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Inhibited autophagy may contribute to heme toxicity in cardiomyoblast cells

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ABSTRACT

Several groups have demonstrated that induction of heme-oxygenase-1 (HO-1) could protect the myocardium against ischemic events; however, heme accumulation could lead to toxicity. The aim of the present study was to investigate the role of autophagy in heme toxicity.

H9c2 cardiomyoblast cells were treated with different dose of hemin or cobalt-protoporphyrin IX ($CoPP_{IX}$) or vehicle. Cell viability was measured by MTT assay. DCF and MitoSOX staining was employed to detect reactive oxygen species. Western blot analysis was performed to analyse the levels of HO-1, certain autophagy related proteins and pro-caspase-3 as an apoptosis marker. To study the autophagic flux, CytoID staining was carried out and cells were analyzed by fluorescence microscope and flow cytometry.

Decreased cell viability was detected at high dose of hemin and $CoPP_{IX}$ treated H9c2 cells in a dose-dependent manner. Furthermore, at concentration of the inducers used in the present study a significantly enhanced level of ROS were detected. As it was expected both treatments induced a robust elevation of HO-1 level. In addition, the Beclin-1- independent autophagy was significantly increased, but caused a defective autophagic flux with triggered activation of caspase-3.

In conclusion, these results suggest that overexpression of HO-1 by high dose of hemin and $CoPP_{IX}$ can induce cell toxicity in H9c2 cells via enhanced ROS level and impaired autophagy.

1. Introduction

Heme oxygenases (HOs) exist in two major isoforms with catalytic activity including the inducible HO-1 and constitutive HO-2 [1]. The expression of HO-1 occurs at baseline level in most tissues under physiological circumstances and it can be highly induced in response to various harmful conditions [2]. HO-1 is increasingly recognized as an important mediator of cellular homeostasis in case of stress and cell injury. It plays a crucial role in cell survival and also inhibits apoptosis through several distinct mechanisms [3]. However, heme is a “double edged sword” [4]; in small amount, it acts as a functional group of heme proteins, and provides indispensable cellular functions; however, when a large amount of heme accumulates, it has been found to be toxic, which manifests as an extremely diverse process in large variety of cell types [5]. In general, hem toxicity is accompanied by enhanced generation of iron-derived reactive oxygen species (ROS), DNA damage, with oxidation of lipids and proteins [6]. For this purpose, former studies have investigated that $CoPP_{IX}$ and hemin were cytotoxic on different cell types. Cai et al. have used series of dose of $CoPP_{IX}$ and found that treatment with 10–40 μM $CoPP_{IX}$ was not toxic for human cardiac stem cells [7]. Additionally, cell culture experiments have shown that 3–30 μM hemin was sufficient to decrease the cell viability of cultured neurons and astrocytes by 30–40% [8]. Interestingly, hemin was not significantly affected the cell viability on CaCo-2 cells at the concentrations up to 100 μM [9]. Furthermore, Gemelli et al. investigated the cytotoxic effect of hemin (0.1–1 mM) in colon epithelial cell line. They found that hemin decreased the cell viability in a dose and time dependent manner [10]. Autophagy is an evolutionarily conserved, dynamic and highly regulated process of self-digestion. There are at least three different

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types of autophagy: macroautophagy (hereafter referred as autophagy), microautophagy and chaperone-mediated autophagy. Autophagy includes a series of sequential steps. Its initiation starts with the activation of the class III PI3K/Vsp34 and Beclin-1 complex. Elongation of isolation membrane by ATGs, LC3B and p62 proteins is then processed with the maturation of autophagosome (double-membrane structure) and followed by fusion with a lysosome generating autolysosome [11].

Overexpression of HO-1 by human HO-1 recombinant plasmid prevented the cardiac dysfunction at high glucose conditions and enhanced the level of autophagy [12]. Higdon et al. has used various concentrations of hemin to induce HO-1 and found that hemin caused mitochondrial dysfunction in endothelial cells by post-translational modification of proteins induced by reactive lipid and oxygen species. Furthermore, hemin exposure also induced mitophagy, but it was not sufficient to prevent cell death [13].

Nevertheless, heat stress induced HO-1 expression suppressed the protective autophagy in cerebellar Purkinje cells [14].

The present study was planned to investigate the role of autophagy-related heme toxicity.

