoporosis[7]. In another study, glucocorticoids attenuated the phosphorylation at Ser-9 to activate GSK3β, which led to the impediment of the G1/S cell cycle transition in osteoblasts[18]. The determination of cell fate by GSK3β has been studied a lot in osteoporosis, but little is known about the mechanisms in osteonecrosis.

In this study, we investigated the function of GSK3β in osteonecrosis. The establishment of

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rat ONFH model was validated by H&E staining. Phosphorylation alteration of GSK3β was detected in rat ONFH model and Dex-treated primary osteoblasts. Inhibitors of GSK3β expression and its function were used to validate the influence of GSK3β in cellular apoptosis. Our study is the first functional investigation of GSK3β in ONFH, and provides a wider perspective for disease treatment.

1 MATERIALS AND METHODS

1.1 Animals

This study was approved by the Institutional Animal Care and Use Committee of Wuhan University, followed by the guidance for the Care and Use of Laboratory Animals (1996). A total of 188 10-week-old male Sprague-Dawley rats weighing 250±20 g were got from the Hubei Provincial Center for Disease Control and Prevention, Wuhan, China. The rats were housed under specific pathogen-free (SPF) conditions. Besides, they were provided access to conventional chow and tap water *ad libitum*. All surgical operations were done under anesthesia, and all attempts were made to minimize the suffering.

1.2 Cell Culture

The primary osteoblasts were isolated from neonatal rats, and cultured according to the standard procedures in our lab. All procedures were performed under sterile conditions. Briefly, rats were soaked in 75% ethanol. After washing by sodium phosphate buffer (SPB, Hyclone, USA), the skulls and periosteum were obtained. Blood vessels and connective tissues were removed carefully. Then skulls were cut into pieces and transferred into a culture flask. Four mL trypsin was added into the flask for 5 min. After digestion was completed, Ham's F-12 Nutrient Mixture (Hyclone, USA) containing 10% fetal bovine serum (FBS) (Gibco, USA) was used to terminate the digestion. The skull pieces were incubated with 0.1% collagenase Ⅰ solution for 30 min, then centrifuged to collect the osteoblasts, which were cultured in Ham's F-12 Nutrient Mixture containing 10% FBS at 37°C with 5% CO_2. One h later, the flask was turned over and the osteoblasts were continuously incubated. Once primary culture cells reached confluence over the entire bottom of the flask, they were ready for passage. Third generation cells were used in these experiments.

1.3 Materials

Lithium chloride (LiCl), LPS (Escherichia coli serotype 055: B5) and Dex were purchased from Sigma-Aldrich (USA). Methylprednisolone (MPS) was purchased from Pfizer Pharmaceutical, China. Phosphorylation-specific antibodies against GSK3β at Ser-9 (catalog no. 9336) and mouse anti-GSK3β monoclonal antibody (catalog no. 9315) were purchased from Cell Signaling (USA). Other primary antibodies used in this study, rabbit anti-Bcl-2 polyclonal antibody (ab196495), rabbit anti-Bax monoclonal antibody (ab32503), rabbit anti-beta-catenin monoclonal antibody (ab32572) were purchased from Abcam Biotechnology (USA). Rabbit anti-caspase-3 polyclonal antibody and mouse anti-GAPDH monoclonal antibody (no. 60004) were purchased from ProteinTech Biotechnology (USA).

1.4 Experimental Design

In animal experiments, all rats were randomly divided into two groups. (1) ONFH model group (n=9): rats were injected with LPS and MPS in the first week. On the day 1 and 2, rats were given an intravenous injection of LPS (1.8 mg/kg). On the day 3 to 7, the animals were given 25 mg/kg MPS intramuscularly to promote the development of femoral head necrosis as described by Okazaki *et al*[19]. Rats were given 0.9% saline over the next 3 weeks. (2) Control group (n=9): all rats were given 0.9% saline during the four weeks. Animals were sacrificed at the end of the fourth week with an overdose injection of pentobarbital sodium. Subsequently, bilateral femoral heads from each rat were excised and put in liquid nitrogen immediately. They were stored at −80°C until analysis.

