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Treatment with hydrogen molecule alleviates TNFα-induced cell injury in osteoblast

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Abstract Tumor necrosis factor-alpha (TNFα) plays a crucial role in inflammatory diseases such as rheumatoid arthritis and postmenopausal osteoporosis. Recently, it has been demonstrated that hydrogen gas, known as a novel antioxidant, can exert therapeutic anti-inflammatory effect in many diseases. In this study, we investigated the effect of treatment with hydrogen molecule (H_2) on TNFα-induced cell injury in osteoblast. The osteoblasts isolated from neonatal rat calvariae were cultured. It was found that TNFα suppressed cell viability, induced cell apoptosis, suppressed Runx2 mRNA expression, and inhibited alkaline phosphatase activity, which was reversed by co-incubation with H_2. Incubation with TNFα-enhanced intracellular reactive oxygen species (ROS) formation and malondialdehyde production increased NADPH oxidase activity, impaired mitochondrial function marked by increased mitochondrial ROS formation and decreased mitochondrial membrane potential and ATP synthesis, and suppressed activities of antioxidant enzymes including SOD and catalase, which were restored by co-incubation with H_2. Treatment with H_2 inhibited TNFα-induced activation of NFκB pathway. In addition, treatment with H_2 inhibited TNFα-induced nitric oxide (NO) formation through inhibiting iNOS activity. Treatment with H_2 inhibited TNFα-induced IL-6 and ICAM-1 mRNA expression. In conclusion, treatment with H_2 alleviates TNFα-induced cell injury in osteoblast through abating oxidative stress, preserving mitochondrial function, suppressing inflammation, and enhancing NO bioavailability.

Keywords Hydrogen molecule · Tumor necrosis factor-alpha · Inflammation · Oxidative stress · Nitric oxide · NFκB

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Introduction

Tumor necrosis factor-alpha (TNFα) is one of several cytokines produced in excess within the joint space in rheumatoid arthritis and postmenopausal osteoporosis, which shift the formation/resorption balance in the skeleton toward resorption and lead to periarticular bone loss [1–4]. The clinical effectiveness of blocking TNFα in treating active rheumatoid arthritis established the pathogenic significance of TNFα in rheumatoid arthritis [5, 6]. Bone loss induced by ovariectomy was markedly decreased in TNFα knock-out mice [4]. TNFα has been shown to decrease osteoblastic bone formation through the suppression of osteoblast proliferation, induction of osteoblast apoptosis, and inhibition of osteoblast differentiation [7–9]. The detrimental role of TNFα in rheumatoid arthritis and osteoporosis has fostered an increased interest in the potential therapeutic anti-inflammatory drugs.

Accumulating reports revealed the potential use of hydrogen gas (H_2) as a therapeutic agent in the therapy of conditions associated with inflammation-related multiple organ dysfunction syndrome. H_2 treatment protected against multiple organ damage in a zymosan-induced generalized

inflammation model [10], ameliorated lipopolysaccharide-induced acute lung injury in mice through reducing inflammation and apoptosis [11], and inhibited carrageenan-induced paw edema through suppressing the production of TNFα by activated macrophages [12]. H_2 exerted marked anti-inflammatory property and decreased the expressions of pro-inflammatory factors including TNFα, IL-6, IL-1β, CCL2 and IL-10, TNF-γ, IL-12, ICAM-1, PGE2, and PGE2 in several reports [13–16].

In this study, we investigated the effect of treatment with H_2 on TNFα-induced cell injury in primary osteoblast. Treatment with hydrogen in vitro usually utilized two methods including inhalation of H_2 or incubation of hydrogen-rich medium (HRM). Compared to H_2, HRM was safe, economical, and easily available, so we chose the latter.

Methods

Animals and materials

Chemicals, drugs, and reagents were obtained from Sigma Chemical (St. Louis, MO, USA) unless otherwise stated. Neonatal Sprague–Dawley (SD) rats were purchased from the Vital-River Animal Ltd (Beijing, China). All the animals were entrained to controlled temperature (22–25 °C), 12-h light and 12-h dark cycles (light 08:00–20:00 h; darkness 20:00–08:00 h), and free access to food and tap water. All the animals used in this study received humane care in compliance with institutional animal care guidelines. All the surgical and experimental procedures were in accordance with institutional animal care guidelines, and were approved by the Local Institutional Committee.

