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ORIGINAL PAPER

Heme oxygenase-1 prevents glucocorticoid and hypoxia-induced apoptosis and necrosis of osteocyte-like cells

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Abstract

Glucocorticoids and hypoxia is considered to promote osteocyte apoptosis and necrosis, which are observed in glucocorticoid-associated osteonecrosis and osteoporosis. Heme oxygenase-1 (HO-1) induced by hemin is reported to have cytoprotective effects in ischemic diseases. The objective of this study was to evaluate the effect of HO-1 on osteocyte death caused by glucocorticoids and hypoxia. We confirmed that hemin induced HO-1 expression in MLO-Y4 mouse osteocytes. MLO-Y4 was cultured with dexamethasone (Dex) under hypoxia (DH group). Furthermore, these cells were cultured with hemin (DH-h group) or hemin and zinc protoporphyrin IX (an HO-1 inhibitor) (DH-h-PP group). The rates of apoptosis and necrosis of these groups were analyzed by flow cytometry and compared with cells cultured under normal condition. Both apoptosis and necrosis increased in the DH group. Hemin administration significantly reduced cell death caused by glucocorticoids and hypoxia in the DH-h group, and its effect was attenuated by the HO-1 inhibitor in DH-h-PP group. Capase-3 activity significantly decreased in the DH-h group. This implied that the cell death inhibition effect due to hemin is mediated by HO-1 and caspase-3. HO-1 induction may be useful in the treatment of glucocorticoid-associated osteonecrosis and osteoporosis.

Keywords Heme oxygenase-1 · Hemin · Osteonecrosis of the femoral head · Osteoporosis · Hypoxia

Introduction

Glucocorticoids have been effectively used for the treatment of various diseases such as rheumatoid arthritis or systemic lupus erythematosus. However, various side effects of glucocorticoid treatment have been reported, and osteoporosis is a typical complication in such cases. Another serious complication is glucocorticoid-associated osteonecrosis of the femoral head (ONFH), an intractable disease that destroys the hip joint function [1]. Once glucocorticoid-associated ONFH sets in and the femoral head collapses, surgical treatment is the only option available. As yet, there are no effective conservative treatments for glucocorticoid-associated ONFH. Thus, the development of novel efficient prophylaxis method is urgently needed.

Although the pathology and etiology of glucocorticoid-associated ONFH have not been completely elucidated, there have been reports of abnormal lipid metabolism, coagulation abnormalities, oxidative stress, and fat embolism inside the bones after glucocorticoid administration [2–9]. For these reasons, it is thought that the cells where ischemia occurs are exposed to hypoxia inside the femoral head [10–13]. The hypoxia increases reactive oxygen species (ROS) production, damaging DNA and RNA [14], and activates apoptotic regulators Bcl-2, Bax in the osteocytes, inducing cell death [15]. Both of glucocorticoids and hypoxia are reported to induce cell death through the activation of caspase [16, 17]. Furthermore, a previous study found that glucocorticoids and hypoxia induced apoptosis and necrosis of cultured osteocyte-like cells [18]. These types of cell death are possibly involved in the onset of glucocorticoid-associated ONFH. Thus, inhibition of apoptotic cell death and ROS production may prevent onset of glucocorticoid-associated ONFH.

Hemin is a substance that induces heme oxygenase-1 (HO-1), which has anti-inflammatory and antioxidation

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properties. The hemin-induced HO-1 catalyzes the rate-limiting step of heme decomposition. HO-1 exerts its cytoprotective effects by promoting the production of iron ions [19], bilirubin [20], and CO [21]. HO-1 has been reported to have tissue-protective effect in ischemic diseases such as myocardial infarction [22]. Thus, it may be a potential candidate for the treatment of glucocorticoid-associated ONFH and osteoporosis, which are ischemic bone diseases [13, 23]. The objective of this study was to evaluate the effect of HO-1 on osteocyte death caused by glucocorticoids and hypoxia.

