

Cytotoxic effects of metal protoporphyrins in glioblastoma cells: Roles of albumin, reactive oxygen species, and heme oxygenase-1

Jyh-Ming Chow^a, Guan-Cheng Huang^b, Hui-Yi Lin^c, Shing-Chuan Shen^c,
Liang-Yo Yang^d, Yen-Chou Chen^{c,e,*}

^a Section of Hematology-Oncology, Department of Internal Medicine, Taipei Municipal Wan-Fang Hospital, Taipei Medical University, Taiwan

^b Department of Internal Medicine, Chi-Mei Medical Center, Tainan, Taiwan

^c Graduate Institute of Medical Sciences, Taipei Medical University, Taipei, Taiwan

^d Department of Physiology and Graduate Institute of Neuroscience, Taipei Medical University, Taipei, Taiwan

^e Cancer Research Center and Orthopedics Research Center, Taipei Medical University Hospital, Taipei, Taiwan

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Abstract

We investigate the cytotoxic effect of metal protoporphyrins including ferric protoporphyrin (FePP; hemin), cobalt protoporphyrin (CoPP), and tin protoporphyrin (SnPP) in glioblastoma cells C6 and GBM8401. Data of MTT assay show that FePP and CoPP, but not SnPP, significantly reduce the viability of glioma cells C6 and GBM8401 in the absence of serum. In the condition with fetal bovine serum (FBS) or bovine serum albumin (BSA), the cytotoxic effect of FePP and CoPP was completely inhibited. Binding of FePP, CoPP, and SnPP with BSA was examined via spectrophotometer analysis, and the protective effect of serum against FePP and CoPP-induced cell death was abolished by BSA depletion. A loss in the integrity of DNA with an occurrence of apoptotic events including DNA ladders, caspase 3 and PARP protein cleavage, and chromatin-condensed cells is observed in FePP-treated or CoPP-treated C6 cells. An increase in intracellular peroxide level was examined in FePP, but not CoPP, -treated C6 cells, and *N*-acetyl-L-cysteine (NAC) addition significantly protected C6 cells from FePP, but not CoPP, -induced cell death with reducing FePP-stimulated reactive oxygen species (ROS) production. Activation of extracellular regulated kinases (ERKs) and c-Jun-N-terminal kinases (JNKs) with an increase in the heme oxygenase-1 (HO-1) protein was observed in FePP-treated or CoPP-treated C6 cells in the absence of FBS or BSA, and adding JNKs inhibitor SP600125 (SP), but not ERKs inhibitor PD98059 (PD), significantly attenuated FePP-induced or CoPP-induced HO-1 protein expression in accordance with reducing JNKs protein phosphorylation. However, PD98059, SP600125, or transfection of C6 cells with antisense HO-1 oligonucleotides show no effect on the cytotoxicity elicited by FePP and CoPP in C6 cells. Effect of serum and BSA on the cytotoxicity of metal protoporphyrins in glioma cells is first demonstrated in the present study, and the roles of ROS, MAPKs, and HO-1 were elucidated.

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1. Introduction

Hemoglobin is an erythrocyte protein, and released into intracellular space through complement-mediated cell lysis in the CNS hemorrhage. Several experimental evidences suggest that hemoglobin may contribute to cell damage after hemorrhage through a spontaneous nonenzymatic oxidation reaction, releases of superoxide, and decreases in the affinity of globin binding with heme (ferroprotoporphyrin IX) moiety (Vanderveldt and Regan, 2004; Wagner et al., 2003; Vollaard et al., 2005). Protoporphyrin (PP) IX is a heme metabolite, and iron protoporphyrin has been shown to possess versatile biological

Abbreviations: Hemin or FePP, ferric protoporphyrin; CoPP, cobalt protoporphyrin; SnPP, tin protoporphyrin; BSA, bovine serum albumin; NAC, *N*-acetyl-L-cysteine; HO-1, heme oxygenase-1; JNKs, c-Jun NH₂-terminal kinases; ERKs, extracellular signal-regulated protein kinases; ROS, reactive oxygen species; DCHF-DA, dichlorodihydrofluorescein diacetate; LDH, lactate dehydrogenase; PARP, poly (ADP-ribose) polymerase; LPS, lipopolysaccharide; LTA, lipoteic acid; iNOS, inducible nitric oxide; NO, nitric oxide.

* Corresponding author at: Graduate Institute of Medical Sciences, College of Medicine, Taipei Medical University, No. 225, Wu-Hsing Street, Taipei 110, Taiwan. Tel.: +886 2 27361661x6152; fax: +886 2 23787139.