Calvarial osteoblast isolation and culture

The osteoblasts were isolated from neonatal SD rat calvariae immediately after dissection, as described previously [17]. Bones were washed in PBS containing 4 mM EDTA for 10 min at 37 °C and then incubated in a HEPES buffer solution (25 mM HEPES, pH 7.4, 70 mM NaCl, 30 mM KCl, 10 mM $NaHCO_3$, 1.5 mM K_2HPO_4, 1 mM $CaCl_2$, 60 mM sorbitol, 27.8 mM D-(+)-glucose, and 1 mg/ml BSA) containing 2 mg/ml collagenase and 90 μM N-α-tosyl-L-lysyl chloromethyl ketone for three sequential 20-min digestion periods at 37 °C in a shaking water bath. At the end of each digestion, released cells were collected and resuspended in the HEPES buffer also containing 1 mM $MgSO_4$, and all three digests were pooled for plating on 60-mm Primaria culture dishes (Falcon, Becton–Dickinson). Medium was changed every 2–3 days.

Hydrogen-rich medium (HRM) preparation

Over a 4-h period, we dissolved H_2 into DMEM under 0.4 MPa pressure as method described by Ohsawa [18]. We dissolved O_2 into a second medium by bubbling O_2 gas at the saturated level, and CO_2 into a third medium by bubbling CO_2 gas. All three media were maintained at atmospheric pressure. Then, the three media (H_2:O_2:CO_2) were combined in the proportion 75:20:5 % (vol/vol/vol) and fetal bovine serum was added (FBS) to achieve a final concentration of 1 %. For culture, combined medium was poured into a culture flask. Then, the culture flask was filled with mixed gas consisting of 75 % H_2, 20 % O_2, and 5 % CO_2 (vol/vol/vol) and cultured cells in the closed culture flask. HRM was freshly prepared every week, which maintained a continuous concentration.

Reactive oxygen species (ROS) production

The cells were seeded in black 96-well plates and cultured for 48 h. The culture medium was replaced with phenol-red-free DMEM containing 2′,7′-dichlorodihydrofluorescein diacetate (10 μM) 30 min before the treatment. Then, the ROS production was measured with a fluorescence reader.

Cell viability assay

MTT assay (reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a purple formazan product) was used to estimate cell viability. After treatment, cells were washed with PBS twice, and 50 μl of 1 mg/ml MTT solution was added to each well and incubated for 4 h at 37 °C in the dark. The media were decanted and then washed twice with PBS. The produced formazan salts were dissolved with dimethyl sulphoxide at 490 nm using an ELISA reader. Results were calculated as the ratio of the absorbance of the control cells.

Alkaline phosphatase (ALP) activity assay

To measure the ALP activity, cells were seeded in a 12-well plate and treated with indicated chemicals for 1, 3, 5, or 7 days. The media was changed every day. The medium was removed and the cell monolayer was gently washed twice with PBS. Cells were then lysed with cell lysis buffer (0.5 ml for a 35-mm dish) and centrifuged at 12,000×g for 10 min. The resulting supernatant was used for the measurement of ALP activity and protein concentration with a commercially available ALP activity assay kit (Cell Biolabs, San Diego, CA, USA) and a BCA-protein assay kit (Bio-Rad, Hercules, CA, USA), respectively. ALP activity was expressed as nmol/min/mg protein.

Flow cytometric analysis for apoptosis

Apoptosis was examined by Annexin V-fluorescein isothiocyanate staining (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. Cells were seeded on 6-well plates and incubated for 2 days. After treatment, the FITC fluorescence intensity was measured using a Becton–Dickinson FACS Caliber flow cytometer (BD Biosciences).

Determination of intracellular NO

Intracellular NO content was measured using a membrane-permeable indicator dye, 4-amino-5-methylamino-2′,7′-difluororescein diacetate (DAF-FM), which reacts with NO to form a green fluorescent product [19]. After treatment, cells were incubated for 30 min at 37 °C in 10 μM DAF-FM in the dark, washed with PBS and incubated for an additional 30 min in medium without the dye. Cells were rinsed with D-Hanks (KCl, 0.2 g/l; KH_2PO_4, 0.2 g/l; NaCl, 8.0 g/l; $Na_2HPO_4{\cdot}2H_2O$, 2.16 g/l). Fluorescence intensity was recorded at the excitation wavelength of 488 nm. The single cell intracellular NO concentration was analyzed as the average intensity of DAF-FM fluorescence.

Determination of NOS activity

The eNOS and iNOS activities were determined with the Nitric Oxide Synthase Assay Kit (Biyotime Institute of Biotechnology, Jiangsu, China).