In cellular experiments, 1×10^8 primary osteoblasts were cultured in 25-cm dishes. Then different dosage of Dex was incubated with the cells for 24 h to induce apoptosis. When LiCl or si-GSK3β was used in the experiment, cells were pretreated with 25 mmol/L LiCl for 1 h or 25 nmol/L si-GSK3β for 24 h before incubation with Dex. Then proteins were extracted immediately and prepared for the following experiment.

1.5 Western Blotting

Osteoblasts were processed by M-PER mammalian protein extraction reagent (Pierce, USA) and protease inhibitor cocktail set Ⅲ (Calbiochem, Germany) plus 5 mmol/L EDTA. A total of 10 μg of protein was loaded and separated by 10% SDS-PAGE. Then, the separated proteins were transferred to a PVDF membrane and blocked with 5% non-fat milk in Tris-buffer saline containing 0.05% Tween-20 (TBST). The membranes were incubated with the corresponding primary antibodies for 1 h at room temperature or overnight at 4°C. Then, the membranes were washed three times and incubated with a horseradish peroxidase-conjugated secondary antibody (1:10 000) in TBST for 1 h at room temperature. All specific bands were visualized by an ECL system kit (Pierce Biotechnology, China). All tests were repeated three times.

1.6 SiRNA Transfection and Real-time PCR Analysis

All siRNAs used were synthesized by GenePharma (China). The sequence of si-GSK3β oligonucleotides used in this study was 5′-AAGTAATCCACCTCTGGC-TAC-3′. Primary osteoblasts were transfected with si-GSK3β or with a negative control siRNA using Lipofectamine RNAiMAX (Invitrogen, USA) according

to the manufacturer's instructions. At 24th h after the transfection, cells were incubated with Dex for another 24 h or harvested to detect the knockdown efficiency. GAPDH was set as an endogenous control. All experiments were repeated at least three times.

2 RESULTS

2.1 Phosphorylation of GSK3β Ser-9 in Rat ONFH Model

Eighteen rats were used in this experiment. Throughout the study, none of the rats was accidentally dead. The mean body weight was nearly the same between the two groups (fig. 1A). After injection of LPS and MPS, all rats in ONFH model group presented severe osteonecrosis in behavior. The animal model establishment was also validated by microscopic morphology (fig. 1B). So we harvested the femoral heads and extracted the proteins for further detection.

Several proteins in cellular apoptosis pathway were utilized here. The proportion of Bcl-2/Bax could regulate apoptosis. When Bcl-2 was decreased and Bax was increased, the downstream caspase-3 was cleaved to initiate apoptosis. Meanwhile, Wnt/β-catenin pathway is involved in apoptosis by regulating target downstream genes. It was reported that β-catenin was decreased in rat ONFH model[17]. Western blotting revealed that cellular apoptosis process was activated in the rat ONFH model (fig. 2). At the same time, the expression of GSK3β was slightly increased while phosphorylation level of GSK3β at Ser-9 was significantly decreased. These results implied an important function of GSK3β phosphorylation at Ser-9 in osteonecrosis.

