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Metformin alleviates hyperglycemia-induced apoptosis and differentiation suppression in osteoblasts through inhibiting the TLR4 signaling pathway

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ABSTRACT

Aims: Metformin was found to protect against hyperglycemia-induced injury in osteoblasts, but the cellular mechanisms involved remain unclear. Therefore, the aim of this study was to determine the effect of metformin on hyperglycemia-induced apoptosis and differentiation suppression in osteoblasts and to explore its relationships with the TLR4 signaling pathway.

Main methods: A mouse osteoblast cell line, MC3T3-E1, and a diabetic rat model were used to survey the protective effects of metformin on hyperglycemia-induced injury. TLR4 expression was altered using small interfering (si)RNA and lentivirus-mediated TLR4 overexpression. LPS was used as a specific TLR4 activator, and CLI-095 was used as a TLR4 inhibitor.

Key findings: Metformin improved osteoblast differentiation, reduced apoptosis in hyperglycemic osteoblasts, and inhibited TLR4, MyD88 and NF-κB expression in a dose-dependent manner. Down-regulating the expression or inhibiting the activity of TLR4 enhanced these protective effects of metformin on osteoblast differentiation, cell viability and cell apoptosis in hyperglycemic conditions, whereas up-regulating the expression or activating the activity of TLR4 had the opposite effects. Activating NF-κB suppressed the protective effects of metformin, while inhibiting NF-κB activity had the opposite effects. Metformin increased ALP and OCN secretion, enhanced BMP-2 expression, improved bone mineral density (BMD), and decreased TLR4, MyD88 and NF-κB levels in the femur tissues of diabetic rats.

Significance: Taken together our experimentation support the hypothesis that metformin may alleviate hyperglycemia-induced apoptosis and differentiation suppression in osteoblasts by inhibiting the TLR4/MyD88/NF-κB signaling pathway.

1. Introduction

According to studies from around the world, both type 1 and type 2 diabetes patients have a higher prevalence of osteoporosis, and fractures due to osteoporosis can result in disability and even death from diabetic complications [1–3]. A pervasive feature of diabetes mellitus is the chronic, low-level state of systemic and sterile inflammation. Epidemiologic studies have established an association between inflammatory biomarkers and the occurrence of diabetes mellitus and complications [4–6]. Previous studies have reported a definite association between diabetic cardiomyopathy and inflammation [7,8]. Hyperglycemia, the major feature of diabetes, can cause osteoblast dysfunction by activating inflammation and apoptosis [9,10]. Therefore, hyperglycemia is one of the main causes of osteoporosis [11,12]. However, the mechanisms of the deleterious effects of hyperglycemia on metabolic bone disease in diabetes mellitus (DM) are still unknown.

Toll-like receptors (TLRs) are a family of receptors that play a critical role in innate and adaptive immune response activation [13]. In individuals with type 2 diabetes, there is a tremendous increase in TLR4 expression, as well as an increase in the levels of endogenous ligands and their activated downstream signaling cascades, such as NF-κB signaling [14,15]. Some previous studies have shown that the effects of TLR4 on bone metabolism are related to inhibiting osteoblast differentiation [16,17]. One study has recently reported that lipopolysaccharide (LPS) could inhibit BMP-2-induced osteoblast differentiation by activating the TLR4/MyD88/NF-κB signaling pathway [16]. Another study showed that osteoblast differentiation was inhibited in a TLR4-dependent manner in an LPS-stimulated inflammatory environment

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[17]. However, the effect of TLR4 on hyperglycemia-induced osteoblast dysfunction has not been determined.

The actions of metformin, which is commonly used to manage type 2 DM, are modulated through multiple pathways [18–20]. Many previous experimental and clinical outcomes have suggested that metformin, apart from its hypoglycemic action, may increase bone mineral density (BMD) to consequently reduce the frequency of fractures in diabetic patients [20–22]. Recently, metformin was also found to protect osteoblasts and improve bone formation ability [23]. However, metformin's mechanism of action and its effects on osteoblasts have not yet been documented. It has been suggested that metformin can attenuate the TLR4 pathway in the skeletal muscle tissue of diabetic rats [24]. Moreover, TLR4 may mediate the mechanism involved in bone formation suppression in diabetic mice [25]. However, there have been no relevant reports suggesting that the protective effects of metformin on hyperglycemic osteoblasts are related to TLR4 activation. Thus, the aim of the present study was to elucidate the relationship between the protective effects of metformin on hyperglycemic osteoblasts and the expression of TLR4, MyD88 and NF-κB.

2. Materials and methods

2.1. Cell culture

Mouse MC3T3-E1 pre-osteoblastic cells were cultured in complete medium [Low glucose Dulbecco modified Eagle medium (LDMEM; 5.6 mmol/L glucose) supplemented with +50 mg/L ascorbic acid + 0.5 mmol/L β-glycerophosphate + 2 mmol/L L-glutamine] supplemented with 10% fetal bovine serum (FBS) in a 5.0% CO_2 humidified atmosphere at 37 °C. The MC3T3-E1 cell line was purchased from Beijing Dingguo Changsheng Biotechnology (China), and all reagents were obtained from Gibco (USA). Cells at passages 15 to 18 were used for the experiments. The cell seeding density was $3 * 10^5$/ml.

2.2. Intervention

2.2.1. High glucose intervention

MC3T3-E1 cells were cultured in 6-well plates with complete medium for 24 h until the cells reached 40% confluence. To obtain high glucose (HG) conditions, the culture medium was removed, and the cells were treated with HG solution [25 mmol/L glucose in Low glucose Dulbecco modified Eagle medium (LDMEM) with 50 mg/L ascorbic acid + 0.5 mmol/L β-glycerophosphate + 10% FBS + 2 mmol/L L-glutamine] for 7 days. Cells treated with complete medium were used as controls.

2.2.2. Metformin intervention

To observe the effects of metformin on HG-induced damage, MC3T3-E1 cells were cultured in HG for 7 days and then treated with metformin [dissolved in 0.1% dimethylsulfoxide DMSO (v/v); BN1504091105; Chia Tai Tiangqing, China] for 7 days. Metformin was dissolved in complete culture medium at a final concentration of 10 mmol/L before use. This prepared metformin solution was then diluted to 25, 50, and 100 μmol/L for treatment. HG-induced cells without metformin and un-induced cells exposed to metformin were used as controls.

2.2.3. LPS intervention

To study whether TLR4 was involved in the ability of metformin to alleviate the hyperglycemia-induced effects, lipopolysaccharide (LPS) was used as a specific activator of TLR4 and served as a positive control. MC3T3-E1 cells were cultured in 6-well plates with complete medium for 24 h until the cells reached 40% confluence. Then, the cells were pretreated with 20 μg/mL LPS (dissolved in 0.1% DMSO (v/v); Sigma-Aldrich) in complete medium for 7 days. The medium was removed, and the cells were washed twice with PBS, and then treated with 100 μmol/L metformin with complete medium for 7 days. LPS-induced cells without metformin and un-induced cells exposed to metformin were used as controls.