Quantitative real-time PCR analysis (qRT-PCR)

The cells were transferred into a tube containing Trizol (Life Technologies Inc., Gaithersburg, USA) and total RNA was isolated, according to the manufacturer's protocol. RT-PCR analysis was performed with a QuantiTectTM SYBR® Green PCR (Tiangen, Shanghai, China) according to the manufacturer's instructions. The sequences of primers are listed in Table 1. The highly specific measurement of mRNA was carried out for IL-6, ICAM-1, IκBα, NF-κB p65, Runx2, and GAPDH using the LightCycler system (Bio-Rad, Carlsbad, USA). Each sample was run and analyzed in duplicate. Target mRNA levels were adjusted as the values relative to GAPDH, which was used as the endogenous control to ensure equal starting amounts of cDNA. When comparison between two groups was performed, the control group was used as the calibrator with a given value of 1, and the other groups were compared with this calibrator.

Measurement of mitochondrial ATP and ROS production

Mitochondria were isolated by differential centrifugation of cellular homogenates. Mitochondrial protein concentration was determined using a DC Protein Assay Kit (Bio-Rad, Hercules, CA). Rates of ATP formation were quantified using a commercially available kit (BioVision, Mountain View, CA, USA). Mitochondrial ROS production was evaluated by lucigenin chemiluminescence. The results were corrected for protein content.

Mitochondrial transmembrane potential (MMP)

The osteoblastic MMP in vitro was measured by flow cytometric analysis performed on a FACSCalibur fitted with an excitation/emission setting of 488/525 nm. The rhodamine-123 (Rh123) was used to detect changes in the MMP. Osteoblasts were incubated with Rh123 (0.1 nM) in a 37 °C water bath for 5 min in PBS. Cellular mean fluorescence intensity was analyzed using Cell Quest software programs (Phoenix Flow Systems, CA, USA).

NF-κB luciferase assay

NF-κB activity was determined using the NF-κB luciferase assay. Cells were seeded on 24-well culture plates at 2×10^4 cells/well. Cells were incubated for 1 h with a total of 170-ng plasmids (Promega, Madison, WI, USA) (85-ng NF-κB-dependent luciferase reporter plasmid-pGL4.32[luc2P/NF-kB-RE/Hygro] and 85-ng pcDNA3-β-gal), 1-μl Tfx-50 reagent (Promega), and 200-μl serum-free RPMI. In all, 800-μl RPMI containing FBS was then added, and incubation continued. The pGL4.32 plasmid was a NF-kB reporter vector. It contained NF-kB response elements and firefly luciferase gene. After 24 h of incubation, cells were treated with indicated chemicals. Luciferase activity was measured using a luciferase assay system and normalized against β-galactosidase activity.

NADPH oxidase assay

NADPH-dependent superoxide production was measured by the lucigenin-enhanced chemiluminescence method as described previously [20]. Cells were washed and pelleted in ice-cold PBS and then prepared in 300-μl lysis buffer (20 mM KH_2PO_4 (pH 7), 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 0.5 μg/ml leupeptin) using a Dounce homogenizer (100 strokes on ice). Homogenates were centrifuged at 800×*g* at 4 °C for 10 min to remove the unbroken cells and debris, and aliquots were used immediately. To start the assay, 100-μl

Table 1 Sequences of oligonucleotides used as primers

Target gene		Sequence (5′–3′)	Accession number[a]
IL-6	Sense	TCCTACCCCAACTTCCAATGCTC	NM_012589
	Antisense	TTGGATGGTCTTGGTCCTTAGCC	
Runx2	Sense	TATGGCACTTCGTCAGGATCC	NC_005108.2
	Antisense	AATAGCGTGCTGCCATTCG	
IκBα	Sense	CCCTGGAAAATCTTCAGACG	NM_001105720
	Antisense	ACAAGTCCACGTTCCTTTGG	
NF-κB p65	Sense	CATCAAGATCAATGGCTACA	NM_199267.2
	Antisense	CACAAGTTCATGTGGATGAG	
ICAM-1	Sense	AGACACAAGCAAGAGAAGAAAAGG	NC_005107.2
	Antisense	TTGGGAACAAAGGTAGGAATGTAT	
GAPDH	Sense	AGACAGCCGCATCTTCTTGT	NM_017008
	Antisense	CTTGCCGTGGGTAGAGTCAT	

[a] Genbank accession number of cDNA and corresponding gene is available at http://www.ncbi.nlm.nih.gov/

homogenates were added to 900-μl 50 mM phosphate buffer (pH 7.0) containing 1 mM EGTA, 150 mM sucrose, 5 μM lucigenin, and 100 μM NADPH. Photon emission was measured in a luminometer every 30 s for 10 min. There was no measurable activity in the absence of NADPH. For each sample, superoxide anion production was expressed as relative chemiluminescence (light) units per milligram protein.