E-mail address: yc3270@tmu.edu.tw (Y.-C. Chen).

functions such as cytotoxic or cytoprotective effects on several different cells. Heme is the prosthetic group of hemoglobin, and high levels of free heme cause cytotoxicity leading to organ and tissue damages through catalyzing the formation of lipid peroxide and DNA damage (Varga et al., 2007; Sesink et al., 1999). Diaconu et al. (2003) indicated that heme performed antiproliferative and apoptosis-inducing effects in hepatoma cells. Arruda et al. (2004) indicated that heme inhibited human neutrophil apoptosis. Hemin (ferric protoporphyrin, FePP), an oxidized form of heme, may be released from hemoglobin and interacts with several acceptors including proteins and lipids in cells. Hemin has been shown to be cytotoxic, and in vitro exposure to hemin enhances the cytotoxicity of H_2O_2 in endothelial cells and breast carcinoma cells (Balla et al., 1991; Croci et al., 2002). However, hemin is a potent inducer of heme oxygenase-1 (HO-1), a key enzyme in catalyzing the rate-limiting step in heme degradation, and antiapoptotic, anti-inflammatory, and antiproliferative actions of HO-1 have been identified (Diaconu et al., 2003; Lin et al., 2005). Hemin inhibits lipopolysaccharide (LPS)-induced or lipoteic acid (LTA)-induced nitric oxide production and iNOS gene expression via inducing HO-1 gene expression. Our previous studies demonstrated that overexpression of HO-1 gene significantly protected glioma cells from H_2O_2 -induced or chemical anoxia-induced apoptosis (Chen et al., 2006a,b). Additionally, cobalt PPIX (CoPP) and tin PPIX (SnPP), iron-exchanged derivatives of hemin, are formed in trace amounts during heme biosynthesis in the condition of iron insufficiency. Both CoPP and SnPP are competitive HO-1 inhibitors, and inhibition of HO-1 enzyme activity by CoPP and SnPP has been shown. CoPP and SnPP also have been shown to induce HO-1 gene expression in several types of cells (Smith et al., 1991; Ho et al., 2000). Busserolles et al. (2006) delineated that CoPP protected Caco-2 cells from serum deprivation-induced apoptosis through HO-1 induction. Kaizu et al. (2003) indicated that SnPP reduces HO activity, and induction of HO-1 protein by high dose SnPP may lead to attenuate apoptotic cell death elicited by ischemia/reperfusion in the rat kidney (Kaizu et al., 2003). The cytotoxic mechanisms of FePP, CoPP, and SnPP against the viability of glia cells are still unclear.

Reactive oxygen species (ROS) are implicated in the pathogenesis of several human diseases such as atherosclerosis, Alzheimer disease, and ischemia/reperfusion (Takano et al., 2003; Huang et al., 2005). Free heme offers cytotoxic effect through oxidative stress by generating ROS, and oxidation of lipid by free heme and hemoprotein has been shown in several previous studies (Zhao et al., 2004; Ryter and Tyrrell, 2000). Beppu et al. (1986) reported that hemin increased hydroperoxide-induced lipid oxidation in human erythrocytes. Hemin is able to oxidize low and high-density lipoproteins to produce cytotoxic products (Camejo et al., 1998). Exposure of neuron-like cells to hemin leads to cell death by a marked increase in ROS production. Goldstein et al. (2003) indicated that the neurotoxic effect elicited by heme is iron and oxidative dependent. An increase in reactive oxygen species (ROS) generation, induction of protein kinase C (PKC) activity, and elevation of IL-8 expression have also been identified in heme-treated neutrophils (Graça-Souza et al., 2002).

Although hemin has been shown to induce ROS generation, it also protects cells from hydrogen peroxide-induced cell death and inhibits lipopolysaccharide (LPS)-induced or lipoteic acid (LTA)-induced NO production through induction of HO-1 gene expression. It suggests that hemin may play as a 2-edged sword in promotion of oxidation and induction of cytoprotection (Balla et al., 2000). The comparative role of ROS in FePP, CoPP and SnPP-treated glioma cells is still undefined.

In the present study, we investigate the cytotoxic effects of three metal protoporphyrins including FePP, CoPP, and SnPP in glioma cells C6 and GBM-8401. Data of the study indicate that FePP and CoPP, but not SnPP, induce cytotoxic effect through apoptosis induction in the condition without serum. The roles of ROS production, HO-1 induction, extracellular regulated kinases (ERKs) and c-Jun-N-terminal kinases (JNKs) activation in FePP and CoPP-induced cell death with the condition with or without serum (FBS) or albumin (BSA) are investigated.

