N-methyl pyrrolidone (NMP) ameliorates the hypoxia-reduced osteoblast differentiation via inhibiting the NF-κB signaling

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ABSTRACT — Ischemic-hypoxic condition for local osteoblasts and bone mesenchymal stem cells during bone fracture inhibits bone repairing. N-methyl pyrrolidone (NMP) has been approved as a safe and biologically inactive small chemical molecule, and might be useful for bone fracture repairing. In the present study, we investigated the effect of NMP on the hypoxia-reduced cellular viability and the expression of differentiation-associated markers, such as bone morphogenetic protein 2 (BMP-2), propeptide of type I procollagen I (PINP), alkaline phosphatase (ALP) or runt-related transcription factor 2 (Runx2) in the osteoblasts, and then we examined the molecular mechanism underlining such effect in the human osteoblastic hFOB 1.19 cells. Our results demonstrated that NMP significantly blocked the hypoxia-induced cell viability reduction and inhibited the hypoxia-caused expression downregulation of BMP-2, PINP, ALP and Runx2 in hFOB 1.19 cells. Then we confirmed the involvement of nuclear factor κB (NF-κB) pathway in the regulation by NMP on the hypoxia-mediated the reduction of osteoblast differentiation. The upregulated expression and transcriptional activity of NF-κB, while the downregulated inhibitory κB expression by the hypoxia treatment was reversed by the treatment with 10 mM NMP. In conclusion, our study found a protective role of NMP in osteoblast differentiation in response to hypoxia, and such protection was through inhibiting the NF-κB signaling. This suggests that NMP might be a protective agent in bone fracture repairing.

Key words: N-methyl pyrrolidone (NMP), Hypoxia, Osteoblast differentiation, NF-κB signaling

INTRODUCTION

The oxygen supply/blood flow deprivation or interruption during bone fracture results in an ischemic-hypoxic condition for local osteoblasts and bone mesenchymal stem cells (MSCs) and inhibits bone repairing (Lu et al., 2013). Particularly, hypoxia exerts a significant influence (Heppenstall et al., 1976), via regulating the expression of transcriptional factors and cytokines (Warren et al., 2001) such as hypoxia-inducible factors (Akeno et al., 2001), vascular endothelial growth factor (Bouletreau et al., 2002) and bone morphogenetic protein 2 (BMP-2) (Bouletreau et al., 2002). And such factors/cytokines then pose regulatory roles on the wound healing or bone regeneration (Naik et al., 2009; Kolar et al., 2010). BMPs, such as BMP-2 (Hu et al., 2013; Gao et al., 2015), alkaline phosphatase (ALP) (Huang et al., 2015), osteopontin (Jiang et al., 2015) and osteocalcin (Huang et al., 2015), have been indicated to be deregulated by hypoxia in the differentiation of MSCs during bone repair. Moreover, sustained hypoxia even leads to the apoptosis and even necrosis of osteoblasts (Sun and Peng, 2015) and MSCs (Piret et al., 2002).

N-methyl pyrrolidone (NMP) has been approved as a safe and biologically inactive small chemical constituent in medical devices. Recently, NMP has been reported to have the pharmaceutical property of enhancing bone regeneration in a rabbit calvarial defect model in vivo (Miguel et al., 2009). Therefore, NMP might be useful for the treatment of osteoporosis or other bone diseases associated with excessive bone resorption. In addition, it exerts an anti-inflammatory potential on the lipopolysaccharide (LPS)-induced inflammatory process (Ghayor et al., 2015). The LPS-promoted levels of TNF-α, IL-1β, IL-6, iNOS and COX-2 were inhibited by NMP in a dose-dependent manner. And the effect of NMP is mediated...
through the downregulation of nuclear factor κB (NF-κB) pathway (Ghayor et al., 2015).

In the present study, we investigated the effect of NMP on the hypoxia-induced apoptosis and differentiation of osteoblasts, and then we examined the molecular mechanism underlining such effect in osteoblasts. Our study suggests that NMP has a protective role in the hypoxia-induced impairment in osteoblast differentiation.