Activity assay of catalase and total SOD

Catalase activity was determined by monitoring the rate of decomposition of H_2O_2 from the decrease in absorbance at 240 nm by the method as described previously [21]. Total SOD activity was assayed by monitoring nitroblue tetrazolium (NBT) reduction according to Spitz and Oberley [22] with some modifications. SOD inhibits NBT reduction caused by O_2 in the aerobic xanthine/xanthine oxidase system, and changes of absorbance at 560 nm within 2 min were recorded. One unit of SOD is defined as the amount of enzyme that causes 45 % inhibition of NBT reduction under the assay conditions described [21].

Malondialdehyde (MDA) assay

After treatment, cells were collected and the MDA concentration, a presumptive marker of oxidant-mediated lipid peroxidation, was determined using a MDA assay kit (Cayman, Ann Arbor, USA). The results were corrected for protein content.

Assay of $OONO^-$ level

The $OONO^-$ production in cells was detected as the method described by Elks [23]. Briefly, samples were incubated at 37 °C with CMH (1-hydroxy-3-methoxycarbonyl-2, 2,5,5-tetramethylpyrrolidine, 200 μM) for 30 min, then CPH (1-hydroxy-3-carboxypyrrolidine, 500 μM) for 30 min for $OONO^-$ measurement.

Study design

Cells were incubated with TNF-α (50 ng/ml) and treated with HRM or not for 4 h, apoptosis rate was determined.

Cells were incubated with TNF-α (5 ng/ml) and treated with HRM or not for 1, 3, 5, or 7 days, ALP activity was determined.

Cells were incubated with TNF-α (5 ng/ml) and treated with HRM or not for 24 h, cell viability, Runx2 mRNA expression, ROS formation, MDA production, NADPH oxidase activity, mitochondrial functions including mitochondrial ROS and ATP formation and MMP, total SOD and catalase activities, IκBα and NF-κB p65 mRNA expression, intracellular NO and $OONO^-$ levels, and eNOS and iNOS activities were determined.

Statistical analysis

All data are presented as mean ± SD A one-way ANOVA with LSD post hoc test was used to detect significant differences between groups. A probability level of less than 0.05 was considered significant. Statistical analysis was performed using SPSS 11.0.0 software (SPSS Inc., Chicago, IL, USA).

Results

Effect of treatment with H_2 on osteoblastic function treated by TNFα

In cultured primary osteoblasts, incubation with TNFα suppressed cell viability (TNFα, 5 ng/ml; Fig. 1a) induced

cell apoptosis (TNFα, 50 ng/ml; Fig. 1b), suppressed Runx2 mRNA expression (TNFα, 5 ng/ml; Fig. 1c), and inhibited ALP activity (Fig. 1d). Co-incubation with H_2-enhanced cell viability suppressed apoptosis, enhanced Runx2 expression and ALP activity, indicating that treatment with H_2 restored the osteoblastic function impaired by TNFα.

Incubation with H_2 under control condition had no significant effect on osteoblastic function.

Effect of treatment with H_2 on oxidative stress induced by TNFα

In cultured primary osteoblasts, incubation with TNFα (5 ng/ml) enhanced intracellular ROS formation (Fig. 2a) and MDA (Fig. 2b) production, increased NADPH oxidase activity (Fig. 2c), impaired mitochondrial function marked by increased mitochondrial ROS formation (Fig. 2d) and decreased mitochondrial membrane potential (Fig. 2e) and ATP synthesis (Fig. 2f), and suppressed activities of antioxidant enzymes including SOD (Fig. 2g) and catalase (Fig. 2h). Co-incubation with H_2 suppressed both ROS and MDA production, decreased NADPH oxidase activity, restored mitochondrial function and activities of antioxidant enzymes, indicating that treatment with H_2 effectively abated TNFα-induced oxidative stress.

Incubation with H_2 under control condition had no significant effect on oxidative stress, NADPH oxidase activity, mitochondrial function, activities of SOD and catalase.