2. Materials and methods

2.1. Cell culture

All cells used in the present studies including glioma C6 and GBM8401 cells, macrophages RAW264.7, and keratinocytes HaCaT are derived from ATCC (American Type Culture Collection; Rockville, MD), and incubated in RPMI-1640 medium supplemented with 2 mM glutamine, antibiotics (100 U/ml of penicillin A and 100 U/ml of streptomycin), and 10% heat-inactivated fetal bovine serum at 37 °C in a humidified incubator containing 5% CO_2 .

2.2. Chemicals

All chemicals including (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (MTT), hydrogen peroxide (H_2O_2), metal protoporphyrins, *N*-acetyl-L-cysteine (NAC), and 2',7'-dichlorodihydrofluorescein-diacetate (DCHF-DA) were obtained from Sigma Chemical (St. Louis, MO). The antibodies for detecting HO-1, caspase 3, PARP and α -tubulin protein were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and antibodies for detecting total and phosphorylated ERKs, JNKs, AKT, and p38 were purchased from Cell Signaling (Beverly, MA). PD98059 (PD) and SP600125 (SP) were obtained from Calbiochem (La Jolla, CA).

2.3. Cell viability

MTT was used as an indicator of cell viability as determined by its mitochondrial-dependent reduction to formazone. Cells were plated at a density of 4×10^5 cells/well in 24-well plates for 12 h, followed by treatment with FePP, CoPP, or SnPP under different conditions for a further 12 h. Cells were washed with PBS three times, and MTT (50 mg/ml) was added to the medium for a further 4 h, and the formazone crystals were dissolved using 0.04 N HCl in isopropanol. The absorbance was read at 600 nm with an ELISA analyzer (Dynatech MR-7000; Dynatech Laboratories).

2.4. Morphological observations

Cells were plated at a density of 4×10^5 cells/well into 24-well plates for 12 h and treated with indicated compounds for a further 12 h. The supernatant was removed and cells were washed twice with PBS. Giemsa solution was added into the cells, and extra-Giemsa solution was removed by PBS. The alternative morphology of cells was detected under microscopy observation.

2.5. LDH release assay

The percentage of LDH released extracellularly was calculated as an indicator of cell death through the oxidation of NADH at 530 nm by an LDH assay kit (Roche Applied Science). The amount of LDH released into the medium under different treatments compared to the total amount of LDH present derived from cells treated with 2% Triton X-100 was described as the term of cytotoxicity. The cytotoxicity (%) was determined by the equation $[(\text{OD530 of the treated group} - \text{OD530 of the control group}) / (\text{OD530 of the Triton X-100-treated group} - \text{OD530 of the control group})] \times 100\%$.

2.6. Western blotting

Total cellular extracts were prepared, and an equal amount of proteins from each group was separated on SDS-polyacrylamide minigels, followed by transfer to immobilized polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were incubated with 1% bovine serum albumin in PBST buffer (blocking solution) for 1 h, and then incubated with indicated antibodies at 4 °C for 16 h. The dilutions of anti-HO-1, caspase 3, PARP, α -tubulin, t-ERKs, t-JNKs, t-p38 and t-AKT antibodies with blocking solution are at the ratio of 1:2000. And, the dilutions of anti-pERKs, pJNKs, p-p38, and p-AKT antibodies with blocking solution are at the ratio of 1:1000. The expression of indicated protein was detected by staining with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma).

2.7. DNA gel electrophoresis

Cells were washed twice with PBS, and lysed in 100 μ l of lysis buffer (50 mM Tris (pH 8.0), 10 mM ethylenediaminetetraacetic acid (EDTA), 0.5% sodium sarkosinate, and 1 mg/ml proteinase K) at 56 °C for 3 h, followed by incubating with RNase A (0.5 mg/ml) for an additional 56 °C for an additional 1 h. DNA was extracted with phenol/chloroform/isoamyl alcohol (25/24/1, v/v) before loading. Samples were mixed with loading buffer (50 mM Tris, 10 mM EDTA, 1% (w/w) low-melting point agarose, and 0.025% (w/w) bromophenol blue) and loaded onto a pre-solidified 2% agarose gel containing 0.1 mg/ml ethidium bromide. DNA in gels was observed and photographed under UV illumination.

2.8. Determination of ROS production

ROS production in FePP-treated or CoPP-treated cells under different conditions was monitored by flow cytometry using DCHF-DA as a fluorescent dye as described in our previous study (Lin et al., 2007). Briefly, cells were treated with FePP for 1 h, followed by changing the medium to remove free FePP before adding DCHF-DA (100 μ M) for an additional 30 min. The 2',7'-dichlorofluorescein (DCF) fluorescence intensity in cells was detected by flow cytometry analysis or observed under fluorescent microscope.