2. Materials and methods

2.1. Materials

Hemin, cobalt-protoporphyrin IX ($CoPP_{IX}$), medium, serum and MTT were purchased from Sigma (St. Louis, MO, USA) DCFDA from Santa Cruz Biotechnology (Dallas, TX, USA); MitoSOX from Life technologies (Paisley, Scotland). The H9c2 cells were obtained from ATCC, CRL-1446, LGC Standards GmbH Wesel, Germany. Stain-Free gels were purchased from Bio-Rad Laboratories (Hercules, CA, USA). HO-1 antibody was obtained from Abcam (Cambridge, UK). Beclin-1, LC3B, p62 and Caspase-3 antibodies were obtained from Cell Signaling Technology (Boston, MA, USA). CYTO-ID Autophagy Detection Kit 2.0 was bought from Enzo Life Sciences, Inc. (Farmingdale, NY, USA).

2.2. Cell culture and treatment

Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% streptomycin-penicillin at 37 °C in a humidified incubator consisting of 5% CO_2 and 95% air. Cells were exposed to eight different conditions: only medium (untreated control group); medium containing 20 mM NaOH (vehicle treated control group); 3 μM hemin; 10 μM hemin; 30 μM hemin; 100 μM hemin; 2.5 μM $CoPP_{IX}$; 25 μM $CoPP_{IX}$ and 100 μM $CoPP_{IX}$ groups for 24 h.

2.3. Cell viability assay

Cell viability was measured by MTT experiments on 96-well plates. After treatment, MTT solution (final concentration of 0.5 mg/ml) was added to each well and incubated for 3.5 h at 37 °C. Then medium was replaced by isopropyl alcohol to dissolve formazan product and incubated for 30 min at 37 °C. Absorbance was measured with a microplate reader (FLUOstar OPTIMA, BMG Labtech) at 570 and 690 nm. The values were expressed relative to untreated control, which was represented as 100% of viability. 1% H_2O_2 were used as positive control. Absorbance values were averaged across 7 replicate wells, and repeated minimum 3 times.

2.4. Protein isolation

After the treatment, samples were lysed in 100 μl isolating buffer (in mM: 25 Tris-HCl, 25 NaCl, 1 orthovanadate, 10 NaF, 10 pyrophosphate, 10 okadaic acid, 0.5 EDTA, 1 PMSF, 1x protease inhibitor cocktail and TritonX-100). Subsequently, samples were centrifuged at 14000 rpm at 4 °C for 10 min in three times freezing-melting cycles. The supernatant fraction were collected and protein concentration was determined using BCA kit (Thermo Scientific, Rockford, IL, USA).

2.5. Western blotting

A total of 25 μg of protein in each sample was separated on TGX Stain-Free™ 12% acrylamide gels. Then, gels were exposed to UV light thereby trihalo compounds contained in stain-free gels covalently bind to tryptophan residues in proteins allowing total protein quantification. After transferring the proteins to PVDF membranes for 1 h at 100 V, membranes were exposed by another brief irradiation and the resulting fluorescence signals were recorded, and the signal intensity is proportional to the total protein volume. After blocking with 5% of non-fat dry milk in TBST, membranes were incubated with primary antibody solution at 4 °C, overnight. The membranes were washed with TBST and incubated with HRP-conjugated secondary antibody solution. After washing, the membranes were incubated with Clarity Western ECL substrate (Bio-Rad Laboratories) to visualize by enhanced chemiluminescence bands according to the recommended procedure (ChemiDoc Touch, Bio-Rad Laboratories). The chemiluminescent bands and each total protein lane intensity were measured by Image Lab software (Bio-Rad Laboratories). During quantification, protein density is measured directly on the membranes and reflected to total loaded proteins. Thus, this type of normalization eliminates the need to select housekeeping protein [15,16].

2.6. DCFDA staining

Cells were seeded in black 96-well plates. After cells adhered, medium was removed and cells were washed with PBS. DCFDA dye was added for 1 h and diffused into cells. At the end of incubation period excess amount of the dye was removed and fresh medium was added back. After 30 min cells were treated with hemin and $CoPP_{IX}$ (100 μM) for 24 h. Then, medium was removed and washed with PBS. Then the intensity of fluorescent compound was detected by fluorescence spectroscopy with excitation and emission spectra of 485 nm and 528 nm respectively.