MATERIALS AND METHODS

Cells and cell culture/treatment

Human osteoblastic hFOB 1.19 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), and were cultured in a 1:1 mixture of Ham’s F12 medium and Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco, Rockville, MD, USA), which was supplemented with 2.5 mM L-glutamine (Sigma-Aldrich, St. Louis, MO, USA), with 0.3 mg/mL G418 (Thermo Scientific, Rockford, IL, USA) and with 10% fetal bovine serum (FBS, Sijiqing, Hangzhou, China). hFOB 1.19 cells were incubated in a humidified incubator with 5% CO₂ at 37°C. For the hypoxia treatment, hFOB 1.19 cells were incubated with 100 μL substrate at dark for 15 min, and then the plate was read at 450 nm with a spectrophotometer.

Enzyme-linked immunosorbent assay (ELISA)

The intracellular levels of BMP-2, procollagen type I N-terminal propeptide (PINP), ALP or Runx2 in hFOB 1.19 cells were quantified with the ELISA kit for BMP2, PINP, ALP or runt-related transcription factor 2 (Runx2) (all from Abcam, Cambridge, UK) according to the product’s manual. The microplate for each marker was incubated with 100 μL antibody solution (against BMP2, PINP, ALP or Runx2) at 37°C for one hour. Hereafter, 100 μL horseradish peroxidase-conjugated secondary antibody was added for an incubation at 37°C for 30 min. Four-time washing with phosphate buffered saline adding Tween 20 (PBST) was performed before each incubation. Finally, the plate was incubated with 100 μL substrate at dark for 15 min, and the plate was read at 450 nm with a spectrophotometer.

RT-qPCR analysis of p65 mRNA and NF-κB luciferase reporter assay

Total cellular mRNA samples from hFOB 1.19 cells were prepared with the Recover All Total Nucleic Acid Isolation Kit (Ambion, Austin, TX, USA) according to the kit’s manual, and were supplemented with 1 μL RNasePlus RNase Inhibitor (Promega, Madison, WI, USA). The p65 mRNA level was quantified with the Takara One Step RT-PCT kit (Takara, Tokyo, Japan), with the p65-specific primers (Forward primer: 5'-tgatcactaaggaagatgtg-3', Reverse primer: 5'-gaaggtcaggtgceccag-3'). The quantification of p65 was relatively presented with β-actin as reference gene, via the ΔΔCT method (Livak and Schmittgen, 2001).

For the NF-κB luciferase reporter assay, hFOB 1.19 cells were plated into 96-well plates and cultured to approximately 85%-confluent. Then hFOB 1.19 cells were transfected with the NF-κB luciferase reporter plasmid (Genomeditech, Shanghai, China) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions.

Protein isolation and western blotting

5 x 10⁵ hFOB 1.19 cells post treatment were subject to the protein isolation with the Nuclear/Cytosol Fractionation Kit (BioVision, San Diego, CA, USA), the cytosolic and the nuclear fraction were collected respectively and were added with Protease Inhibitor Cocktail (Sigma-Aldrich). Then each fraction was quantified with BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA). To analyze the p65 and inhibitory κB (IκB) levels, each protein sample was separated by electrophoresis with 12% SDS-PAGE gradient gel, and then was transferred to nitrocellulose membrane. The membrane was then incubated with 2% BSA (4°C overnight) to block out non-specific binding sites, was incubated (4°C overnight) with the rabbit polyclonal antibody to p65 (Abcam, Cambridge, MA, USA), to IκB (Santa Cruz Biotechnology, SantaCruz,CA,USA)ortoβ-actin(Sinobio,Beijing,China), and then was incubated (room temperature for 1 hr) with the horseradish peroxidase-linked secondary goat-anti-rabbit antibody (Bio-Rad Laboratories). Three-time washing with 1x PBST was performed before each inoculation. And the specific p65, IκB or β-actin band was then quantified with enhanced chemiluminescence (ECL) (Thermo Scientific).