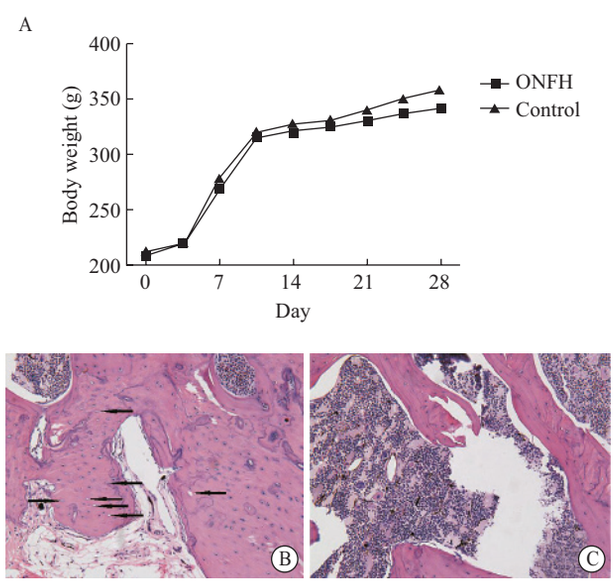

Fig. 1 Validation of rat ONFH model
A: body weight over time of rats in the ONFH model group and control group. ONFH: ONFH model group. Control: control group. B and C: histological appearance of the femoral head in ONFH model group (B) and control group (C). The arrows indicate the empty lacunae. The original magnification of H&E staining: ×200.

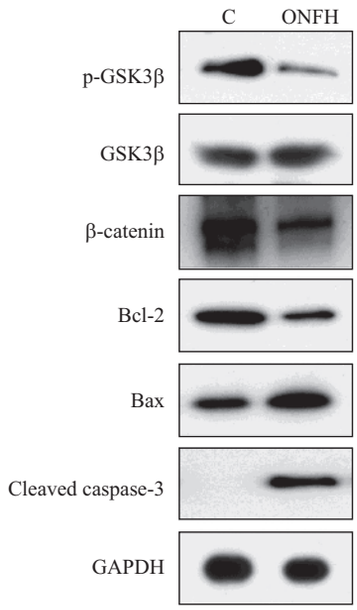

Fig. 2 Protein expression or phosphorylation changes in rat ONFH model
The expression levels of phospho-GSK3β, GSK3β, β-catenin, Bcl-2, Bax and cleaved caspase-3 were detected by Western blotting in ONFH and normal rats. GADPH was utilized to prove equal amount of protein loading in each lane. ONFH: ONFH model group. C: control group

2.2 Phosphorylation of GSK3β Ser-9 in Primary Osteoblasts Incubated with Dex

Prior to the cellular experiments, we detected the cytotoxic effects of Dex in different dosages. Previous study revealed that Dex at a concentration range from 1 to 10 μmol/L had no effect on cell viability in 48 h. So we incubated primary osteoblasts with Dex in a dose-dependent and time-dependent manner.

In the dose-dependent manner, 1×10^8 primary osteoblasts were treated with 10, 100 nmol/L and 1 μmol/L Dex for 24 h. During Dex-induced apoptosis, Bax and cleaved caspase-3 were increased, and Bcl-2 and β-catenin decreased (fig. 3A). Phosphorylation of GSK3β at Ser-9 was also decreased, indicating the activation of GSK3β, which was coordinated with the initiation of apoptosis. In the time-dependent manner, osteoblasts were incubated with 1 μmol/L Dex for 4, 8, 16 and 24 h. The phosphorylation of GSK3β at Ser-9 was decreased as early as 4 h (fig. 3B). This implied that the influence of Dex on GSK3β was at early stage of apoptosis.

2.3 Knockdown of GSK3β in Primary Osteoblasts Incubated with Dex

The above experiments demonstrated that Dex could alter the phosphorylation of GSK3β. But it was still unknown whether the Dex-induced apoptosis was