2.7. MitoSOX staining

Cells were seeded on coverslip. After the treatment with hemin and $CoPP_{IX}$ (100 μM) for 24 h, medium was removed and cells were washed 3 times with PSB. MitoSOX™ Red was added for 10 min at 37 °C in dark. Then, dye was removed and cells were washed 3 times with PBS. Nucleus was stained by DAPI. Cells were fixed with 4% formaldehyde then washed again. The coverslips were placed to a slide and examined the staining by fluorescence microscopy. Images were captured by Zeiss Axio Scope. A1 fluorescent microscope and analyzed with ZEN 2011 v.1.0.1.0. Software (Carl Zeiss Microscopy GmbH, München, Germany). Mean intensity of red color was quantified by Image J software (NIH, Bethesda, Maryland, USA). 100 cells/group were measured in 3 different experiment.

2.8. CYTO-ID staining

CYTO-ID Autophagy Detection Kit 2.0 was performed to measure autophagic vacuoles and monitor autophagic living cells were seeded overnight and treated with hemin and $CoPP_{IX}$ (100 μM) for 24 h. Rapamycin (5 μM) was used as the positive control. Autophagic process was inhibited by chloroquine (10 μM, for 18 h). After treatments, cells were collected by centrifugation and washed with

1x assay buffer. The cell pellets were resuspended in fresh 1x assay buffer. 250 μL of the diluted CYTO-ID Green stain solution was added to each sample and mixed, then incubated for 30 min at 37 °C in dark. Cells were washed with 1 × assay buffer and fixed with 1% formaldehyde, then were immediately analyzed with a FC-500 flow cytometer (Beckman Coulter, Pasadena, CA, USA). The results were analyzed by CPX Analysis Software (Beckman Coulter). Autophagic flux was measured by ΔMFI in each group (ΔMFI: $MFI_{\text{with Chloroquine}} - MFI_{\text{without Chloroquine}}$).

Cells were seeded on coverslips and treated with hemin and $CoPP_{IX}$ (100 μM) for 24 h. Rapamycin (5 μM) was used as the positive control. Autophagic process was inhibited by chloroquine (10 μM, 18 h). After treatments, the medium was removed and cells were washed with 1x Assay Buffer. A total of 100 μL of Microscopy Dual Detection Reagent was added to each sample and incubated for 30 min at 37 °C. Cells were washed with 1 × assay buffer and fixed with 4% formaldehyde then washed again. The coverslips were placed to a slide and examined the staining by fluorescence microscopy (Zeiss Axio Scope. A1).

2.9. Statistical analysis

The data were expressed as mean ± SEM. Statistical analysis were performed with GraphPad Prism version 5 (La Jolla, CA, USA). One-way analysis of variance (ANOVA) test followed by Dunnett multiple comparison tests, which identified the significant difference between control and treated groups. A probability value of $P < 0.05$ was used as the criterion for statistical significance. When significant ($p < 0.05$), *, **, and *** represent $p < 0.05$, $p < 0.01$, and $p < 0.001$ at the Dunnett's post-test, respectively.

3. Results

3.1. Effects of high doses of HO-1 inducers on viability of H9c2 cells and hemeoxygenase-1 expression

As it is shown in Fig. 1 panel a and b, treatment with hemin or $CoPP_{IX}$ decreased the viability of H9c2 cardiomyocytes in a dose-dependent manner. At hemin concentration of 25 and 100 μM the toxic effect was profound compared to untreated sample. Additionally, similar alteration in cell viability was detected if $CoPP_{IX}$ was used. Average percentage of cell viability for 25 and 100 μM $CoPP_{IX}$ were as the follows: 79.3 ± 4.9%; 39.1 ± 4.8%, respectively (Fig. 1b). Western blot analysis using an antibody against HO-1 indicated a significant increase in HO-1 expression in H9c2 cells (Fig. 1c and d).

3.2. Effect of hemin or $CoPP_{IX}$ treatment on ROS level

In order to study the role of ROS in the toxic effect induced by high dose of hemin or $CoPP_{IX}$ treatments in H9c2 cells MitoSOX and DCFDA staining were carried out. MitoSOX detect mitochondrial superoxide, whereas DCFDA offers general ROS detection. As it can be seen in Fig. 2a and b both hemin and $CoPP_{IX}$ treatments enhanced ROS, which could play an important role in the toxic effect of the inducers and hem.