2.2.4. TLR4 agonist and inhibitor interventions

To study the effect of TLR4 on the protective effects of metformin in HG-induced osteoblast damage, MC3T3-E1 cells were pretreated with high glucose solution for 7 days, and then exposed to 4 μmol/L TLR4 inhibitor [CLI-095, dissolved in 0.1% DMSO (v/v); Sigma-Aldrich] + 100 μmol/L metformin or 20 μg/mL TLR4 agonist [LPS, dissolved in 0.1% DMSO (v/v); Sigma-Aldrich] + 100 μmol/L metformin with complete medium for 7 days. HG-induced cells without CLI-095 or LPS and un-induced cells exposed to CLI-095 or LPS were used as controls.

2.2.5. NF-κB agonist and inhibitor interventions

To study the effect of NF-κB on the protective effects of metformin in HG-induced osteoblast damage, MC3T3-E1 cells were pretreated with high glucose solution for 7 days, and then exposed to 10 ng/mL NF-κB agonist [TNF-α, dissolved in 0.1% DMSO (v/v); Sigma-Aldrich] + 100 μmol/L metformin or 20 μmol/L NF-κB inhibitor [PDTC, dissolved in 0.1% DMSO (v/v); Sigma-Aldrich] + 100 μmol/L metformin with complete medium for 7 days. HG-induced cells without TNF-α or PDTC and un-induced cells exposed to TNF-α or PDTC were used as controls.

2.3. TLR4 small interfering (si)RNA, TLR4 cloning construct, and adenovirus preparation

siRNA targeting mouse TLR4 mRNA (GenBank Accession Number: 021297.2; CCACCUCUCUACCUUAAUA) was designed and synthesized by GenePharma Co, Ltd. (Shanghai, China), and multiple siRNA oligonucleotides, including negative siRNA, were created using primer design principles. An RNAi lentiviral vector and a lentiviral vector carrying the cDNA of TLR4 were prepared and packaged by Genechem Co (Shanghai, China). MC3T3-E1 cells were infected with the RNAi lentiviral vector or TLR4-expressing lentiviral vector at 80 multiplicity of infection (MOI). After 12 h of incubation, the medium was exchanged for fresh DMEM medium containing 0.3% FBS, and the cells were incubated for 72 h. TLR4 protein expression was detected by Western blot, and the cells were then used for the experiments.

2.4. Animal experiments

Specific pathogen-free (SPF) grade, five-week-old male Sprague-Dawley (SD) rats (210 ± 10 g) were provided by the laboratory animal facility at Fujian Medical University (animal certification number SCXK (HU)2011-0031) and maintained in a pathogen-free environment with a 12 h light/dark cycle and free access to food and water. This study was approved by the Ethics Committee of Biomedical Research of the First Affiliated Hospital of Fujian Medical University. After acclimatization, the rats were divided randomly into two groups fed different diets ($n = 20$ for each group): a normal control diet (NC, kcal %: 10% fat, 20% protein, and 70% carbohydrate; 3.85 kcal/g)) and a high fat diet (HFD, kcal %: 45% fat, 20% protein, and 35% carbohydrate; 4.73 kcal/g). The control rats were subdivided into two groups, half of which were treated with metformin (900 mg/kg/d; $n = 10$; Chia Tai Tianqing, China), while the other half remained untreated (n = 10). All control rats were fed the normal rodent diet for 24 weeks. The HFD rats received a HFD for 16 weeks to induce obesity, and diabetes was induced in the rats by streptozotocin (STZ, 30 mg/kg) injection. Blood glucose levels were determined using blood collected from the tail vein after 72 h, and levels higher than 16.7 mmol/L for three consecutive days were considered standard for the diabetic model. The rats in the NC group were injected intraperitoneally with a similar dose of normal saline. A total of 20 diabetic rats were divided into a metformin-treated