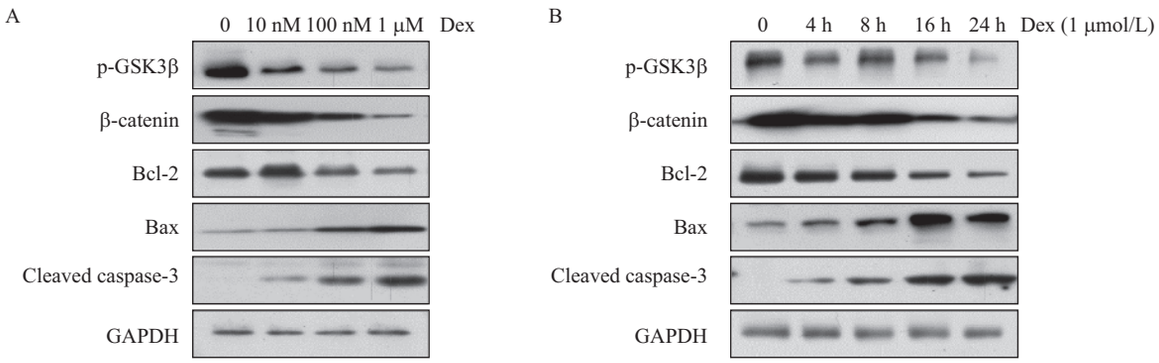

Fig. 3 Dosage-dependent and time-dependent influences of Dex on primary osteoblast apoptosis
A: 1×10^8 primary osteoblasts were treated with 10 nmol/L, 100 nmol/L and 1 μmol/L Dex for 24 h. B: Osteoblasts were incubated with 1 μmol/L Dex for 4, 8, 16 and 24 h. The expression levels of phospho-GSK3β, β-catenin, Bcl-2, Bax and cleaved caspase-3 were detected by Western blotting. GADPH was utilized to prove equal amounts of protein loading in each lane.

mediated by GSK3β. So we synthesized chemical siRNA targeting GSK3β, and transfected it into the osteoblasts before Dex treatment.

The knockdown efficiency was validated by Western blotting. As shown in fig. 4, cells were treated with 1 μmol/L Dex for 24 h. When intracellular GSK3β was knocked down, the expression of Bax and cleaved caspase-3 was decreased, and that of Bcl-2 was increased. This illustrated that GSK3β was upstream of Bcl-2/Bax in Dex-induced apoptosis.

2.4 Selective Inhibition of GSK3β Function by LiCl

The regulation of GSK3β on osteonecrosis could be contributed to the expression alteration or post translational modification profile change. To further investigate the determinant factor of the influence, we used a well-known specific GSK3β inhibitor, LiCl. LiCl promotes the phosphorylation of GSK3β at Ser-9 but not the other phosphorylation sites, and neither affects the expression of background GSK3β.

Primary osteoblasts were pretreated with 25 mmol/L LiCl for 1 h to inhibit the activity of GSK3β, and then incubated with 1 μmol/L Dex for additional 24 h. When LiCl was added, the phosphorylation level of GSK3β at Ser-9 was significantly upregulated. Compared to the Dex group, LiCl counteracted the activation of caspase-3 induced by Dex. The results validated the vital role of phosphorylation of GSK3β at Ser-9 in modulating osteonecrosis.

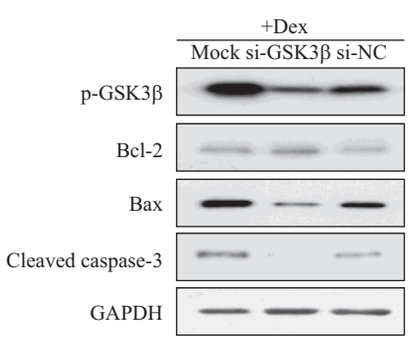

Fig. 4 Knockdown of GSK3β in Dex-treated osteoblasts
Cells were transfected with siRNA before Dex treatment, and then treated with 1 μmol/L Dex for 24 h. The expression levels of phospho-GSK3β, Bcl-2, Bax and cleaved caspase-3 were detected by Western blotting. GADPH was utilized to prove equal amounts of protein loading in each lane. si-GSK3β: siRNA targeting GSK3β; si-NC: nonsense siRNA

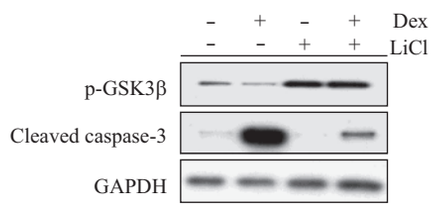

Fig. 5 Promotion of GSK3β Ser-9 phosphorylation by LiCl in Dex-treated osteoblasts
Cells were pretreated with 25 mmol/L LiCl for 1 h, and then incubated with 1 μmol/L Dex for additional 24 h. The expression levels of phospho-GSK3β and cleaved caspase-3 were detected by Western blotting. GADPH was utilized to prove equal amounts of protein loading in each lane.