3.3. Effect of hemin or $CoPP_{IX}$ treatment on autophagy

As it is shown in Fig. 3a and b the expression level of Beclin-1 remained unchanged after HO-1 induction with hemin or $CoPP_{IX}$. Significantly increased LC3B-II (Fig. 3c and d) and p62 (Fig. 3e and f) expressions were measured by Western blotting. These results showed that hemin treatment markedly upregulated LC3B-II and p62 protein levels in cardiomyocytes, indicating that autophagy was induced, however, the level of p62 remained elevated suggesting the lack of its function. We further investigated these autophagic markers after HO-1 induction by $CoPP_{IX}$. The results supported the upregulation of autophagy pathway. Similarly, we found significantly increased expression level of LC3B-II and p62 in $CoPP_{IX}$ 100 group compared to untreated cells. To confirm our Western blot results, Cyto-ID Green staining were carried out and samples were analyzed by microscopy and flow cytometry. Monitoring autophagic flux, cells were treated with chloroquine, which is a known autophagic flux inhibitor.

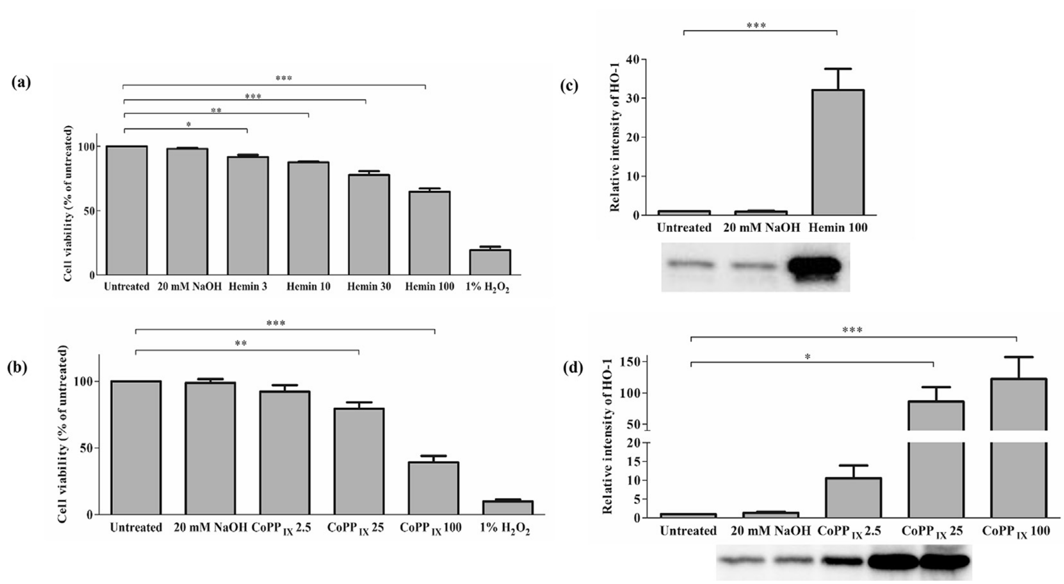

Fig. 1. Effect of HO-1 inducers on viability of H9c2 cells and HO-1 expression. Cells were treated with various concentrations of hemin (a) and $CoPP_{IX}$ (b). 20 mM NaOH was used as treated control. Cell viability was measured by MTT assay. Viability was reported as percentages of cell surviving hemin (n = 4) or $CoPP_{IX}$ (n = 6) exposure compared to the untreated group. *, **, and ***represent $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. Analysis of protein level of HO-1 on H9c2 cell lysate following treatment with hemin (c) or $CoPP_{IX}$ (d) by Western blot. Values were normalized to the total protein level, and expressed as the mean ± SEM, n = 6–8 in each group. *, *** represent $p < 0.05$, $p < 0.001$, respectively, compared to the untreated control.