Materials and methods

Cell culture and treatment

We obtained the MLO-Y4 mouse osteocyte-like cells from Kerafast, Inc (originally from the laboratory of Lynda Bonewald). The cells were cultured as previously described [24]. Briefly, the culture plates were coated with collagen type I-C, and a-MEM, to which 5% (v/v) FBS, 5% (v/v) FCS, streptomycin (100 μg/mL), and penicillin (100 units/mL) were added. Dexamethasone (Dex) (MSD, Tokyo, Japan) was used as the glucocorticoid. Hypoxia environment was set by adjusting to 5% (v/v) CO_2 and 1% (v/v) O_2 concentrations with N_2 using a CO_2/tri-gas incubator (Astec, Fukuoka, Japan). Hemin was added as an HO-1 inducer 18 h before exposure to the glucocorticoids and hypoxic conditions. Zinc protoporphyrin IX (ZnPPIX) was added as an HO-1 inhibitor 2 h before hemin administration. The hemin was directly diluted with the culture fluid. The ZnPPIX was diluted with DMSO and then diluted with the culture fluid, adjusting the DMSO concentration to 0.1% (v/v).

Cell viability assay

Cell viability was determined using the RealTime-Glo MT Cell Viability Assay Kit (Promega, Madison, WI, USA). Cells were plated in a poly-L-lysine (PLL)-coated 96-well plate at a density of 1000 cells per well in 100 μL of medium containing 10% FBS. After 24 h, MT cell viability substrate and Nanoluc® Enzyme were added to the medium and the cells were exposed to various concentrations of hemin (0, 1, 5, 10, 20, 30 μM) and ZnPPIX (0, 1, 5, 10, 20, 30 μM). The CentroXS3 LB 960 system (Berthold Technologies, Oak Ridge, TN, USA) was used to measure light emission.

Total RNA extraction and real-time RT-PCR analysis

Cells were harvested and total RNA was extracted using ISOGEN (Nippon Gene, Osaka, Japan). Extracted RNA was reverse-transcribed using PrimeScript™ RT Master Mix (Takara Bio, Shiga, Japan), according to the manufacturer's instructions. Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) was performed using the Biosystem 7300 (Applied Biosystems, Carlsbad, CA, USA). Sequences of the primers used were as follows: human HO-1 (forward primer, 5′-AGGCTAAGACCGCCTTCCT-3′; reverse primer, 5′-TGTGTTCCTCTGTCAGCATCA-3′) and 18S ribosomal RNA (forward primer, 5′-ATGAGTCCACTTTAAATCCTTTAACGA-3′; reverse primer, 5′-CTTTAATATACGCTATTGGAGCTGGAA-3′). Each 25 μL reaction mixture contained 2 μL of cDNA (100 ng) and 12.5 μL of the TaqMan Gene Expression PCR Master Mix (TOYOBO, Osaka, Japan) for the target gene. The amplification protocol consisted of 40 cycles of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min. Relative changes in gene expression were normalized against the 18S ribosomal RNA as the internal control.

Western blotting

The cells were washed twice with PBS and then harvested and lysed in RIPA buffer (Nacalai Tesque) that was diluted tenfold in ultrapure water. Samples were frozen at − 80 °C for 10 min, thawed at 37 °C, and then incubated on ice for 30 min. The samples were centrifuged at 20,600×*g* for 5 min and the supernatant was aspirated. Protein concentrations of the supernatants were measured using bicinchoninic acid (BCA) assay, and equal amounts of total protein were subjected to SDS–PAGE. Samples containing 5 μg of protein were separated by 10% Bis-Tris Gel NuPAGE® electrophoresis using 5% MOPS SDS Running Buffer (Thermo Fisher Scientific, Waltham, MA, USA). Separated proteins were dry-blotted onto nitrocellulose iBlot®gel transfer stacks in iBlot Gel transfer devices (Thermo Fisher Scientific) for 7 min. The nitrocellulose membrane was blocked by shaking in Blocking One solution (Nacalai Tesque) for 60 min at room temperature. The blots were subsequently shaken overnight at 4 °C in the same solution containing the following specific primary antibodies: anti-mouse HO-1 rabbit monoclonal antibody (1:2000 dilution; Abcam, Tokyo, Japan; catalog no. ab68477) and mouse anti-human β-actin antibody (1:4000 dilution; Sigma-Aldrich; catalog no. A2228). The β-Actin was used as a loading control.

Blots were washed three times with TBST and shaken for 60 min at room temperature with peroxidase-conjugated secondary antibodies: goat anti-rabbit IgG antibody (1:4000 dilution; Sigma-Aldrich; catalog no. A0545), and anti-mouse IgG antibody (1:4000 dilution; Sigma-Aldrich; catalog no. A4416). The blots were then again washed three times with TBST, and chemiluminescence emission was visualized using Chemi-Lumi One Super (Nacalai Tesque). Protein band intensities were assessed using the ECL Select LAS500 (GE Healthcare, Buckinghamshire, England).