2.9. Absorption spectroscopy

The absorptive spectra of FePP, CoPP, and SnPP in the SF, 5% FBS, and 0.2% BSA condition in 1 cm quartz cuvettes were recorded using a slit of 5 nm and a scan speed of 250 nm/min. The absorption spectra were performed at pH7.

2.10. BSA depletion

BSA depletion from FBS performed by Albumin Removal Kit (Digital-gene). Using dilution buffer diluted serum to a final volume 400 μ l and added to the column at room temperature for 2 min. Centrifuged at 10,000 rpm for 1 min and collected pass through solution was the BSA depletion serum fraction. Adding 400 μ l of wash buffer to column and centrifuged at 10,000 for 1 min to remove buffer, twice. Adding 400 μ l elution buffer to column and centrifuged at 10,000 rpm for 1 min to collect pass through solution was the BSA fraction. Equal amount (10 μ l) samples of the BSA removal and bound fractions were separated by SDS-PAGE and stained with Coomassie blue.

2.11. Transfection of HO-1 antisense and sense oligonucleotides

HO-1 sense and antisense oligonucleotides were directed against the flanking translation initiation codon and 6 base pairs on either side to the mouse HO-1 cDNA, and modified with phosphorothioate (Lee et al., 2002; Lin et al., 2007). The sequence of the HO-1-specific antisense oligonucleotides was 5'-ACGCTCCATCACCG-3', and the sense oligonucleotides was 5'-CCGGTGATGGAGCGT-3'. Briefly, cells were placed in serum-free medium and then transfected with the phosphorothioated HO-1-specific sense or antisense oligonucleotides (1 μ g) for 12 h. After 12 h of incubation, cells were treated with indicated compounds for an additional 12 h, and the viability of cells was analyzed by MTT assay.

2.12. Plasmid digestion assay

pBR322 plasmid DNA was used to examine the ROS-producing activity of FePP and CoPP. In order to analyze the ROS-producing activity of FePP or CoPP, pBR322 plasmid DNA (0.25 μ g) was incubated with different concentrations of FePP or CoPP for 30 min in the presence of H₂O₂ (40 μ M). At the end of both reactions, samples were incubated with 5 $\times$ tracking dye (40 mM EDTA, 0.05% bromophenol blue and 50 vol.% glycerol) to stop the reaction, and the conformation of pBR322 plasmid DNA was analyzed by agarose electrophoresis via ethidium bromide staining, and visualized under UV transilluminator.

2.13. Statistical analysis

Values are expressed as the mean $\pm$ S.E. from three-independent experiments. The significance of the difference from the respective controls for each experimental test condition was assayed using Student's *t*-test for each paired experiment. A *p* value of <0.01 or <0.05 was regarded as indicating a significant difference. The measurements are derived from three-independent cultures, and there are three replicate wells in each time.

3. Results

3.1. FePP reduction of the viability of glioma C6 and GBM-8401 cells in the serum-free condition

MTT assay was used to examine the cytotoxic effect of FePP in glioblastoma cells including glioma cells C6 and GBM-8401, macrophages RAW264.7, and keratinocytes HaCaT. As illustrated in Fig. 1A, FePP, at the doses of 20 and 40 μ M, significantly reduces the viability of glioma cells C6 in the condition without fetal bovine serum (SF). In the presence of 10% fetal bovine serum (FBS), the cytotoxic effect of FePP against the viability of glioma cells C6 was completely blocked. Similarly, FBS, at the doses from 2% to 10%, fully suppresses the cytotoxicity of FePP in glioma cells C6 (Fig. 1B). As the same part of experiments, the cytotoxic effect of FePP in C6 cells is completely inhibited in the presence of 0.2% bovine serum albumin (BSA) (Fig. 1C). However, FePP shows no cytotoxic effect in RAW264.7 and HaCaT cells in the presence or absence of FBS (Fig. 1D and E). In order to elucidate if FePP preferentially reduces the viability of glioma cells, GBM-8401 cells were used in the present study. Data of Fig. 1F indicate that FePP, at the dose of 20 μ M, significantly reduces the viability of GBM8401 cells in SF, but not 5% FBS and 0.2% BSA, condition (Fig. 1F). The cytotoxic effect of FePP in the absence of FBS or BSA was identified in glioma cells.