MTT assay for cellular viability

Viability of hFOB 1.19 cells was examined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bro-
mide (MTT) assay kit (Invitrogen). Briefly, hFOB 1.19 cells, post normoxia, hypoxia or (and) NMP treatment, were incubated with the MTT reagent at 37°C for 3 hr. Optical densities (OD) at 450 nm were measured by spectrophotometer (Crystaleye, Olympus, Tokyo, Japan). Cellular viability was presented as average OD_{450} value.

Statistical analysis
Statistical difference was analyzed with SPSS17.0 software (IBM SPSS, Armonk, NY, USA). The difference between two groups was analyzed by Student's t test. A p value less than 0.05 was considered to be significant.

RESULTS

Hypoxia reduces the expression of osteoblast differentiation-associated markers
Osteoblast differentiation is characterized by the high expression of markers such as BMP-2, PINP, ALP and Runx2 (Bandow et al., 2010). Recently, human osteoblastic hFOB cell line has been indicated to be suitable as an osteoblast-like cell line (Che et al., 2015). We selected this cell line for our study. We firstly examined the expression of BMP-2, PINP, ALP and Runx2 with ELISA in the hFOB 1.19 cells under normoxia or hypoxia. As indicated in Figure 1A, the BMP-2 level was markedly reduced in the hFOB 1.19 cells under hypoxia than under normoxia (p < 0.05 or p < 0.01 for 6, 12 or 24 hr post treatment). And such downregulation by hypoxia was also found in the expression of PINP (p < 0.05 or p < 0.01, Fig. 1B), of ALP (p < 0.05, p < 0.01 or p < 0.001, Fig. 1C) and of Runx2 (p < 0.05 or p < 0.01, Fig. 1D).

NF-κB signaling was promoted in the hypoxia-mediated reduction of osteoblast differentiation
NF-κB pathway is induced by hypoxia in neurons (Angelo et al., 2014), in T lymphocytes (Bruzzese et al., 2014), in microglia cells (Guo et al., 2014), and also in various types of tumor cells (Cheng et al., 2014; Li and Li, 2015). In order to identify the hypoxia-mediated...
reduction of osteoblast differentiation-associated markers, we then explored the activation of NF-κB pathway in the hFOB 1.19 cells under normoxia or hypoxia. We found that there was a high p65 mRNA level in the hypoxia-treated hFOB 1.19 cells (p < 0.01 or p < 0.001 for 6, 12 or 24 hr post treatment, Fig. 2A). Western blotting assay (Fig. 2B) also indicated an upregulation of p65 in protein level in the hypoxia-treated hFOB 1.19 cells (p < 0.01 respectively for 12 or 24 hr post treatment, Fig. 2C). However, IκB, an inhibitor of NF-κB pathway, was markedly downregulated by hypoxia in hFOB 1.19 cells (p < 0.05 or p < 0.01, Fig. 2D). To substantiate the activation of NF-κB pathway by hypoxia, we exposed hFOB 1.19 cells to hypoxia, and then assessed the NF-κB

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**Fig. 2.** Activation of NF-κB signaling in hFOB 1.19 cells under normoxia or hypoxia. hFOB 1.19 cells were incubated with maintaining medium for 6, 12 or 24 hr under normoxia or hypoxia, then the mRNA (A) level of p65 was examined with real-time quantitative PCR, and the protein levels of p65 and IκB were examined with western blotting assay (B-D). E: Construction of an NF-κB luciferase reporter; F: the NF-κB transcriptional activity with the NF-κB luciferase reporter in the hFOB 1.19 cells under normoxia or hypoxia. Data represented the average level of quartic independent experiments with S.E.M. indicated. Statistical significance was shown as *p < 0.05, **p < 0.01, ***p < 0.001 or ns: no significance.
activation using an NF-κB luciferase reporter, which contains four copies of NF-kB binding site (Fig. 2E). Fig. 2F demonstrated that there was approximately 2-fold upregulation of luciferase activity in the hypoxic hFOB 1.19 cells, compared to the normoxia-treated hFOB 1.19 cells (p < 0.01 or p < 0.001). These data indicated that hypoxia induced p65 expression and its activation.