3 DISCUSSION

ONFH has become more and more prevalent around the world. Many investigators had tried to explain the pathogenesis from the aspect of cellular apoptosis. The PI3K/Akt-Bax/Bcl-2/caspase3 pathway is one of the most studied apoptotic signaling pathways in orthopedic diseases[20–22], and phosphorylation of GSK3β is the crucial step. In our study, phosphorylation of GSK3β at Ser-9 was validated in rat ONFH model and primary osteoblasts. When Dex initiated cellular apoptosis, phosphorylation at Ser-9 was decreased to activate GSK3β. We further demonstrated that GSK3β mediated Dex-induced osteonecrosis by knocking

down the GSK3β expression and inhibiting its activity. Taken together, we provided evidence of anti-apoptosis function of Ser-9 phosphorylation in femoral heads.

Although GSK3β is predominant in cytosol, researchers have proved that GSK3β exists in mitochondria and nucleus as well. When GSK3β locates in cytosol or resides on mitochondrion, it may initiate intrinsic apoptotic signaling cascade by DNA damage, oxidative stress and endoplasmic reticulum stress[23]. This is mediated by Bcl-2 family of proteins and results in the disruption of mitochondria, which causes the activation of caspases and cellular apoptosis[24]. Besides, nuclear GSK3β exhibits a greater activity than cytosolic GSK3β[25, 26]. As is known, many transcription and translation factors including p53 and cyclic AMP response element binding protein (CREB) could be phosphorylated by active GSK3β. This contributes to the intrinsic apoptotic signaling pathway initiated by differential gene expression. In our study, GSK3β was regulated soon after Dex treatment. This could be explained by the apoptotic signal transduction on mitochondrion. But whether the nuclear factors participate in osteonecrosis at the early stage requires for the subcellular localization detection of GSK3β at 4 h post-treatment.

When primary osteoblasts are incubated with Dex, the oxidative stress pathway is also activated. This takes place on mitochondrion, which may crosslink with GSK3β. The downregulation of GSK3β Ser-9 could initiate the apoptotic pathway independent of oxidative stress, at the same time, GSK3β with higher activity increases the intracellular oxidative stress. Reactive oxygen species (ROS), is the key product of oxidative stress. It can also activate the apoptotic pathway independent of GSK3β. Whether GSK3β and ROS could interact in osteonecrosis has become an interesting problem. In obese osteoarthritis patients, GSK3β inactivation is responsible for ROS production, triggering oxidative stress and DNA damage response[27]. Similarly, Li *et al* had revealed that lithium could increase the generation of ROS and lead to decreased cell survival and proliferation via the ROS/GSK3β/NF-κB pathway[28]. On the contrary, a study on apoptosis in granulocyte-differentiated cells implied that ROS initiates GSK3β-dependent apoptosis. It is hypothesized that the interaction of GSK3β and ROS may be various in different diseases and cell lines. Thus it is worth a greater effort to investigate the interaction in osteonecrosis.

In conclusion, our study has demonstrated the protective role of GSK3β Ser-9 phosphorylation in Dex-induced apoptosis. The decrease of GSK3β expression and its activity inhibited the initiation of Bax/Bcl-2-mediated cellular apoptosis. Our work is the first one to illustrate the GSK3β phosphorylation and cell fate determination in rat ONFH model and primary osteoblasts. It could be a promising therapeutic strategy for clinical treatment.

Conflict of Interest Statement

There are no conflicts to declare.

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