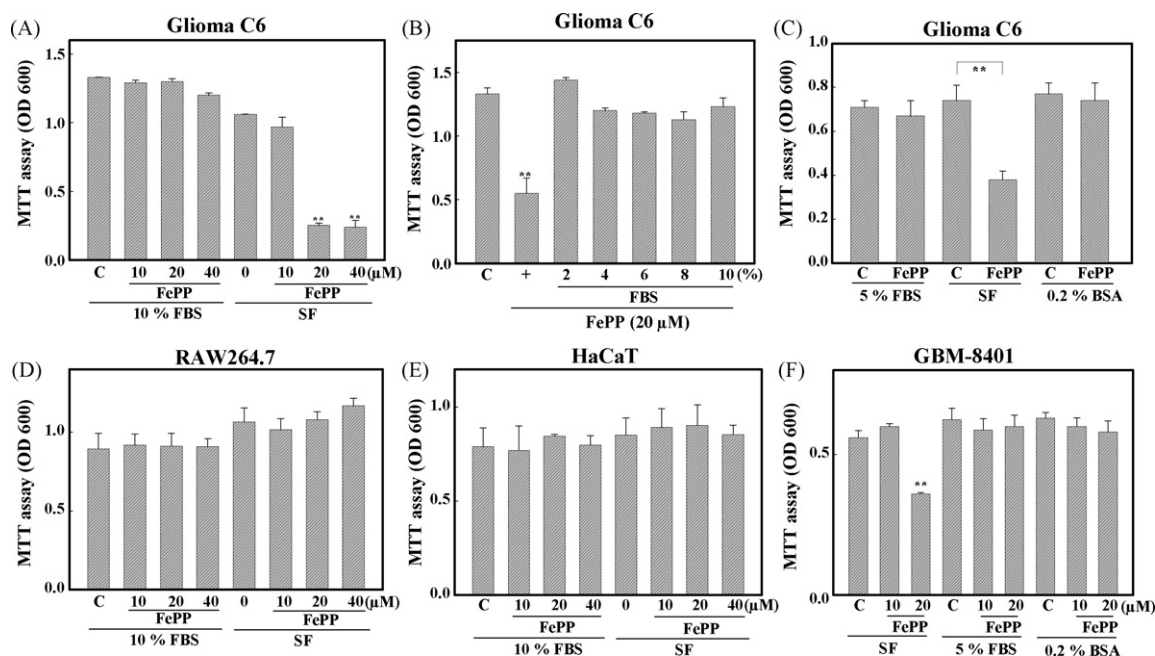


Fig. 1. FePP induction of cell death in glioblastoma cells C6 and GBM-8401, but not in RAW264.7 macrophages and keratinocytes HaCaT in the serum-free (SF) condition. (A) FePP reduces the viability of glioma cells C6 in SF, but not 10% fetal bovine serum (FBS), condition. C6 cells were treated with different doses of FePP (10, 20, and 40 μ M) for 12 h in the SF or 10% FBS condition, and the viability of cells was measured by MTT assay. (B) FBS addition completely inhibits FePP-induced cytotoxicity in glioma cells C6. Cells were treated with FePP in the presence or absence of different percentages (2%, 4%, 6%, 8%, and 10%) of FBS, and the viability of cells was detected by MTT assay. (C) FePP-induced cytotoxicity is blocked by FBS or BSA addition in glioma cells C6. Cells were treated with FePP (20 μ M) in the condition of 5% FBS, SF, or 0.2% BSA for 12 h, and the viability of cells was examined by MTT assay. (D and E) As described in (A), the viability of macrophages RAW264.7 (D) and human keratinocytes HaCaT (E) was examined by MTT assay. (F) FePP induces cytotoxic effect in another glioma cells GBM8401. As described in (C), GBM8401 cells were treated with FePP (10 and 20 μ M) in the condition of SF, 5% FBS, or 0.2% BSA, and the viability was examined by MTT assay. Data derived from three independent experiments are expressed as the mean $\pm$ S.E., and $**p < 0.01$ indicates a significant difference from control (C) group.

3.2. FePP and CoPP, but not SnPP, reduce the viability of glioblastoma cells with a loss in the integrity of DNA and an occurrence of chromatin-condensed cells