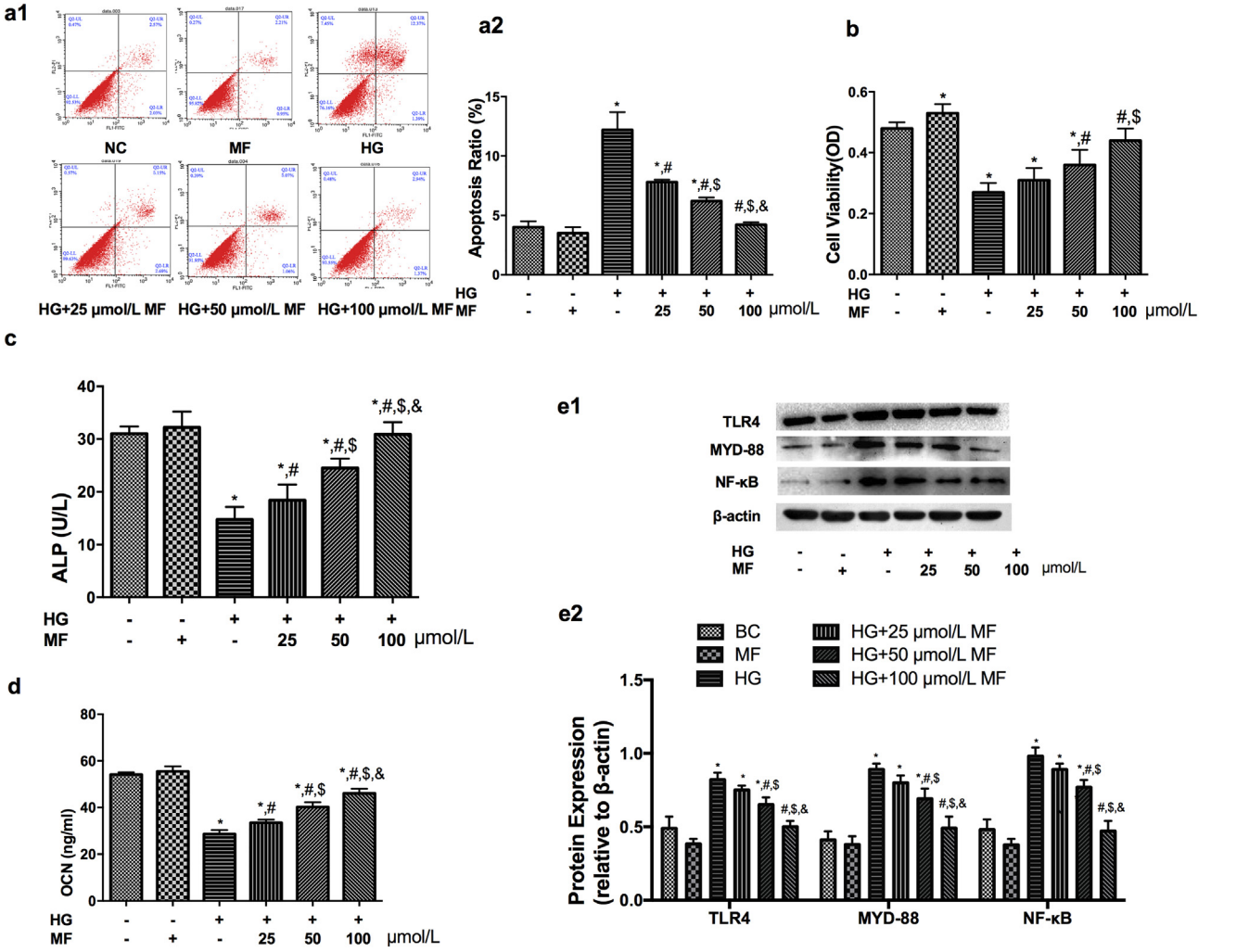

Fig. 1. The protective effects of metformin on hyperglycemic osteoblasts. MC3T3-E1 cells were exposed to 25 mmol/L glucose for 24 h and then cultured with different concentrations (25, 50, or 100 μmol/L) of metformin for 72 h. All data are presented as the mean ± SD of three independent experiments. (a) Flow cytometric analysis of apoptotic cells stained with Annexin V and propidium iodide (PI). (a1) Representative FACS images in each group. Shown is one of three representative experiments that yielded essentially similar results. (a2) Collective analyses of all three independent experiments. (b) Osteoblast viability was assessed by MTT assays. (c, d) Bone turnover markers were measured using ELISA kits. (e) TLR4, MyD88, NF-κB and BMP-2 expression levels were detected by Western blotting. (e1) Representative Western blot images of each group. (e2) Ratio of the target proteins to β-actin. $*P < 0.05$ vs. the BC group (without glucose or metformin), $\#P < 0.05$ vs. the HG group (25 mmol/L glucose), $\$P < 0.05$ vs. the HG + 25MF group (25 mmol/L glucose + 25 μmol/L metformin), $\&P < 0.05$ vs. the HG + 50MF group (25 mmol/L glucose + 50 μmol/L metformin).

group (900 mg/kg·d for 16 weeks, $n = 10$) or a type 2 DM group (intragastric administrationof the same volume of saline, n = 10) using a random number table. Body weights and lengths were recorded once per week for the duration of the study.

2.5. Detection of bone mineral density

After receiving their corresponding interventions, the rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (0.03 mL/kg), and then BMD of femur tissue was detected via dual-energy X-ray absorptiometry (DAXE, prodigy, GE LUNAR, USA).

2.6. Enzyme-linked immunosorbent assay (ELISA)

2.6.1. Serum bone turnover markers

The control and experimental rats were fasted overnight for 8 h and then anesthetized with an intraperitoneal injection of 10% chloral hydrate (0.03 mL/kg). Abdominal aorta blood samples were clotted for 2 h at room temperature and then centrifuged at 3000 rpm for 10 min. The serum samples were assayed immediately or aliquoted and stored at ≤ −20 °C to avoid repeated freeze-thaw cycles. Osteocalcin (OCN), ALP and tartrate-resistant acid phosphatase (TRAP)5b were measured by ELISA kits (Cusabio, China) according to the manufacturer's instructions.

2.6.2. Supernatant ALP and OCN levels

Cell culture supernatant samples collected from both the control and intervention groups were tested for OCN and ALP levels by ELISA kits (Cusabio, China) according to the manufacturer's instructions.

2.7. Flow cytometric analysis

MC3T3-E1 cells were seeded in 6-well plates. Cells were collected after treatment with 0.25% EDTA-free trypsin and then centrifuged at 10,000 rpm for 5 min. The cells were washed with PBS, and an Annexin V-FITC/PI Apoptosis Detection Kit (BD Biosciences, USA) was used to estimate cell apoptosis. MC3T3-E1 cells were harvested by centrifugation, and labeling was performed by serial addition of annexin V and propidium iodide in the dark at room temperature, according to the instruction of manufacturer. Cell apoptosis was detected using a