**NMP ameliorates the hypoxia-reduced osteoblast differentiation**

In order to examine the possibility that NMP might modulate the hypoxia-mediated reduction of osteoblast differentiation, the osteoblast differentiation-characterized markers, such as BMP-2, PINP, ALP and Runx2 were re-evaluated in the NMP-treated hFOB 1.19 cells under hypoxia. We first investigated the effect of NMP on the viability of hFOB 1.19 cells under hypoxia. As shown in Fig. 3A, NMP exerted no influence on the cellular viability of hFOB 1.19 cells under normoxia. However, the NMP treatment markedly ameliorated the hypoxia-mediated viability reduction of hFOB 1.19 cells (p < 0.05 or p < 0.01, Fig. 3B). And such amelioration was dose-dependent (p < 0.05 respectively, Fig. 3C).

We then examined the BMP-2, PINP, ALP and Runx2 levels with ELISA in the NMP-treated hFOB 1.19 cells under hypoxia. Figure 4A indicated that the BMP-2 reduction was significantly ameliorated by 10 mM NMP at 12 or 24 hr post treatment (p < 0.05 or p < 0.01). And such amelioration by the NMP treatment was also found in the PINP reduction at 12 or 24 hr post treatment (p < 0.05 or p < 0.01, Fig. 4B). In addition, the NMP-mediated amelioration was also found from 6 or at 24 hr post treatment for the ALP reduction or for the Runx2 reduction (p < 0.05 or p < 0.01, Fig. 4C or 4D). Taken together, these results indicate that NMP ameliorates the hypoxia-induced downregulation of cellular viability and osteoblast differentiation.

**NMP inhibited the hypoxia-promoted NF-κB signaling in hFOB 1.19 cells**

In order to investigate the regulation by NMP on the hypoxia-mediated reduction of osteoblast differentiation, we then re-evaluated the activation of NF-κB pathway in the hFOB 1.19 cells under hypoxia, with or without the treatment with 10 mM NMP. As indicated in Fig. 5A, the hypoxia-promoted p65 mRNA level was markedly inhibited at 12 or 24 hr post treatment (p < 0.05 or p < 0.01). And the western blotting results (Fig. 5B) demonstrated that the protein level of p65 (Fig. 5C) was inhibited, whereas the IκB level (Fig. 5D) was upregulated by 10 mM NMP in the hypoxia-treated hFOB 1.19 cells (p < 0.01 or p < 0.001). Moreover, the NF-κB luciferase reporter assay indicated that the promoted luciferase activity was inhibited by the 10 mM NMP in the hypoxic hFOB 1.19 cells (p < 0.05 or p < 0.01, Fig. 5E). Therefore, NMP inhibited the hypoxia-promoted NF-κB signaling.
NMP has recently been reported to be protective in the bone regeneration in a rabbit model (Miguel et al., 2009). And NMP inhibits osteoclast differentiation and attenuates bone resorption, via inhibiting the receptor activator of nuclear factor κB ligand (RANKL)-induced osteoclastogenesis and bone resorption (Liu et al., 2015). NMP inhibits the expression of matrix metalloproteinase-9, cathepsin K, nuclear factor of activated T cells, cytoplasmic 1 and c-Fos, while decreases the activation of activator protein 1 and ERK pathway activation in osteoclasts (Liu et al., 2015). In the present study, we firstly recognized the regulation by NMP on the osteoblast differentiation in vitro. It could significantly block the hypoxia-induced viability reduction of human osteoblastic hFOB 1.19 cells. And the hypoxia-caused expression downregulation of osteoblast differentiation markers (BMP-2, PINP, ALP and Runx2) has also been ameliorated by NMP in the hFOB 1.19 cells. Therefore, the regulation potency of osteoblast differentiation by NMP for the treatment of osteoporosis or other bone diseases associated with excessive bone resorption.