We further examine the cytotoxic effect of metal protoporphyrins CoPP and SnPP in glioma cells C6 and GBM-8401. As illustrated in Fig. 2A, reduction of the viability of glioma cells C6 by FePP and CoPP, not SnPP, was examined by MTT assay in the SF condition, and that was prevented by adding 5% or 10% FBS. Similarly, the cytotoxicity elicited by CoPP was blocked by adding 0.1% and 0.2% BSA (Fig. 2B). Data of LDH release assay show that FePP and CoPP treatment induce the release of LDH into the medium in the SF, but not 5% FBS (FBS) or 0.2% BSA (BSA), condition (Fig. 2C). In GBM-8401 cells, CoPP reduction of the viability in SF condition was blocked by adding 5% FBS and 0.2% BSA (Fig. 2D). Additionally, the integrity of DNA under FePP and CoPP treatment was analyzed in the present study. As illustrated in Fig. 2E, FePP or CoPP treatment induces the loss of DNA integrity of glioma cells C6 in the absence of serum. In the presence of FBS or BSA, the loss of DNA integrity elicited by FePP and CoPP was completely suppressed (Fig. 2E and F). Data of morphological observations show that FePP and CoPP-induced chromatin-condensed cells via Giemsa staining were observed microscopically in SF, but not FBS or BSA, condition. It suggests that FePP and CoPP reduction of the viability in the SF condition is mediated by

apoptosis induction, which is blocked by FBS and BSA addition.

3.3. Interaction of FePP, CoPP, and SnPP with BSA by spectrometry analysis

In order to investigate if FePP, SnPP, and CoPP possess ability to interact with BSA, the UV-vis spectra of the free FePP, CoPP, and SnPP with or without 5% FBS or 0.2% BSA are shown in Fig. 3. As illustrated in Fig. 3A, BSA, FePP, CoPP, and SnPP show absorptive peaks around 279, 370, 415, and 385 nm, respectively. In the presence of BSA and indicated metal protoporphyrins, the absorptive patterns of FePP, CoPP, and SnPP are shifted to right side relative to constituent peaks in the SF condition, and the absorptive peaks of FePP, SnPP, and CoPP were changed to 405, 415, and 430 nm, respectively (Fig. 3A; left panel). Similar results are obtained in FePP, CoPP, or SnPP with 5% FBS (Fig. 3A; right panel). Additionally, BSA-depleted FBS (–BSA) was prepared by a gel filtration method as described in Section 2. Data of SDS-PAGE show that the content of BSA in the BSA-depleted FBS was reduced to around 13%, compared with that in FBS (Fig. 3B). We further analyze the effect of FBS and BSA-depleted FBS on the cytotoxic effects of FePP and CoPP in glioma cells C6. As shown in Fig. 3C, the preventive effect of FBS against the cell death elicited by FePP and CoPP is attenuated after BSA depletion by MTT assay. These