Effect of treatment with H_2 on NFκB pathway activated by TNFα

In cultured primary osteoblasts, incubation with TNFα (5 ng/ml) decreased IκBα mRNA (Fig. 3a) expression and enhanced NFκB mRNA expression (Fig. 3b) and NFκB activity (Fig. 3c). Co-incubation with H_2 increased IκBα mRNA expression and decreased NFκB mRNA expression and activity, indicating that treatment with H_2 inhibited TNFα-induced activation of NFκB pathway.

Incubation with H_2 under control condition had no significant effect on IκBα-NFκB pathway.

Effect of treatment with H_2 on NO formation stimulated with TNFα

In cultured osteoblast, incubation with TNFα (5 ng/ml) enhanced intracellular NO formation (Fig. 4a) and $OONO^-$ production (Fig. 4b), suppressed eNOS activity (Fig. 4c) and enhanced iNOS activity (Fig. 4d). Co-incubation with H_2 decreased NO and $OONO^-$ production, increased eNOS activity and suppressed iNOS activity.

Incubation with H_2 increased NO formation and upregulated eNOS activity, but had no significant effect on $OONO^-$ production and iNOS activity under control condition.

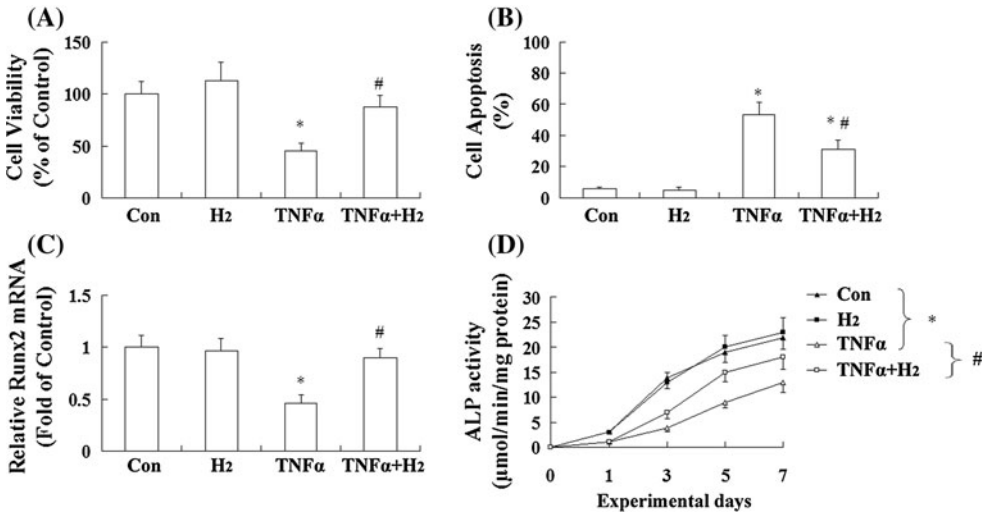

Fig. 1 Effect of treatment with H_2 on osteoblastic function impaired by TNFα. Primary osteoblasts were stimulated with TNFα and treated with H_2 or not. The cell viability (5 ng/ml) (**a**), apoptosis (50 ng/ml) (**b**), Runx2 mRNA level (5 ng/ml) (**c**), and ALP activity (5 ng/ml) (**d**) were assessed by MTT assay, flow cytometric analysis, qRT-PCR and ALP activity assay, respectively. *ALP* alkaline phosphatase; *Runx*2 runt-related transcription factor 2. The in vitro experiments were performed at least three times and each experiment was performed with replicates; $*P < 0.05$ versus the control group; $\#P < 0.05$ versus the TNFα-treated group