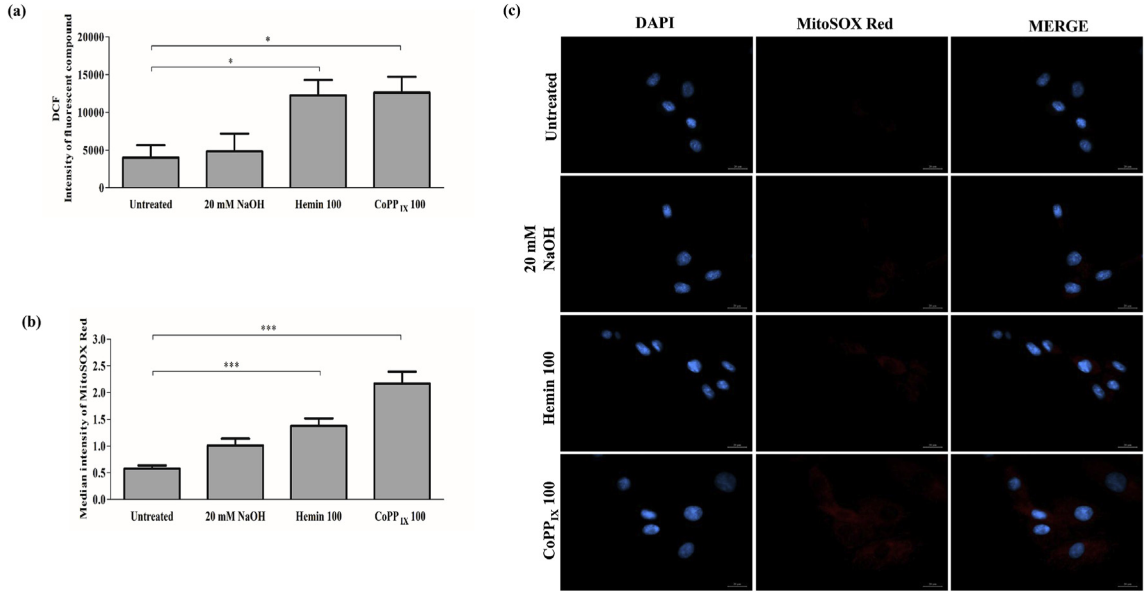

Fig. 2. Effect of HO-1 inducers on ROS level. Cells were treated with hemin or $CoPP_{IX}$. 20 mM NaOH was used as treated control. ROS production were measure by DCF (a) and MitoSOX staining (b), representative pictures (c). Results of DCF staining (five repetition) are expressed as the intensity of florescent compound. Results of MitoSOX staining are expressed as median intensity of MitoSOX Red Results of 100 cells per group repeated three-times. *, and ***represent $p < 0.05$, $p < 0.001$, respectively.

The number of autophagic vacuoles were elevated in untreated and 20 mM NaOH cells in the presence of chloroquine indicating a functioning autophagic flux (Fig. 3g, h, and 3i). Rapamycin was used as positive control, these pictures show numerous vacuoles in the presence or absence of chloroquine. In Fig. 3g, green fluorescent signals and punctate structures were detected. However, after the $CoPP_{IX}$ treatment we found some autophagic vacuoles with decreased number compared to the untreated group. Comparing hemin and Hemin + Q groups no significant alteration was observed. Same results were seen in $CoPP_{IX}$ treated groups in the presence or absence of chloroquine. To quantify the autophagic flux, flow cytometric analysis was carried out. The ΔMFI in each group was assessed (Fig. 3h). The results from this measurement supported the microscopic data. ΔMFI were 20.9 and 22.5 in untreated and 20 mM NaOH treated group supporting the existence of normal autophagic flux. However, after hemin treatment ΔMFI was actually zero. Additionally, this value also significantly decreased in the $CoPP_{IX}$ treated group. As expected, a significant increase was found in ΔMFI (43.5) in rapamycin treated cells. The findings indicate that autophagic process is incomplete when high amount of hemin or $CoPP_{IX}$ was used for HO-1 induction. Thus, our finding shows that both hemin and $CoPP_{IX}$ induced Beclin-1 independent or non-canonical autophagy; however, it was not functioning.

3.4. Apoptosis activation by influence of high concentration of hemin and $CoPP_{IX}$

A diminished level of pro-caspase-3 and enhanced level of cleaved caspase-3 after the hemin or $CoPP_{IX}$ treatment was detected in comparison with controls (Fig. 4), suggesting that the apoptotic machinery is activated after high dose of hemin or $CoPP_{IX}$ treatment.

4. Discussion

The current study shows that high doses of different HO-1 inducers possess a toxic effect on H9c2 cells. The toxic effect is accompanied by malfunctioning autophagy. Several lines of evidence indicated that different concentrations (0.1–1000 μM) of hemin could increase the level of HO-1 expression [10]; however, it may have positive outcome [17] or cytotoxic effects [4] depending on the dose used. Recently, it has been shown that induction of HO-1 by 20 μM $CoPP_{IX}$ protected H9c2 cells against H/R evidenced by decreased apoptosis [17]. However, in the present study, a higher dose of $CoPP_{IX}$ was used, which exhibited a toxic effect. The cardiovascular system has number of cytoprotective pathways developed to prevent the cytotoxic effects of heme and iron, such as HO-1 or hemopexin [18]. Heme toxicity may occur under different pathological conditions including ischemia/reperfusion [6]. When the amount of free heme exceeds the capacity of heme detoxifying enzymes, free heme exerts its toxic effect, which could be mediated via enhanced ROS generation and inflammation. As expected, in our experiments, an enhanced level of HO-1 was detected upon treatment with HO-1 inducer in a dose-dependent manner. However, our cell viability assay indicated an increased toxic effect. In line with the literature an enhanced level of ROS was detected in both treated groups, indicating that under the experimental circumstances used in this study the elevated ROS level contribute to the cell death.