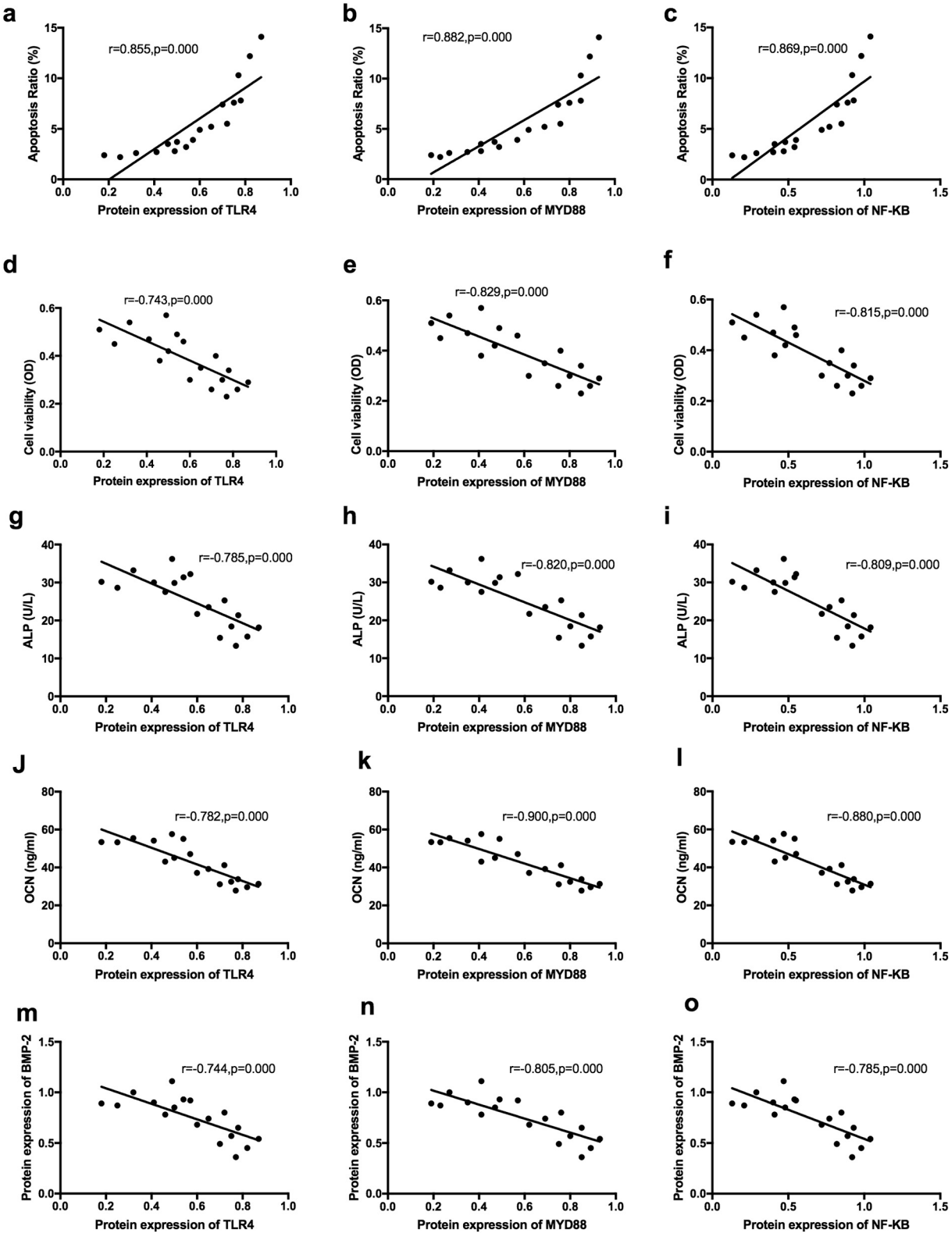

Fig. 2. The protective effect of metformin on hyperglycemic osteoblasts is related to the inhibition of TLR4, MyD88 and NF-κB expression. (a–c) Relationships between apoptosis rate and the expression of TLR4/MyD88/NF-κB. (d–f) Relationships between cell viability and the expression of TLR4/MyD88/NF-κB. (g–i) Relationships between ALP levels and the expression of TLR4/MyD88/NF-κB. (j–l) Relationships between OCN levels and the expression of TLR4/MyD88/NF-κB. (m–o) Relationships between BMP-2 expression and the expression of TLR4/MyD88/NF-κB.

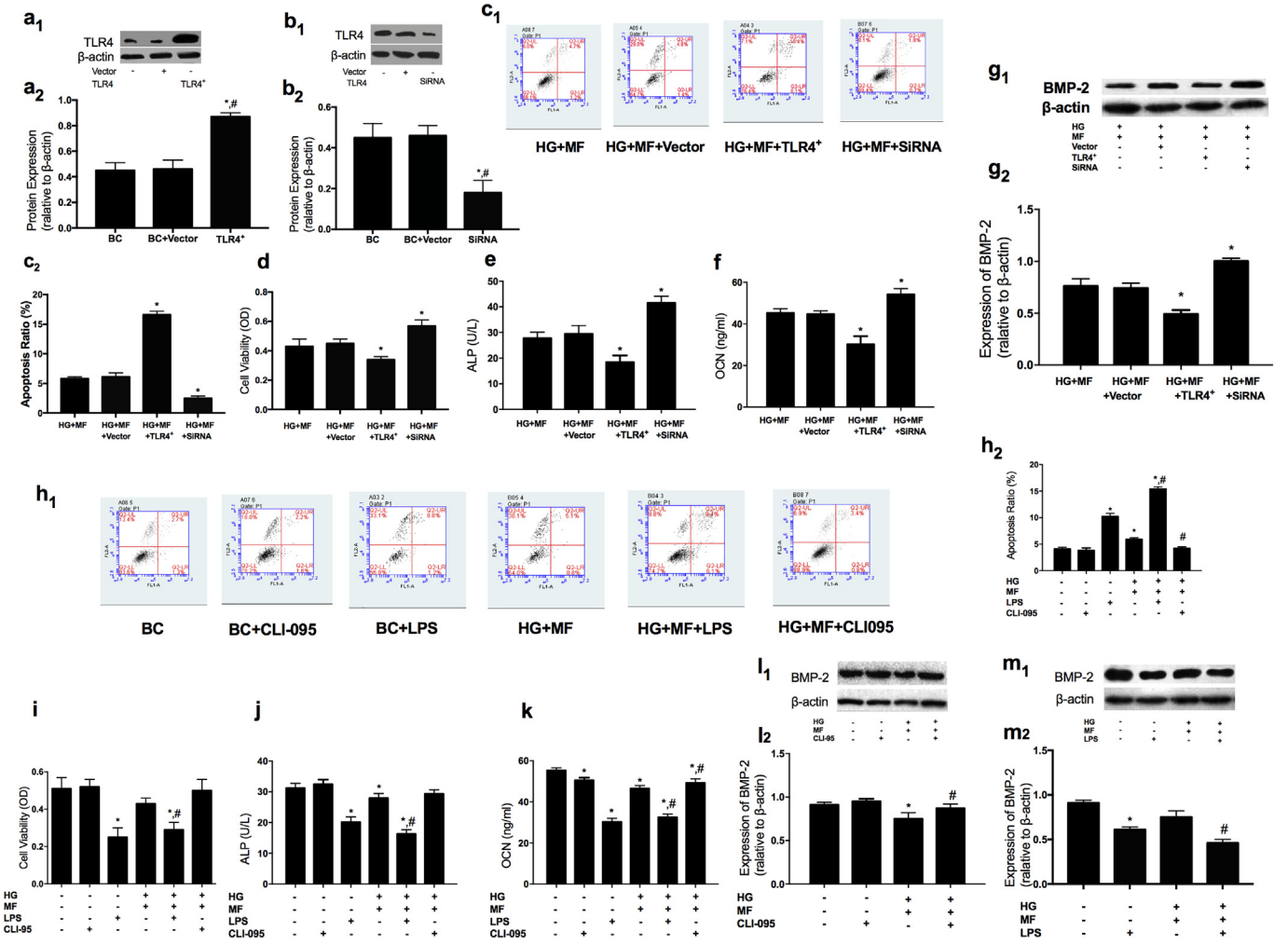

Fig. 3. The effect of TLR4 on metformin-mediated hyperglycemia-induced injury attenuation in osteoblasts. (a) TLR4 expression was increased by lentiviral overexpression. (a1) Representative Western blot images for each group. (a2) The ratio of the target protein to β-actin. $^{*}P < 0.05$ vs. BC group, $^{\#}P < 0.05$ vs. BC + Vector group. (b) TLR4 expression was decreased by TLR4 siRNA. (b1) Representative Western blot images for each group. (b2) The ratio of the target protein to β-actin. $^{*}P < 0.05$ vs. BC group, $^{\#}P < 0.05$ vs. BC + Vector group. (c–g) Relationship between the protective effect of metformin and TLR4 expression. (c) Flow cytometric analysis of apoptotic cells stained with Annexin V and PI. (d) Osteoblast viability was assessed by MTT assays. (e, f) Bone turnover markers were measured using ELISA kits. (g) BMP-2 expression was detected by Western blotting. $^{*}P < 0.05$ vs. HG + MF group. (h–m) Relationship between the anti-glucotoxicity effect of metformin and TLR4 activity. (h) Flow cytometric analysis of apoptotic cells stained with Annexin V and PI. (i) The osteoblast viability was assessed by MTT. (j, k) The influence on the bone turnover markers was measured using ELISA kits. (l) The expression of BMP-2 was detected by Western blotting. (l1) Representative Western blot images in each group. (l2) The ratio of the target protein to β-actin. (m) The expression of BMP-2 was detected by Western blotting. (m1) Representative Western blot images in each group. (m2) The ratio of the target protein to β-actin. $^{*}P < 0.05$ vs. the BC group (complete medium), $\#P < 0.05$ vs. the HG + MF group (25 mmol/L glucose + 100 μmol/L metformin).