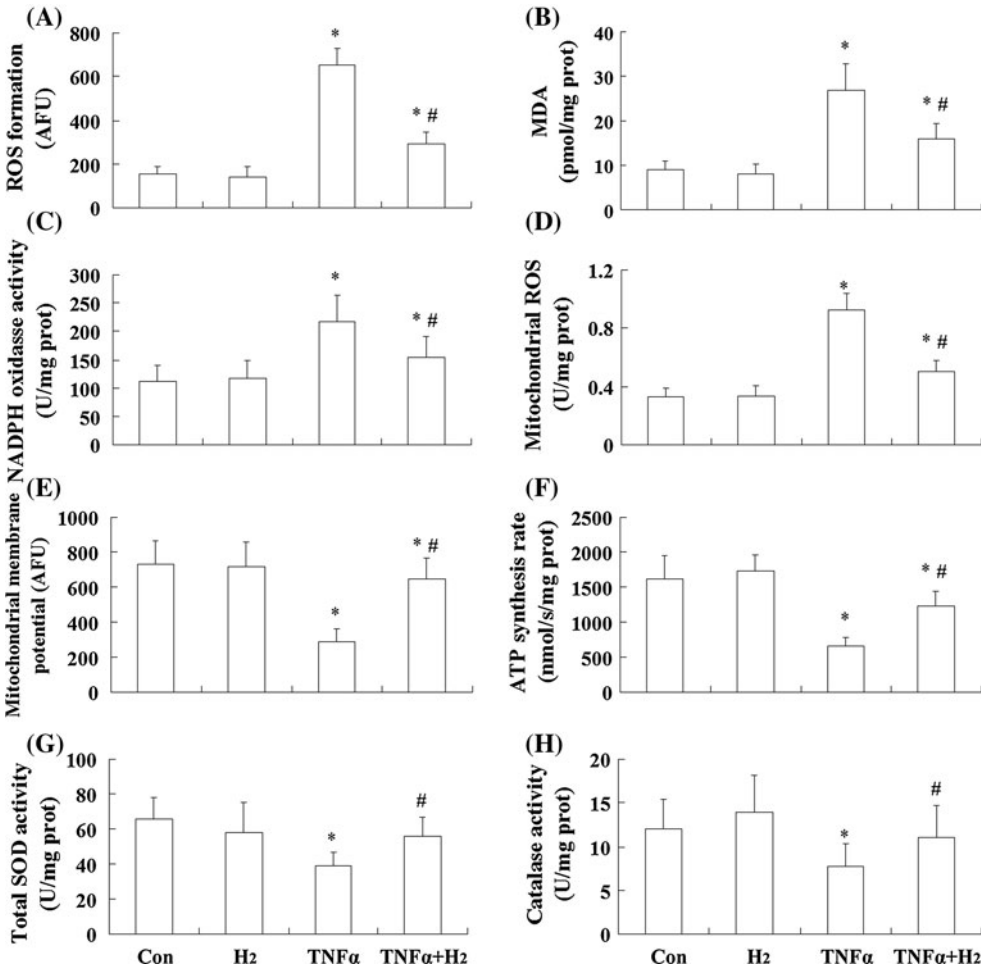

Fig. 2 Effect of treatment with H_2 on oxidative stress induced by TNFα. Primary osteoblasts were stimulated with TNFα (5.0 ng/ml) and treated with H_2 or not. Cellular ROS (**a**), MDA concentration (**b**), NADPH oxidase activity (**c**), mitochondrial ROS formation (**d**), mitochondrial membrane potential (**e**), ATP synthesis rate (**f**), total SOD activity (**g**), and catalase activity (**h**) were determined. *ROS* reactive oxygen species; *AFU* arbitrary fluorescence units; *MDA* malondialdehyde; *SOD* superoxide dismutase; the in vitro experiments were performed at least three times and each experiment was performed with replicates; $*P < 0.05$ versus the control group; $\#P < 0.05$ versus the TNFα-treated group

Effect of treatment with H_2 on IL-6 and ICAM-1 expression induced by TNFα

In cultured osteoblast, incubation with TNFα (5 ng/ml) enhanced IL-6 (Fig. 5a) and ICAM-1 (Fig. 5b) mRNA expression. Co-incubation with H_2 decreased IL-6 and ICAM-1 mRNA expression.

Incubation with H_2 under control condition had no significant effect on IL-6 and ICAM-1 mRNA expression in primary osteoblasts.

Discussion

Recently, Sun et al. [24] revealed that treatment with H_2 alleviated microgravity-induced bone loss in vivo and in vitro and first reported the protective effect of molecular hydrogen on bone. In this study, treatment with H_2 significantly alleviated TNFα-induced cell injury in osteoblast including restoring cell proliferation and suppressing apoptosis. In addition, treatment with H_2 enhanced Runx2 mRNA expression and ALP activity (two biomarkers of osteoblastic differentiation), indicating that H_2 alleviated TNFα-induced reduction of osteoblastic differentiation.