Several studies investigated the connection between HO-1 and autophagy in different tissues. Resveratrol induced HO-1 expression attenuates neurotoxicity through increased autophagolysosome formation [19]. Additionally, the HO-1 is upregulated in liver sepsis, which could be an adaptive response to metabolize free intracellular heme release and suggested a pro-survival induction of autophagy [20]. Revelation of connection with autophagy in cardiomyocytes could give a new dimension to studies involving HO-1. The precise role of autophagy in cardiovascular system has always elicit controversy; there are evidence to support its role as a saviour [21] and also as a killer [22]. Our results show that autophagy is induced since the level of LC3B-II was significantly higher in the presence of HO-1 inducers [23]. It is known that during autophagy, the cytosolic LC3B-I is conjugated to phosphatidylethanolamine to form LC3B-II, which is incorporated to the

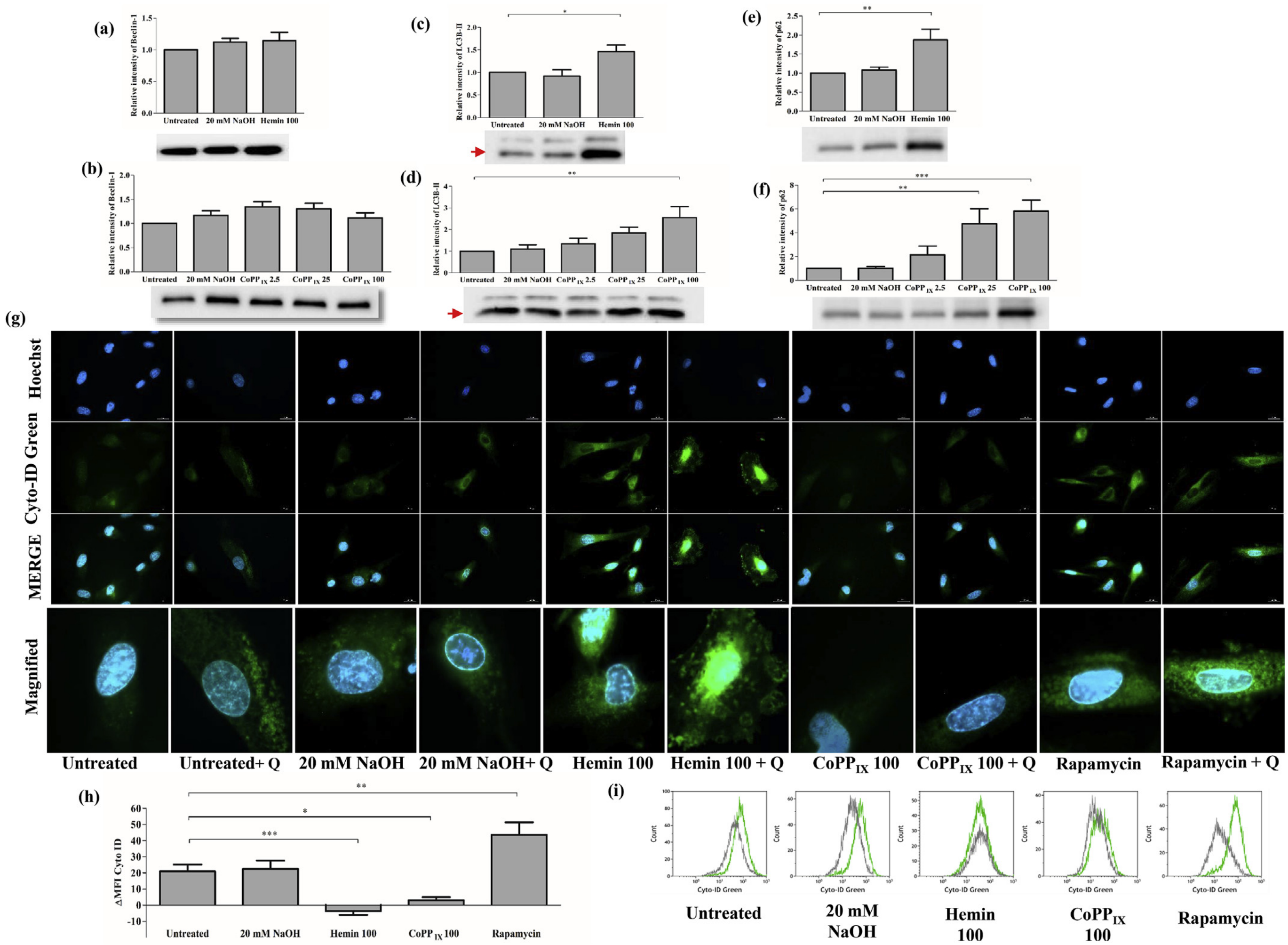

Fig. 3. Effects of HO-1 induction with hemin or $CoPP_{IX}$ on autophagy and autophagic flux. Analysis of protein levels of Beclin-1, LC3B-II and p62 were performed after treatment with hemin (a, c, e), or $CoPP_{IX}$ (b, d, f) by Western blot. Values were normalized to the total protein level, and expressed as the mean ± SEM, n = 6–15. Cells were treated with vehicle, hemin, $CoPP_{IX}$ and rapamycin in the presence or absence of chloroquine. Cells were stained with Cyto-ID Green before microscopic and flow cytometric analysis. Representative pictures of Cyto-ID staining (g). Flow cytometric analysis after Cyto-ID Green staining (h). Data are expressed by ΔMFI in each group where ΔMFI means $MFI_{\text{with Chloroquine}} - MFI_{\text{without Chloroquine}}$. The values are expressed as the mean ± SEM, n = 7, in the case of positive control n = 4. Representative histogram of flow cytometric analysis after Cyto-ID Green staining (i). Grey histograms represent MFI without chloroquine and green histograms are with chloroquine. *, **, and ***represent $p < 0.05$, $p < 0.01$, and $p < 0.001$ compared to the untreated control.