Annexin-V/propidium iodide assay

Cells were stained with 5 µL Annexin V-FITC and 5 µL PI solution (Nacalai Tesque) for 15 min at room temperature in the dark. Following incubation, binding buffer was added and the cells were analyzed by flow cytometry using a FACS Vantage Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). For each sample, 10,000 events were recorded.

Caspase-3 activity

The activity of caspase-3, an apoptosis-related factor, was assessed using the Caspase-Glo 3/7 Assay (Promega). Cells were sub-cultured in a PLL-coated 96-well plate (1×104 cells/well) and were then treated with cisplatin for 24 h. After this treatment, the Caspase3/7 Reagent was added to the cells for 1 h at room temperature. A CentroXS3 LB 960 system was used to measure light emission.

Statistical analysis

All duplicated and triplicated experiments yielded almost identical results. All experimental data are expressed as mean ± SD. Parametric one-way ANOVA was used to test for any differences among the groups. If the result was significant, the Tukey–Kramer test was used to determine specific differences between the groups. In all analyses, $p < 0.05$ was defined as statistically significant.

Results

Effect of hemin and ZnPPIX on MLO-Y4 cell proliferation

Cells were cultured with the indicated concentrations of hemin (0, 1, 5, 10, 20, 30 µM) for 18 h, after which the cell viability was measured every 12 h, for a total of 48 h after end of hemin treatment. At 30 µM concentration of hemin, a significant decrease in cell viability was observed at each hour from 0 to 48 h ($p < 0.05$), while a 20 µM concentration induced a significant decrease in cell viability only at 24 h after end of hemin stimulation ($p < 0.01$) (Fig. 1).

Similarly, cells were cultured with the indicated concentrations of ZnPPIX (0, 1, 5, 10, 20, 30 µM) for 2 h, after which the cell viability was measured every 12 h, for a total of 72 h, after end of ZnPPIX stimulation. No significant change in cell viability was observed at any concentration at any time point examined (Fig. 2).

Evaluation of HO-1 expression after hemin treatment

The MLO-Y4 cells were divided into a total of six groups: a control group cultured in a 20% oxygen environment, and five groups cultured with hemin administered at different concentrations (namely, 1, 5, 10, 20, 30 µM) under a 20% oxygen environment. After culturing the cells for 18 h at the respective conditions, the groups were compared for HO-1 gene and protein expression. The gene expression of HO-1 increased in a dose-dependent manner with hemin (Fig. 3a). In the 1- and 5-µM hemin administration groups, there was an increasing trend in HO-1 gene expression; however, the difference was not significant compared with the control group. In the 10, 20, and 30 µM hemin administration groups, there was a significant increasing trend in the

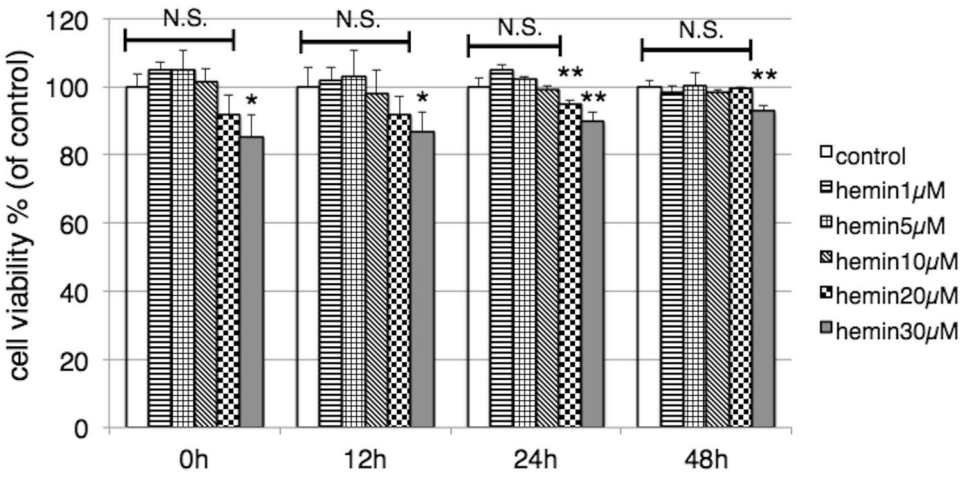

Fig. 1 Cell viability of MLO-Y4 after hemin treatment. MLO-Y4 cells were cultured for 18 h under administration of hemin at different concentrations (0, 1, 5, 10, 20, 30 µM). Cell viability was measured at 0, 12, 24, and 48 h after culturing. Significance was tested by the Tukey–Kramer test and significant differences with the control are indicated as $*p < 0.05$ and $**p < 0.01$