Cytomics™ FC500 Flow Cytometer (Beckman Coulter, Brea, CA, USA).

2.8. Cell viability assay

MC3T3-E1 cells in 96-well plates (2–3 × 10^3 cells/well) were treated with the corresponding intervention. Osteoblast viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays as previously reported [26].

2.9. Protein extraction and Western blotting

The femur tissues were crushed in liquid nitrogen, and 100 mg of bone tissue was added to 1 ml of protein lysate (containing PMSF). Then, the supernatants were removed after low temperature and high-speed centrifugation. Femur or MC3T3-E1 cells were lysed in radioimmune precipitation assay buffer containing 50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS with protease inhibitors on ice for 30 min. Protein concentrations were determined with a bicinchoninic acid (BCA) protein assay kit (Beyotime Biotechnology, China). Equal amounts of protein were separated by 5% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Sigma, USA). The membranes were blocked with 5% non-fat milk in TBST for 1 h at room temperature and then incubated with anti-TLR4(1:500), anti-p-NF-κB p65(1:500), anti-MyD88(1:500), anti-BMP-2(1:500), or anti-β-actin antibodies(1:200) (all purchased from Abcam, UK) overnight at 4 °C. The membranes were incubated with a horseradish-peroxidase-labeled secondary antibody (1:500) for 1 h at room temperature. The protein signals were visualized using an enhanced chemiluminescence detection system. The gray values of the blots were quantified using Image-Pro Plus 6.0 software and normalized to the corresponding β-actin values as an internal control.

2.10. Statistical analysis

All statistical calculations were carried out using SPSS for Windows

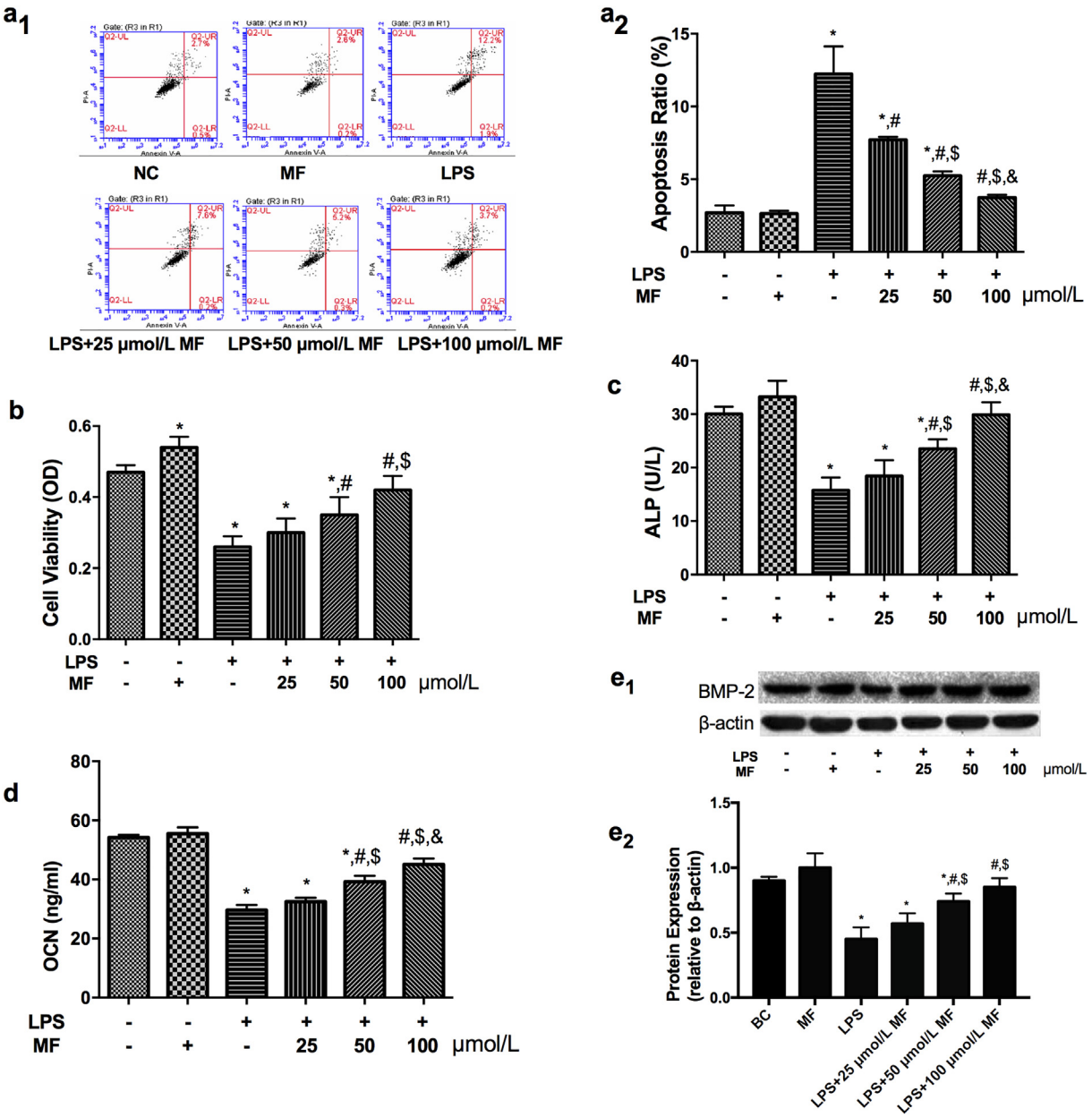

Fig. 4. The protective effects of metformin on LPS-induced osteoblast apoptosis and differentiation suppression. MC3T3-E1 cells were exposed to 20 μg/mL LPS for 24 h and then cultured with different concentrations (25, 50, or 100 μmol/L) of metformin for 72 h. All data are presented as the mean ± SD of three independent experiments. (a) Flow cytometric analysis of apoptotic cells stained with Annexin V and PI. (a1) Representative FACS images for each group. Shown is one of three representative experiments that yielded essentially similar results. (a2) Collective analyses of all three independent experiments. (b) Osteoblast viability was assessed by MTT assays. (c, d) Bone turnover markers were measured using ELISA kits. (e) BMP-2 expression was detected by Western blotting. (e1) Representative Western blot images for each group. (e2) The ratio of the target protein to β-actin. *$P < 0.05$ vs. the BC group (without LPS or metformin), #$P < 0.05$ vs. the LPS group (20 μg/mL LPS), \$$P < 0.05$ vs. the LPS + 25 μmol/L MF group (20 μg/mL LPS + 25 μmol/L metformin), &$P < 0.05$ vs. the LPS + 50 μmol/L MF group (20 μg/mL LPS + 50 μmol/L metformin).