autophagosomal membrane [24]. p62 is another widely used marker, which physically links autophagic cargo to the autophagic membrane [25]. p62 binds directly to LC3 and GABARAP family proteins and is selectively degraded by autophagy processes. Since p62 accumulates when autophagy is inhibited/impaired [26], decreased levels can be observed when autophagy is induced by oxidative stress [27]. Thus, p62 serves as an indicator of autophagic degradation and used as a marker to study autophagic flux. Impaired autophagic flux by HO-1 dependent autophagy was previously found [19]. In line with this study an enhanced level of p62 was found upon HO-1 induction, indicating that the autophagy is malfunctioning. Recently, it has been shown that genetic overexpression of HO-1 protects against hypoxia/reoxigenisation via induction of autophagy in H9c2 [28]. Similarly, the authors found elevated levels of LC3-II in cells overexpressing HO-1 after challenging with H/R. Contrary, this enhanced level of LC3-II was accompanied by decreased level of p62, decreased level of ROS and enhanced mitochondrial stability. However, the difference may be arisen from the different model, since in the current study, toxicity of high dose of HO-1 inducers was examined, while in the publication of Chen and co-authors, the effect of H/R was tested. Another major difference between Chen et al. and our results is the level of HO-1. Here a robust enhancement in the level of HO-1 were observed in our study, while in the other study mild induction of HO-1 was found.

Autophagy flux is a dynamic process that includes initiation, elongation, maturation and degradation. Interestingly, autophagosome formation was independent of Beclin-1, indicating non-canonical autophagy activity in hemin and $CoPP_{IX}$-treated cells (Fig. 3a and b). Recent findings suggest that autophagosome biogenesis occurs also in the absence of Beclin-1 [29]. To further study the autophagic flux, cells were treated with chloroquine, which induces the accumulation of autophagic vacuoles. The fluorescent microscopy images and flow cytometry results revealed an enhanced number of autophagic vacuoles in control and rapamycin treated cells. However, in line with our Western blot data, we failed to find any differences between signals in HO-1 inducers treated cells in the presence or absence of chloroquine, further supporting that malfunctioning autophagy contributes to heme toxicity. Several studies published that autophagy is necessary process to