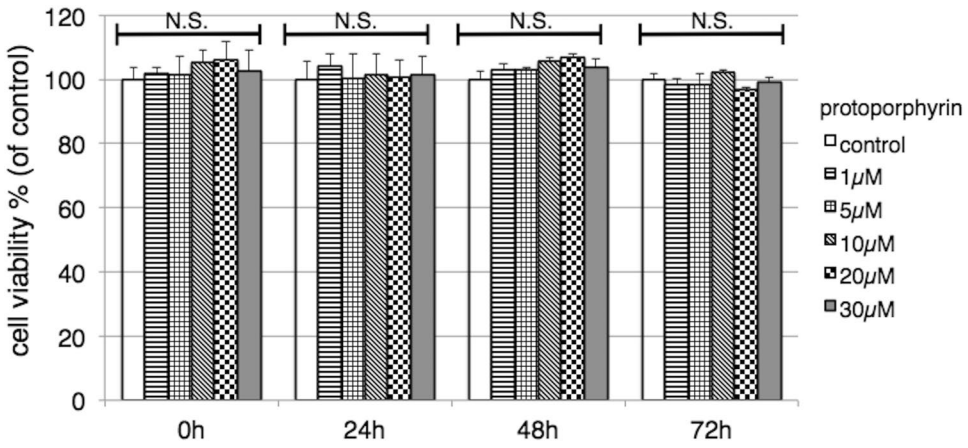

Fig. 2 Cell viability of MLO-Y4 after ZnPPIX treatment. MLO-Y4 cells were cultured for 2 h under administration of ZnPPIX at different concentrations (0, 1, 5, 10, 20, 30 μM). Cell viability was measured at 0, 24, 48, and 72 h after culturing. Significance was tested by the Tukey–Kramer test

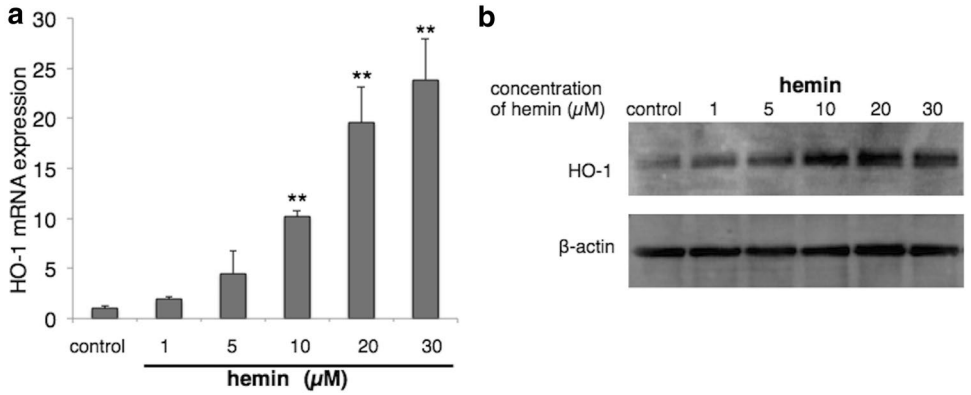

Fig. 3 Effect of hemin treatment on HO-1 mRNA and protein levels. MLO-Y4 cells were cultured for 18 h with hemin at different concentrations (0, 1, 5, 10, 20, 30 μM). **a** HO-1 mRNA expression is shown measured using real-time PCR. The Tukey–Kramer test was performed and significant differences with the control are indicated as $^{**}p < 0.01$. **b** Protein expression of HO-1, measured by western blotting, is shown

expression of HO-1 gene over the control group ($p < 0.01$) (Fig. 3a). Likewise, an increased protein expression of HO-1 over the control group was observed in the 10-, 20-, and 30-μM hemin administration groups (Fig. 3b). Based on these observations, further experiments were conducted using 10 μM hemin concentration, which was capable of effectively inducing HO-1 without affecting cell viability.