version 21.0 (SPSS Inc., USA). A Shapriro-Wilk's test and a visual inspection of normal Q-Q plots showed that the date was normally distributed. The results are expressed as the mean ± standard deviation (SD). One-way ANOVA and Student's unpaired *t*-test were used for the statistical analyses. Pearson's correlation was used to examine the relationship between TLR4 protein expression and the osteoblast apoptosis rate and ALP, OCN and BMP-2 levels. For all tests, $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Metformin attenuated hyperglycemia-induced injury in osteoblasts and inhibited the TLR4 signaling pathway

Metformin significantly reduced apoptosis (Fig. 1a) in hyperglycemic osteoblasts in a dose-dependent manner and increased cell viability (Fig. 1b), ALP (Fig. 1c) and OCN (Fig. 1d) secretion. The results also showed that the protein levels of TLR4, MyD88 and NF-κB in osteoblasts were decreased by metformin treatment in a dose-dependent manner (Fig. 1e).

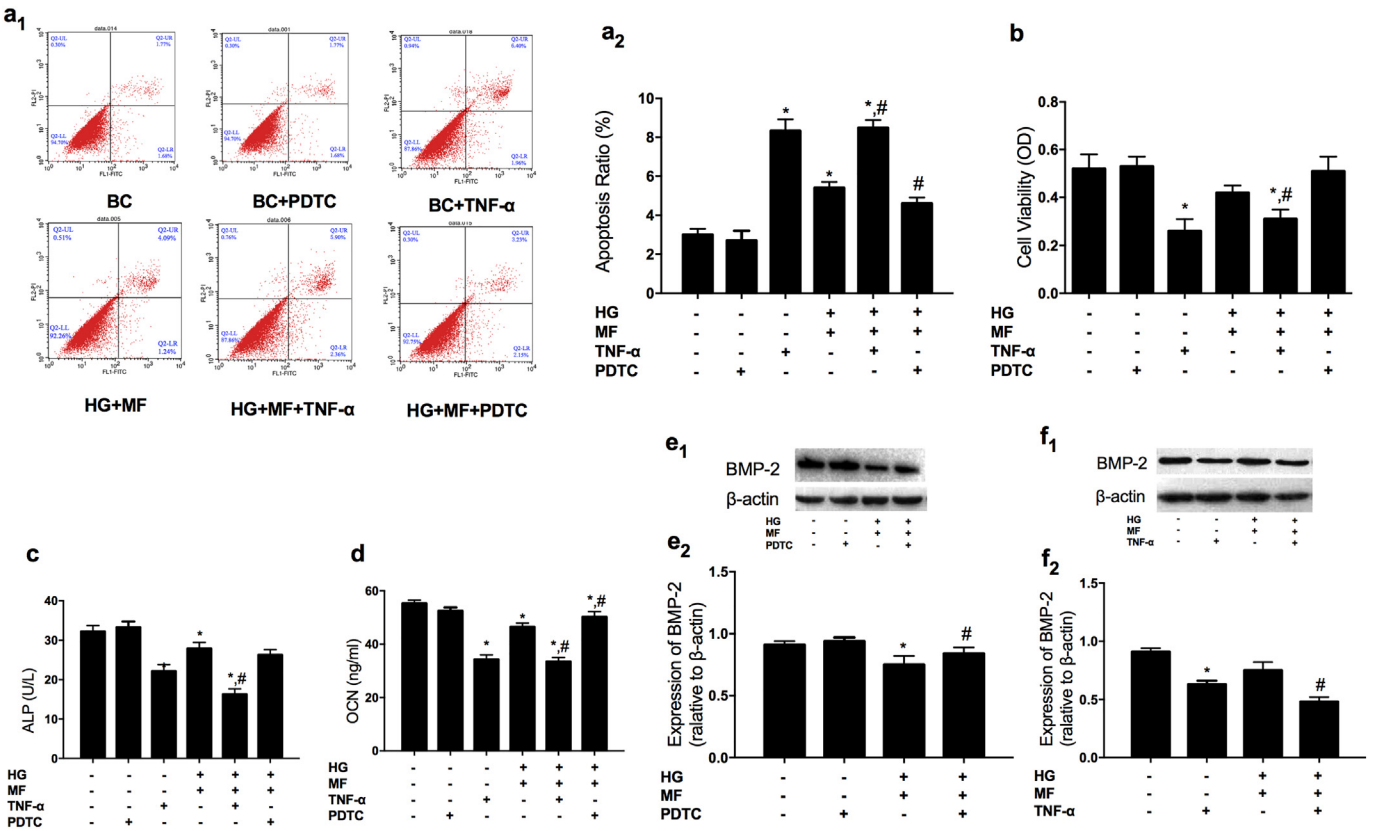

Fig. 5. The effect of TLR4 downstream signaling (NF-κB) on metformin-mediated hyperglycemia-induced injury attenuation in osteoblasts. (a–f) Relationship between the anti-glucotoxicity effects of metformin and NF-κB activity. (a) Flow cytometric analysis of apoptotic cells stained with Annexin V and PI. (a1) Representative FACS images for each group. Shown is one of three representative experiments that yielded essentially similar results. (a2) Collective analyses of all three independent experiments. (b) Osteoblast viability was assessed by MTT assays. (c, d) Bone turnover markers were measured using ELISA kits. (e) BMP-2 expression was detected by Western blotting. (e1) Representative Western blot images for each group. (e2) The ratio of the target protein to β-actin. (f) BMP-2 expression was detected by Western blotting. (f1) Representative Western blot images for each group. (f2) The ratio of the target protein to β-actin.*$P < 0.05$ vs. the BC group (complete medium), #$P < 0.05$ vs. the HG + MF group (25 mmol/L glucose + 100 μmol/L metformin).

3.2. The protective effects of metformin on hyperglycemic osteoblasts were related to TLR4/MyD88/NF-κB expression inhibition

Bivariate correlation analysis was carried out to further investigate the relationship between the protective effects of metformin and the expression of TLR4/MyD88/NF-κB. Increases in TLR4, MyD88 and NF-κB protein expression levels were directly proportional to the occurrence of osteoblast apoptosis. The correlation coefficient R-values were 0.855, 0.882 and 0.869 for TLR4 (Fig. 2a), MyD88 (Fig. 2b) and NF-κB (Fig. 2c), respectively. However, TLR4, MyD88 and NF-κB protein expression levels were inversely proportional to osteoblast viability (R-values: −0.743, −0.829 and −0.815, respectively, Fig. 2def), ALP secretion (R-values: −0.785, −0.820 and −0.809, respectively, Fig. 2ghi), OCN secretion (R-values: −0.782, −0.900 and −0.880, respectively, Fig. 2jkl) and BMP2 expression (R-values: −0.744, −0.805 and −0.785, respectively, Fig. 2mno).