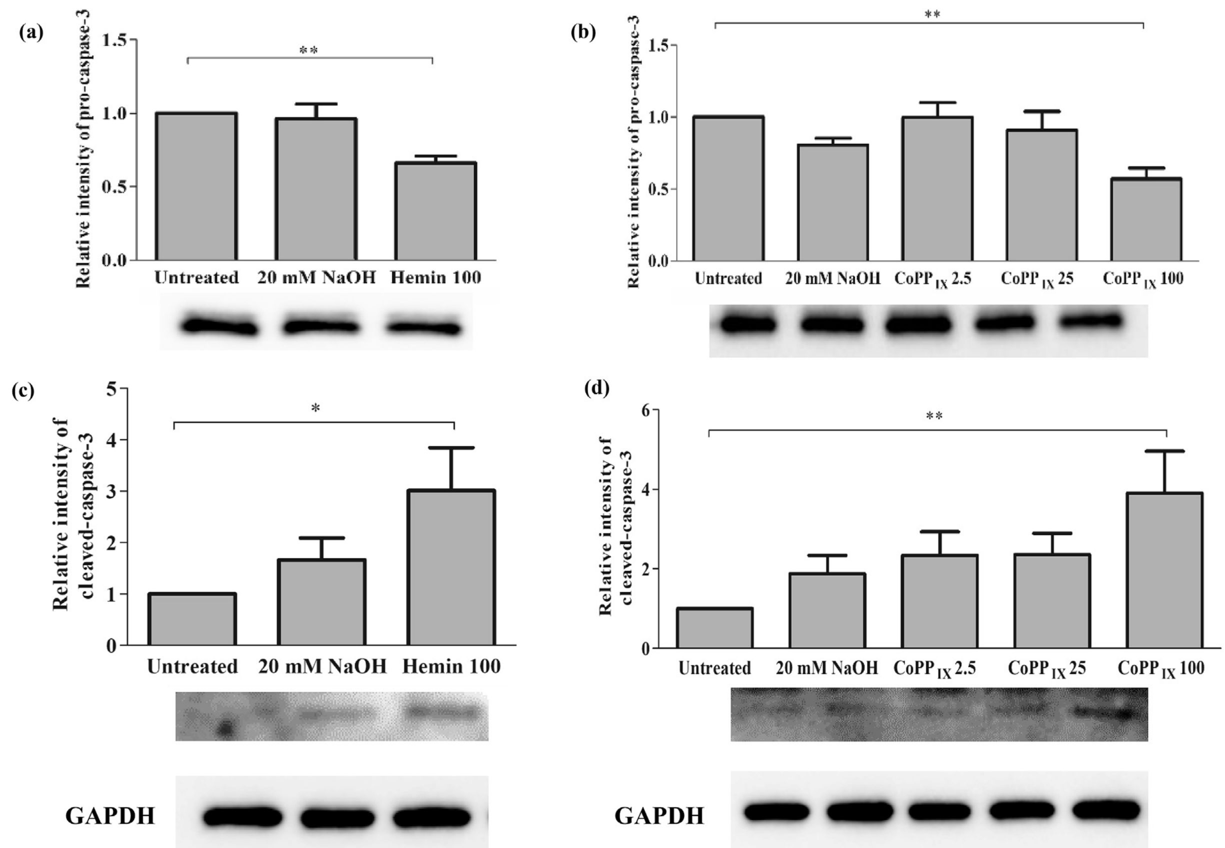

Fig. 4. Effects of HO-1-induction on caspase-3 level. Analysis of protein level of pro-caspase-3 and cleaved-caspase-3 after hemin (a), $CoPP_{IX}$ (b) treatment by Western blot. Values were normalized to the total protein level, and expressed as the mean ± SEM, n = 6 and n = 9. **represent p < 0.01 compared to the untreated control.

remove damaged organelles. If this process fails or overwhelmed, these damaged organelles trigger an apoptotic cell death [30]. Our Western-blot results indicate a lower level of pro-caspase-3 and enhanced level of cleaved-caspase-3 indicating the activation of apoptosis. Taken together, we demonstrated that overexpression of HO-1 by high dose of hemin and $CoPP_{IX}$ induce cell toxicity in H9c2 cells, in which malfunctioning autophagy and enhanced ROS level plays a role.

5. Limitations of the study

In the current study both inducers were used in high concentrations, thus, we cannot rule out that direct toxicity of the inducers may contribute to the harmful effect. However, as it was reported that 20 μM of $CoPP_{IX}$ protected H9c2 cell [28]. Furthermore, we did not used HO-1 inhibitors, which could have confirmed our findings.

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Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.02.140.

Data availability

All data of this study are available from the first author upon request.

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