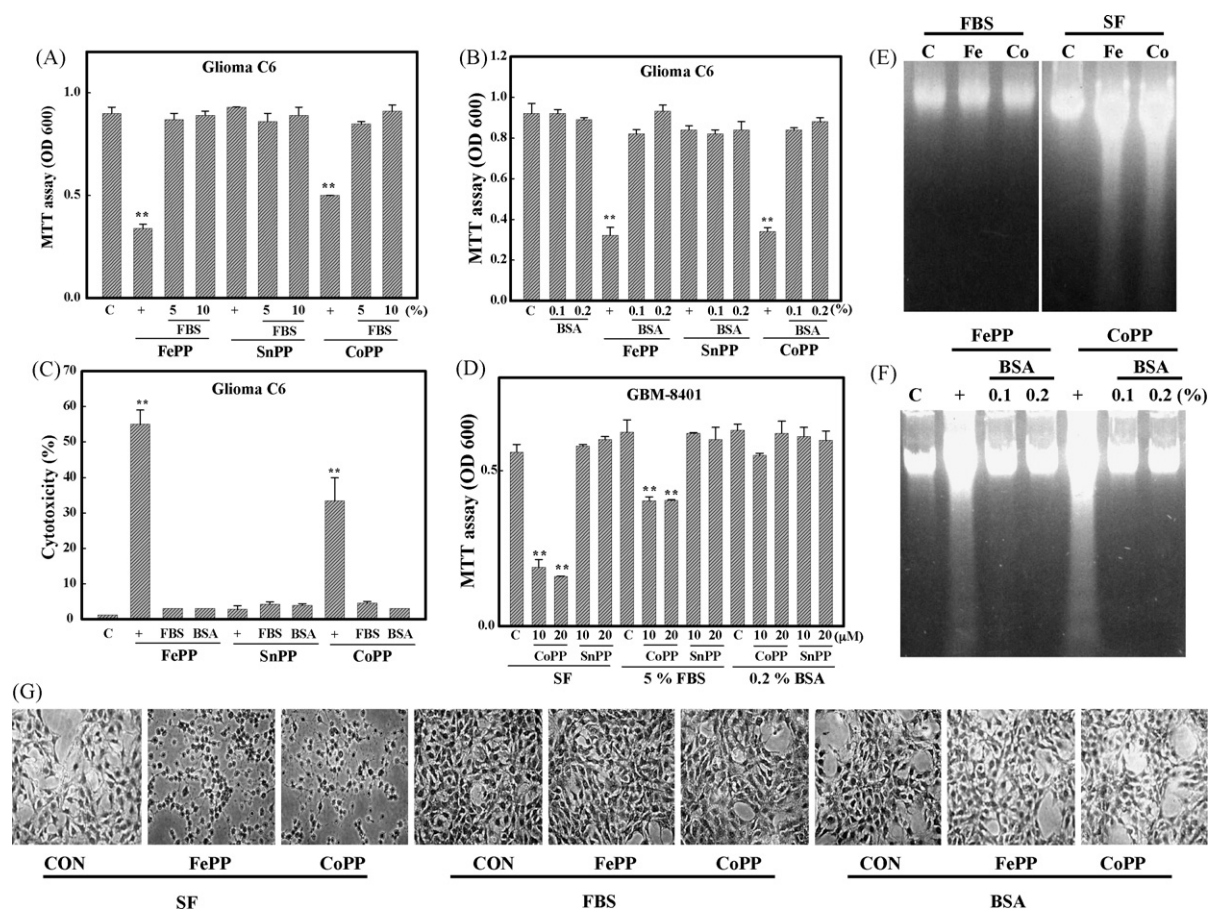


Fig. 2. Differential cytotoxic effects of FePP, CoPP, and SnPP with an occurrence of apoptotic characteristics including a loss in the integrity of DNA and an increase in chromatin-condensed cells. (A) FePP and CoPP, but not SnPP, reduces the viability of glioma cells C6 in the SF condition, which is prevented by FBS addition. C6 cells were treated with FePP, CoPP, or SnPP (20 μ M) with or without FBS, and the viability of cells under different treatments was measured by MTT assay. (B) BSA addition inhibits CoPP and FePP-induced cell death in C6 cells. C6 cells were treated with FePP, SnPP, CoPP (20 μ M) in the presence or absence of BSA (0.1% and 0.2%) for 12 h, and the viability of cells was detected by MTT assay. (C) FBS and BSA inhibit LDH release elicited by FePP and CoPP. C6 cells were treated with FePP, SnPP, CoPP (20 μ M) in the presence or absence of 5% FBS (FBS) or 0.2% BSA (BSA) for 12 h, and the amount of LDH in the medium was detected by LDH release assay as described in Section 2. (D) CoPP, but not SnPP, induces cytotoxic effect in GBM8401 cells. As described in Fig. 1F, the viability of cells was measured by MTT assay. (E) FePP (Fe) and CoPP (Co) induce a loss in the integrity of DNA in the SF, but not FBS, condition. C6 cells were treated with FePP or CoPP (20 μ M) for 12 h with or without FBS, and the integrity of chromosomal DNA was analyzed. (F) BSA addition prevents DNA ladders stimulated by FePP and CoPP. C6 cells were treated with FePP or CoPP (20 μ M) for 12 h with or without BSA (0.1% and 0.2%), and the integrity of chromosomal DNA was analyzed. (G) FBS and BSA protect C6 cells from FePP and CoPP-induced chromatin-condensed cells via Giemsa staining. Cells were treated as described before, and the chromatin-condensed cells were detected by Giemsa staining under microscopic observations. Data derived from three independent experiments are expressed as the mean $\pm$ S.E., and $^{**}p < 0.01$ indicates a significant difference from control (C) group.

data provide evidences to suggest that FBS reduction of FePP and CoPP-induced cytotoxicity is through the BSA–FePP or BSA–CoPP interaction.

3.4. Cytotoxicity of FePP and CoPP in rat glioma cells C6 through ROS-dependent and -independent manner

In order to examine the intracellular ROS level in FePP and CoPP-treated glioma cells C6, DCHF-DA was used in the present study. As illustrated in Fig. 4A, FePP, but not CoPP, treatment induces intracellular DCF fluorescence intensity in glioma cells C6 under fluorescent microscopic observation. Data of flow cytometry analysis show that a significant increase in intracellular DCF intensity was observed in FePP, but not CoPP, treated C6 cells, and FePP-induced intracellular peroxide level was significantly attenuated by adding FBS, BSA, or chemi-

cal antioxidant *N*-acetyl-L-cysteine (Fig. 4B). Furthermore, in vitro plasmid digestion provides an effective method to analyze the prooxidant of chemicals. In the presence of ROS production, plasmid DNA is damaged and conversion of the plasmid conformation from a supercoiled form (SC) to an open circle (OC) occurs. As shown in Fig. 4C, H_2O_2 plus FePP induces the conformation change of pBR322 plasmid from SC to OC, characterized by a decrease in SC and an increase in OC plasmid intensity. No significant SC to OC conversion was detected in H_2O_2 plus CoPP-treated pBR322 plasmid. We further examine the effect of NAC on FePP and CoPP-induced cell death. Data of MTT assay indicate that NAC addition significantly attenuates FePP-induced cytotoxicity in C6 cells, however no preventive effect on CoPP-induced cell death is observed (Fig. 4D). Similarly, NAC dose-dependently inhibits the cytotoxicity elicited by FePP, and a complete suppression of FePP-induced cell death