3.3. TLR4 expression and activity levels influenced the protective effect of metformin in hyperglycemic osteoblasts

To further investigate the effects of TLR4 on the protective effects of metformin in hyperglycemic osteoblasts, we cloned the TLR4 gene using lentiviral-induced TLR4 expression (Fig. 3a) and also silenced TLR4 mRNA expression (Fig. 3b). Down-regulating TLR4 expression enhanced the inhibitory effect of metformin on osteoblast apoptosis (Fig. 3c), improved osteoblast viability (Fig. 3d) and increased the secretion of ALP (Fig. 3e), OCN (Fig. 3f) and BMP-2 (Fig. 3g). TLR4 up-regulation weakened the inhibitory effect of metformin on osteoblast apoptosis (Fig. 3c), reduced osteoblast viability (Fig. 3d) and decreased the secretion of ALP (Fig. 3e), OCN (Fig. 3f) and BMP-2 (Fig. 3g).

Simultaneously, the TLR4 inhibitor CLI-095 and LPS activator were applied to selectively block or activate TLR4 activity. Inhibiting TLR4 activity suppressed cell apoptosis (Fig. 3h), improved cell viability (Fig. 3i), and increased the secretion of ALP (Fig. 3j), OCN (Fig. 3k) and BMP-2 (Fig. 3lm) in hyperglycemic osteoblasts treated with metformin, while TLR4 activation had the opposite effects.

3.4. Metformin alleviated LPS-induced osteoblast apoptosis and differentiation suppression

To determine the effects of metformin on osteoblast differentiation, cell viability and apoptosis following LPS stimulation, cells were treated with 20 μg/mL LPS for 8 h prior to exposure to different concentrations (0, 25, 50 and 100 μmol/L) of metformin for 24 h. The results showed that metformin significantly reduced apoptosis (Fig. 4a) in osteoblasts and increased osteoblast viability (Fig. 4b), ALP (Fig. 4c) and OCN (Fig. 4d) secretion, and BMP2 expression (Fig. 4e) upon LPS stimulation.

3.5. TLR4 downstream activity (NF-κB) influenced the protective effects of metformin in hyperglycemic osteoblasts

NF-κB is a well-known classic downstream target of the TLR4 signaling pathway [27]. To determine whether the activation of the downstream TLR4/MyD88/NF-κB signaling pathway could alter the protective effects of metformin on osteoblast differentiation, cell

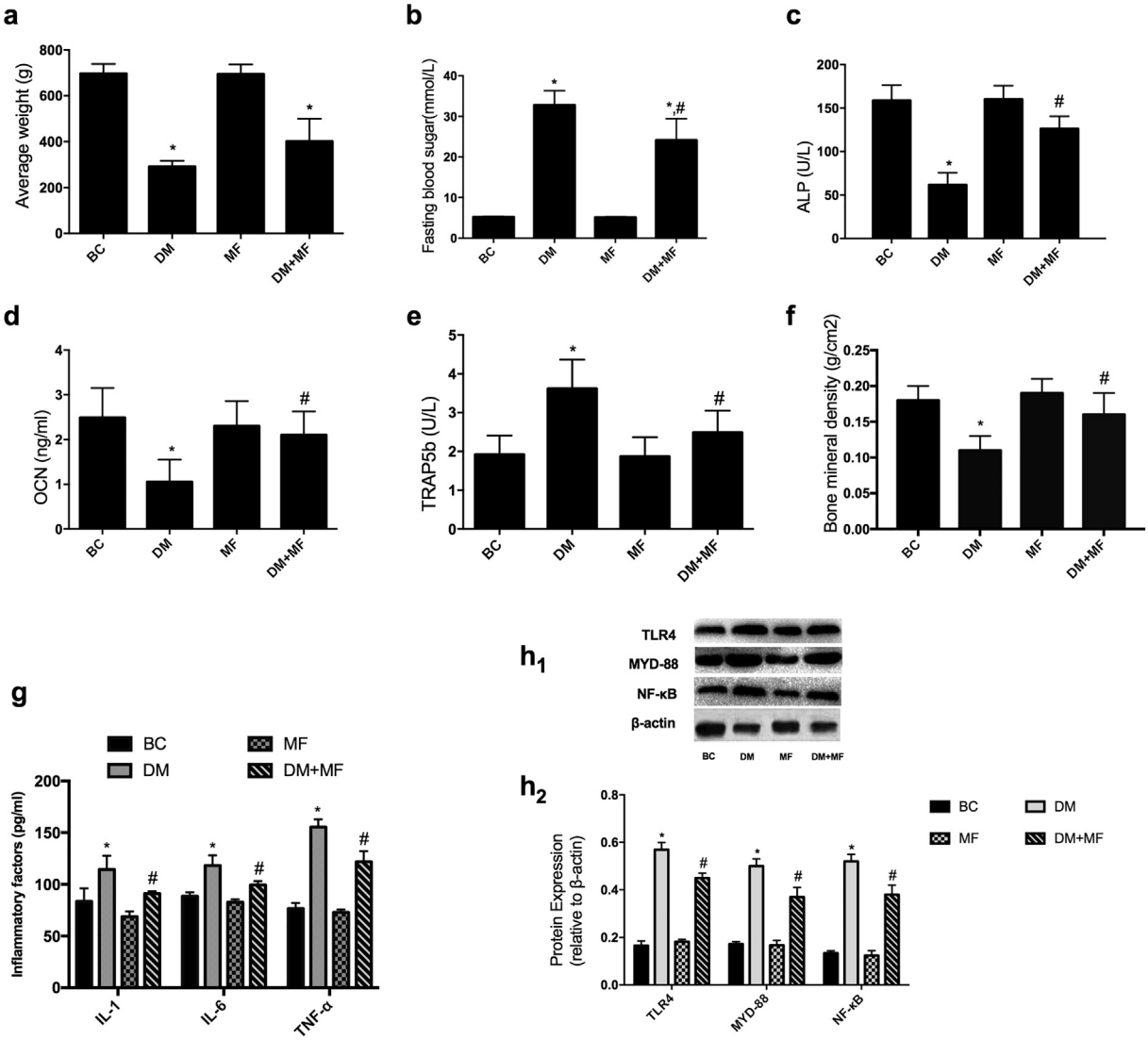

Fig. 6. The effects of metformin on femur and serum inflammation in diabetic rats.
All data are presented as the mean ± SD of three independent experiments. (a) The levels of fasting blood glucose in each group. (b) The average weight of each group. (c, d, e) The effects of metformin on bone turnover markers were measured using ELISA kits. (f) The effect of metformin on BMD was detected by DEXA. (g) Serum IL-1, IL-6 and TNF-α levels were measured using ELISA kits. (h) TLR4, MyD88, and NF-κB expression levels in femur tissues were detected by Western blotting. (h1) Representative Western blot images for each group. (h2) The ratio of the target protein to β-actin. *$P < 0.05$ vs. the BC group, #$P < 0.05$ vs. the DM group.

viability and apoptosis induced by hyperglycemia, NF-κB agonists and inhibitors were used to selectively activate or block the activity of NF-κB. The results showed that NF-κB activation weakened the inhibitory effects of metformin on osteoblast apoptosis (Fig. 5a), reduced osteoblast viability (Fig. 5b) and decreased the secretion of ALP (Fig. 5c), OCN (Fig. 5d) and BMP-2 (Fig. 5ef). Simultaneously, NF-κB inhibition had the opposite effects.