Evaluation of HO-1 expression after ZnPPIX treatment and hemin treatment

The MLO-Y4 cells were divided into a total of five groups: a control group cultured in a 20% oxygen environment, and four groups (namely, Groups PP0, PP10, PP20, and PP30) to which ZnPPIX was administered at various concentrations (0, 10, 20, and 30 μM, respectively) under a 20% oxygen environment and cultured for 2 h, after which 10 μM hemin was administered and cells were cultured for another 18 h. The gene expression of HO-1 was significantly elevated in the group administered only hemin (10 μM) compared with the control group ($p < 0.01$). However, the hemin-induced HO-1 expression gradually decreased as the dose of ZnPPIX increased (Fig. 4). In the PP0 group and PP10 group, although HO-1 gene expression was significantly elevated compared with the untreated control ($p < 0.01$), in the PP20 and PP30 groups the HO-1 expression was inhibited and was similar to the levels in the control group (Fig. 4a). Similarly, the HO-1 protein level was elevated in the PP0 group, while in the PP20 group it was inhibited to levels as in the control group (Fig. 4b).

Evaluation of apoptosis and necrosis of MLO-Y4

The MLO-Y4 cells were divided into the following four groups and examined for cell death: (1) control group, continuously cultured under 20% oxygen environment, (2) DH group, in which after having been cultured under 20% oxygen environment, the cells were administered with 1 μM Dex and cultured for 24 h [25, 26] under 1% oxygen environment [27, 28], (3) DH-h group, in which 10 μM hemin-treated

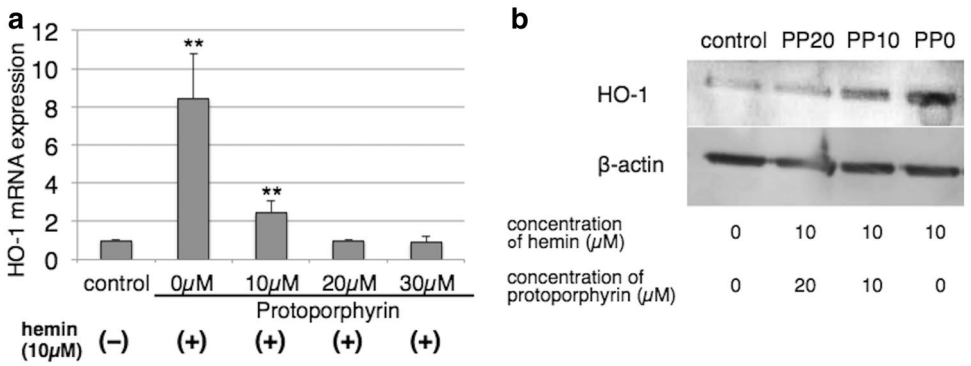

Fig. 4 Effect of ZnPPIX treatment on hemin-induced HO-1 mRNA and protein. Hemin-treated MLO-Y4 cells were cultured for 2 h after administering ZnPPIX at different concentrations (0, 1, 5, 10, 20, 30 μM). **a** HO-1 mRNA expression is shown measured using real-time PCR. The Tukey–Kramer test was performed and significant differences with the control are indicated as $**p<0.01$. **b** Protein expression of HO-1, measured by western blotting, is shown

cells were cultured for 18 h under 20% oxygen environment, followed by 1 μM Dex administration and further culturing for 24 h under 1% oxygen environment, and (4) DH-h-PP group, in which ZnPPIX-administered cells were cultured for 2 h under 20% oxygen environment, after which the cells were treated with 10 μM hemin and cultured for 18 h under 20% oxygen environment, followed by 1 μM Dex administration and further culturing for 24 h under 1% oxygen environment. After having cultured the four groups under the respective conditions, we measured and compared the proportions of apoptosis and necrosis relative to the total cells per well by flow cytometry.

The apoptosis rate was significantly elevated in the DH group (14.95%) compared with the control group (4.63%) ($p<0.01$). Hemin administration significantly reduced the apoptosis in the DH-h group (11.93%), compared with the DH group ($p<0.05$). This apoptosis inhibition effect was attenuated in the DH-h-PP group (15.08%), which had no significant difference with the DH group (Fig. 5a, b).