Oxidative stress is thought to play an important role in the pathogenesis of inflammation. Treatment with H_2 effectively abated TNFα-induced oxidative stress. NADPH oxidase, as the major source of intracellular ROS involved in the regulation of osteoblast viability and differentiation [25, 26], was found upregulated when treated with TNFα in this study. Mitochondria is the another major source of intracellular ROS, which was found impaired when treated with TNFα, marked by increased ROS formation and reduced MMP and ATP synthesis, which indicated that mitochondrial damage was also involved in the TNFα-induced oxidative stress. Treatment with H_2 not only suppressed activity of NADPH oxidase located in cytoplasm but also restored mitochondrial function. Compared with normal antioxidants such as vitamin C and vitamin E, H_2 is electrically neutral and much smaller, so it is able to easily

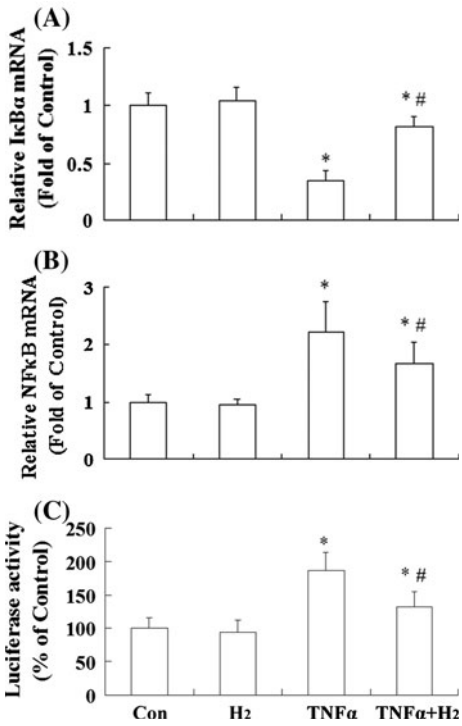

Fig. 3 Effect of treatment with H_2 on NFκB pathway activated by TNFα. Primary osteoblasts were stimulated with TNFα (5.0 ng/ml) and treated with H_2 or not. The mRNA levels of IκBα (**a**), NFκB (**b**), and NFκB activity (**c**) were measured by qRT-PCR method and luciferase assay, respectively. The in vitro experiments were performed at least three times and each experiment was performed with replicates; $*P < 0.05$ versus the control group; $\#P < 0.05$ versus the TNFα-treated group

penetrate membranes and enter cells and organelles such as the nucleus and mitochondria, where most commonly used antioxidant cannot arrive [18].

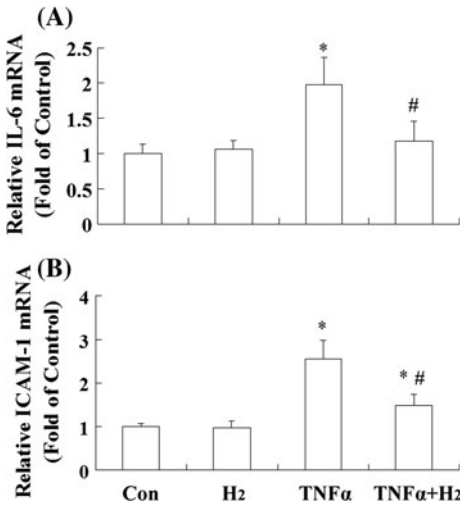

Fig. 5 Effect of treatment with H_2 on IL-6 and ICAM-1 expression induced by TNFα. Primary osteoblasts were stimulated with TNFα (5.0 ng/ml) and treated with H_2 or not. The mRNA levels of IL-6 and ICAM-1 were determined by qRT-PCR method. ICAM-1, intercellular cell adhesion molecule-1. The in vitro experiments were performed at least three times and each experiment was performed with replicates; $*P < 0.05$ versus the control group; $\#P < 0.05$ versus the TNFα-treated group

SOD and catalase (two important antioxidant enzymes), act by catalyzing the conversion of superoxide radicals to hydrogen peroxide, which can then be further reduced to water by catalase [27, 28]. Mice deficient in SOD1 exhibited deficient in bone formation [29]; catalase administration was shown to prevent oophorectomy-induced bone loss [30]. SOD and catalase activities were found down-regulated when treated with TNFα. Treatment with H_2 restored the activities of SOD and catalase. Therefore,