3.6. Effects of metformin on inflammation in femur and serum samples from diabetic rats

To further explore the effects of metformin on hyperglycemia, diabetic rats fed a high sucrose-HFD and administered streptozotocin were treated with metformin, and their bone metabolism and inflammatory responses were then observed. The results revealed that metformin decreased fasting blood sugar (Fig. 6b). However, there was no significant difference observed for weight (Fig. 6a) between the treatment and non-treatment groups ($P > 0.05$). Metformin increased ALP (Fig. 6c) and OCN (Fig. 6d) secretion, decreased TRAP5b secretion (Fig. 6e), improved BMD (Fig. 6f), and decreased TLR4, MyD88 and NF-κB expression (Fig. 6h) in femur tissues. At the same time, metformin significantly reduced serum IL-1, IL-6 and TNF-α levels (Fig. 6g).

4. Discussion

In the present study, we demonstrated that metformin could inhibit the expression of TLR4/MyD88/NF-κB, and these inhibitory effects were positively correlated with the protective effect of metformin against hyperglycemia-induced injury in osteoblasts. The regulation of TLR4 or its downstream expression or activity affected the protective effect of metformin on osteoblasts. Simultaneously, metformin alleviated LPS-induced osteoblast injuries. Furthermore, metformin inhibited inflammatory cytokine expression in diabetic rats, and it also inhibited TLR4/MyD88/NF-κB gene expression in the femur tissues of diabetic rats.

During the process of DM, bone strength decreases, and bone fragility increases; these changes are induced by HG and increase the incidence rate of diabetic osteoporosis and fragility fractures [28,29]. As the most commonly used oral antidiabetic drug, metformin reportedly protects against bone loss [30,31]. In this study, we demonstrated that

under hyperglycemic conditions, metformin improved osteoblast differentiation and reduced apoptosis in osteoblasts in a dose-dependent manner. Similarly, the results of some current studies also support the idea that metformin has a protective effect on hyperglycemic osteoblasts. A recent in vitro study showed that metformin attenuated the suppressive effects of HG on osteoblast proliferation and gene expression [32]. Another two studies have shown that metformin could significantly reduce osteoblast apoptosis and inhibit osteoclast differentiation [33,34]. These findings indicate that metformin is a potential therapeutic agent for treating hyperglycemia-associated osteoblast dysfunction and apoptosis.

Furthermore, we tried to investigate the mechanisms underlying the protective effects of metformin on hyperglycemia-associated osteoblast apoptosis and differentiation suppression. Metformin can exert a protective effect on hyperglycemic osteoblasts by regulating AMPK signaling [35–37]. In fact, this effect may be governed by other signaling pathways. Recent research has confirmed that metformin could suppress the formation of fat by regulating Runx2 and PPARγ activity, and this effect appears to be independent of AMPK activity [38]. In the present study, treatment with metformin remarkably alleviated TLR4 signaling pathway activation, revealing that the TLR4-NF-κB signaling pathway may be important in the protective effects of metformin on hyperglycemia-induced osteoblast dysfunction and apoptosis. As such, we speculated that the protective effects of metformin on hyperglycemia-induced injury may be related to the TLR4/NF-κB signaling pathway. We verified these assumptions based on five observations: First, we observed that TLR4/MyD88/NF-κB expression levels were negatively correlated with the protective effects of metformin in hyperglycemic osteoblasts. Second, we found that silencing TLR-4 expression enhanced the protective effects of metformin in hyperglycemic osteoblasts, and TLR4 overexpression weakened the beneficial effects of metformin. Third, we revealed that TLR4 inhibition increased the protective effects of metformin, while TLR4 activation decreased the protective effects of metformin. Fourth, regulating downstream TLR4/NF-κB signaling pathway activity (NF-κB) affected the protective effects of metformin in hyperglycemic osteoblasts. Activating NF-κB suppressed the protective effects of metformin, while inhibiting NF-κB increased the effects of metformin in hyperglycemic osteoblasts. Fifth, we found that metformin improved osteoblast differentiation and suppressed osteoblast apoptosis not only under hyperglycemic conditions but also under LPS stimulation conditions. Based on the observations above, we hypothesized that the beneficial effects of metformin might be closely related to the expression and activity of TLR-4, indicating that the protective effects of metformin in hyperglycemic osteoblasts might occur by suppressing the TLR4 signaling pathway. Recent studies also support that the effects of metformin are relevant to TLR4 expression in inflammatory responses. A recent study reported that metformin exhibits cardioprotective effects in sepsis by suppressing TLR4 activity [39]. Another study also showed that metformin could protect lung tissues against LPS-induced TLR4 activation [40]. Additionally, metformin attenuates the TLR4 inflammatory pathway in the skeletal muscle of diabetic rats [24].

Finally, our study aimed to further prove the effects of metformin on osteoblasts in vitro. Thus, we determined the effects of metformin on bone metabolism and inflammatory responses in diabetic rats. We revealed that metformin promoted osteogenesis, suppressed the TLR4/NF-κB signaling pathway in femur tissues, and inhibited serum inflammatory cytokines in diabetic rats. The results of in vitro experiments support our conclusions from the in vivo experiments. In addition, we also found that metformin improved the bone metabolic index of diabetic rats, and the increase in BMD was significant.

5. Conclusion

In conclusion, our results demonstrate that metformin could alleviate the damage induced by hyperglycemia in osteoblasts by suppressing the TLR4/MyD88/NF-κB signaling pathway both in vivo and in vitro. The results of the present study help to not only distinguish the role played by metformin in protecting against hyperglycemia-induced injury in osteoblasts but also provide experimental evidence of the molecular mechanism underlying metformin's alleviation of hyperglycemia-induced apoptosis and differentiation suppression in osteoblasts. The primary limitation of this study is that it investigates the effects of metformin on TLR4 expression at only the cellular level. Gene knockout and transgenic animal experiments should be conducted to provide a comprehensive picture of the relationship between the protective effects of metformin and TLR4 expression. Moreover, we examined apoptosis in only MC3T3-E1 cells and femurs, and apoptosis in osteoblasts from the femur was not addressed.

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Compliance with ethical standards

The authors declare that there are no conflicts of interest.

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