Similarly, the necrosis rate was significantly elevated in the DH group (7.56%) compared with the control group (4.28%) ($p<0.01$). Hemin administration significantly reduced the necrosis in the DH-h group (3.02%) compared with the DH group ($p<0.01$). Further, the rate of necrosis in

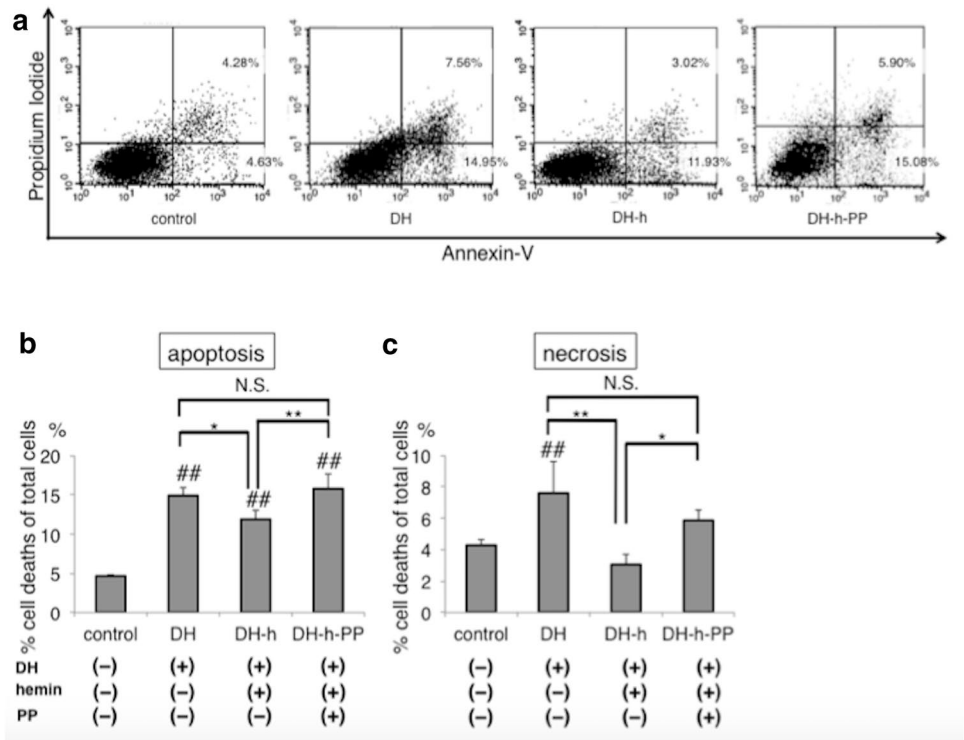

Fig. 5 Evaluation of apoptosis and necrosis of MLO-Y4. **a** Cell apoptosis and necrosis were measured by flow cytometry by the Annexin V-EGFP/PI staining assay. The lower right section of four different quadrants represents apoptosis and the upper right represents necrosis. **b, c** The rates of apoptosis and necrosis were measured by flow cytometry. Comparison was made between four groups (control, DH, DH-h, and DH-h-PP). The Tukey–Kramer test was performed and significant differences between the three groups (DH, DH-h, DH-h-PP) are indicated as $*p<0.05$ and $**p<0.01$. Significant differences with the control are indicated as $\#\#p<0.01$. DH: dexamethasone (1 μM) and hypoxia (1% O_2) for 24 h. hemin: hemin (10 μM) for 18 h. PP: ZnPPIX (20 μM) for 2 h

the DH-h group did not differ significantly from the control group and the necrosis inhibition effect was attenuated in the DH-h-PP group (5.90%), which displayed no significant difference compared with the DH group (Fig. 5a, c).

Analysis of caspase-3 activity

We performed a comparative study of the caspase-3 activity in the control, DH, and DH-h groups after having cultured them for 0, 6, 12, and 24 h under glucocorticoids and hypoxia. At 6 h after the start of glucocorticoids and hypoxia stimulation, there was no significant difference in caspase-3 activity between all three groups. After 12 h, though, the caspase-3 activity was significantly higher in the DH group compared with the control group ($p < 0.01$), as well as the DH-h group, which tended to show the least activity. After 24 h, the caspase-3 activity of the DH-h group was significantly lower than that in the control ($p < 0.05$) as well as DH groups ($p < 0.01$) (Fig. 6).