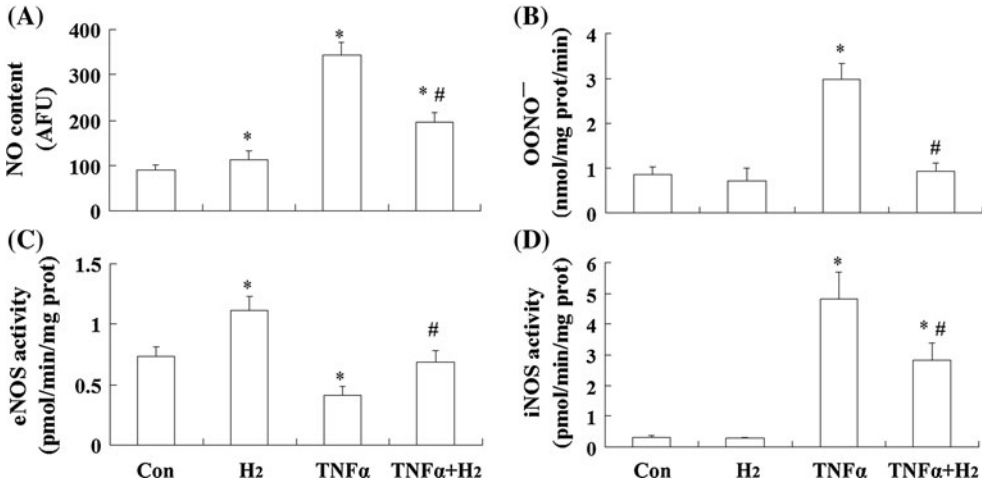

Fig. 4 Effect of treatment with H_2 on NO formation stimulated with TNFα. Primary osteoblasts were stimulated with TNFα (5.0 ng/ml) and treated with H_2 or not. Intracellular NO content (**a**) and $OONO^-$ (**b**) content, and eNOS (**c**) and iNOS (**d**) activities were measured. *NO* nitric oxide; *AFU* arbitrary fluorescence units; *eNOS* endothelial nitric oxide synthase; $OONO^-$ peroxynitrite; *iNOS* inducible nitric oxide synthase; The in vitro experiments were performed at least three times and each experiment was performed with replicates; $*P < 0.05$ versus the control group; $\#P < 0.05$ versus the TNFα-treated group

treatment with H_2 abated TNFα-induced oxidative stress though suppressing ROS formation form NADPH oxidase and mitochondria and restoring intracellular antioxidant defences in osteoblast.

Besides antioxidant property, H_2 exerted its protective effect against TNFα-induced cell injury in osteoblast through suppressing NFκB activity. In this study, H_2 decreased both mRNA expression of IκBα and NFκB and activity of NF-κB induced by TNFα. Several in vivo and in vitro studies revealed that inhibition of NFκB activation promoted bone formation and could be as an important target in the treatment of osteoporosis [31, 32]. Increased expression and activity of NF-κB induces gene transcription of pro-inflammatory cytokines, such as IL-6 and ICAM-1, to increase their production [33], which were also found augmented in osteoblast when treated with TNFα. H_2 suppressed TNFα-induced NFκB activation and subsequent expression of IL-6 and ICAM-1 to directly protect osteoblast against inflammation.

Another important downstream protein of NF-κB is iNOS, which was found upregulated in osteoblast when treated with TNFα. Excess local production of NO derived from iNOS aggravates bone destruction in inflammation-induced osteoporosis [34]. It not only mediated cytokine-induced apoptosis [35] but also had potent inhibitory effects on osteoblast growth and differentiation [36]. In addition, reaction of NO and superoxide anion formed the peroxynitrite ($OONO^-$), which was the most cytotoxic chemicals of ROS. It was reported that treatment with H_2 could reduce the peroxynitrite content selectively [18], which might resulted from that it could downregulated iNOS activity which was observed in this study.

There was another interesting finding that in control condition, treatment with H_2 upregulated eNOS activity and NO formation. Under physiological condition, NO derived from eNOS is one of the key local mediators and second messengers of systemic hormones including calcitonin gene-related peptide, parathyroid hormone, and sex steroids, particularly estrogen, which is involved in the regulation of bone function [37]. Circulating NO level was reported reduced and related with osteoporosis in aged rats and ovariectomized rats [38]. Therefore, our result suggested that H_2 might be used to treat osteoporosis in aged rats and ovariectomized rats.

Conclusion

Treatment with hydrogen molecule alleviates TNFα-induced cell injury in osteoblast, at least in part, through abating oxidative stress, suppressing inflammation, and downregulating iNOS activity. Our results suggest that hydrogen molecule may be used as one novel therapeutic approach for treating bone loss in rheumatoid arthritis and postmenopausal osteoporosis.

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