**DISCUSSION**

NF-κB is a transcriptional protein complex that is composed by RelA (p65), RelB, cRel, NF-κB1 (p50) and NF-κB2 (p52) (Gilmore, 2006), which are usually inactivated in the cytoplasm by a family of inhibitors called Inhibitor of κB (IκBs) (Gilmore, 2006). And the inhibitory κB kinase-mediated phosphorylation promotes the proteasomal degradation of IκB, enabling the active NF-κB to translocate to the nucleus (Vallabhapurapu and Karin, 2009). Upon the stimulation by different stresses, NF-κB was activated via a number of pathways (Perkins, 2012). Hypoxia activates NF-κB through promoting the phos-

**Fig. 4.** Expression of osteoblast differentiation-associated markers in hypoxia-treated hFOB 1.19 cells, post the NMP treatment. Human osteoblastic hFOB 1.19 cells were incubated with maintaining medium (2% FBS) containing 0 or 10 mM NMP for 0, 2, 6, 12 or 24 hr under hypoxia, then the intracellular levels of bone morphogenetic protein 2 (BMP-2) (A), procollagen type I N-terminal propeptide (PINP) (B), alkaline phosphatase (ALP) (C) and Runt-related transcription factor 2 (Runx2) (D) were assayed with ELISA kit. Data were indicated as mean ± S.E.M. for quartic independent experiments. Statistical significance was shown as *p < 0.05, **p < 0.01 or ns: no significance.
phorylation and degradation of IκB on tyrosine residues (Koong et al., 1994). Our study confirmed the upregulated NF-κB level and the increased transcriptional activity of NF-κB, and confirmed the downregulated IκB expression in the hypoxia-treated hFOB 1.19 cells. The downregulation by NMP on the NF-κB pathway has been rec-

Fig. 5. Activation of NF-κB signaling in hypoxia-treated hFOB 1.19 cells, post the NMP treatment. hFOB 1.19 cells were incubated with maintaining medium containing 0 or 10 mM NMP for 6, 12, 24 or 36 hr under hypoxia, then the mRNA (A) level of p65 was examined with real-time quantitative PCR, and the protein levels of p65 and IκB were examined with western blotting assay (B-D). E: the NF-κB transcriptional activity with the NF-κB luciferase reporter in the NMP-treated hFOB 1.19 cells under hypoxia. Data represented the average level of quartic independent experiments with SEM indicated. Statistical significance was shown as *p < 0.05, **p < 0.01, ***p < 0.001 or ns: no significance.
ognized in macrophages (Ghayor et al., 2015) and in osteoclasts (Liu et al., 2015). And the NF-κB pathway is indicated to regulate the expression of multiple genes involved in inflammatory and immune response (McKay and Cidlowski, 1999; Caamaño and Hunter, 2002). And the IKK (inhibitory kB (IκB) kinase)-mediated phosphorylation-induced proteasomal degradation of IκB inhibitor, enabling the active NF-κB to translocate to the nucleus (Vallabhapurapu and Karin, 2009). However, IκB could sequester NF-κB subunits and terminate the transcription activity (Potoyan et al., 2015). In our study, we confirmed the involvement of NF-κB pathway in the regulation by NMP on the hypoxia-mediated the reduction of osteoblast differentiation. The hypoxia-deregulatedNF-κB (activated) and IκB (inactivated) was reversed by the treatment with 10 mM NMP. Interestingly, we did not find marked regulation by NMP (less than 10 mM) on the NF-κB signaling in hFOB 1.19 cells under normoxia. Therefore, the amelioration of the hypoxia-reduced osteoblast differentiation by NMP was via inhibiting the NF-κB signaling. We did determine what up-streamers of IκB / NF-κB signaling were regulated by NMP in the hypoxia-treated hFOB 1.19 cells. Additional studies are required to identify the functional role of NMP in such regulation on the NF-κB signaling in the context of hypoxia.

In conclusion, our study found that NMP has a protective role in osteoblast differentiation in response to hypoxia, and such protection was through inhibiting the NF-κB signaling. This suggests that NMP might be a protective agent in bone fracture repairing.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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