Discussion

In this study, we confirmed that HO-1 was induced by hemin in MLO-Y4 cultured osteocytes. Hemin administration significantly reduced cell death due to glucocorticoids and hypoxia, and this effect was attenuated by the HO-1 inhibitor, ZnPPIX. These observations imply that the hemin-induced cell death inhibition is mediated by HO-1. This study is the novel report to show that hemin-induced HO-1 has an osteocyte protection effect.

HO-1 is known to inhibit the production of ROS [29] and exhibit a cytoprotective effect by exercising an antioxidant and anti-inflammatory effect though the inhibition of cytokines such as TNF-α, IL-6, and IL-1β [30]. Although the hemin-induced HO-1-mediated alleviation of tissue disorders such as ischemic heart disease [18, 22] and renal ischemia [31] is known, there are few reports concerning bone tissue.

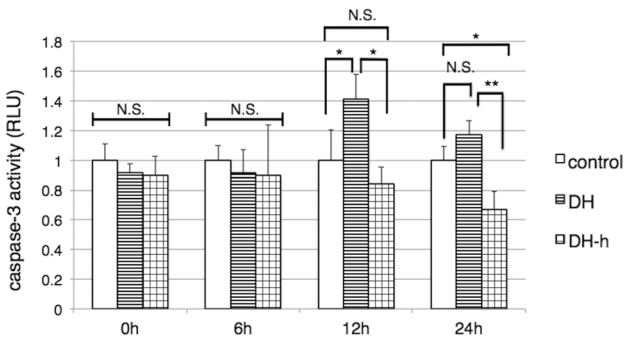

Fig. 6 Evaluation of caspase-3 activity of MLO-Y4. Caspase-3 activity was measured, and comparison over time (0, 6, 12, 24 h) was made between three groups (control, DH, DH-h). The Tukey–Kramer test was performed and significant differences between the three groups are indicated as $*p < 0.05$ and $**p < 0.01$

A previous report suggests that the apoptosis of osteocytes and osteoblasts is involved in the onset process of glucocorticoid-associated ONFH [32]. In addition, studies have revealed that glucocorticoids directly induce apoptosis of osteocytes and osteoblasts [33, 34]. Further, apoptosis of osteocytes and osteoblasts has been associated with osteoporosis [35]. Ischemia within the femoral head generated by vascular endothelial cell disorders due to glucocorticoid administration is thought to be involved in the pathology of ONFH [36, 37]. Induction of osteocyte apoptosis has also been confirmed in vitro under the hypoxic conditions [18]. In this study, we have demonstrated that HO-1 induction not only reduces osteocyte necrosis, but also the rate of apoptosis induced by glucocorticoids and hypoxia. Thus, HO-1 may be able to inhibit osteocyte apoptosis that is important in the onset process of glucocorticoid-associated ONFH, ultimately preventing or limiting osteonecrosis.

Glucocorticoids are reported to have multiple negative effects such as promoting ROS production, inducing DNA and RNA damage [38, 39], and inducing cell death through the activation of caspase [16]. In hypoxic environments, decreased ATP production, increased ROS production, DNA and RNA damage inside the cells [14], and the occurrence of cell death due to activation of caspase [17], are known. In this study, caspase-3 activation due to glucocorticoids and hypoxia could be inhibited by HO-1 induction, again implying the potential use of HO-1 to inhibit the activation of caspase, and thereby reduce osteonecrosis due to glucocorticoids and hypoxia.

Hemin is a clinically established therapeutic agent for treatment of porphyria. In addition, cell viability upon hemin administration (as an HO-1 inducer) exhibited no significant difference over time compared with control at the concentrations used. Hemin can, thus, be safely administered as a treatment drug for glucocorticoid-associated ONFH and osteoporosis.

A limitation of this study is that the inhibitory effect of HO-1 on the apoptosis and necrosis induced by glucocorticoids and hypoxia could only be confirmed in osteocytes. In the future, confirmation of the same effect in studies with osteoblasts and endothelial cells or tissues will be needed. In addition, the mechanism of the inhibitory effect mediated by HO-1 on apoptosis and necrosis induced by glucocorticoid and hypoxia was not completely confirmed.

Conclusion

Our study shows that hemin has an inhibitory effect on apoptosis and necrosis of osteocyte induced by glucocorticoid administration and hypoxia. This inhibitory effect

could be attenuated by an HO-1 inhibitor, implying that the effect of hemin is mediated by induction of HO-1. We propose that hemin-induced HO-1 may be a potent candidate in the prevention of glucocorticoid-associated ONFH and osteoporosis.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest.

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