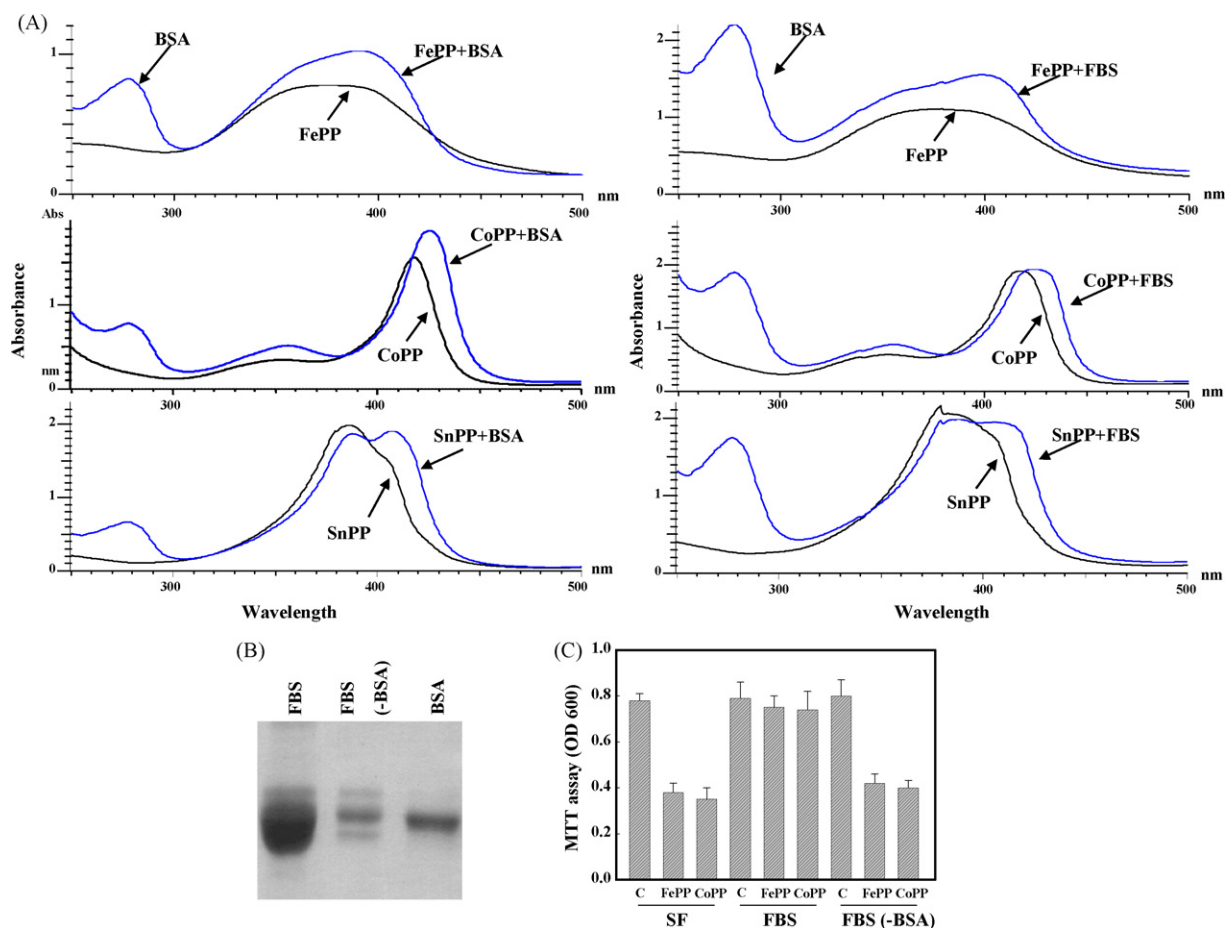


Fig. 3. Interaction of BSA with FePP, CoPP, and SnPP by a spectrometric analysis. (A) UV-vis absorption spectra of free FePP, CoPP, SnPP, and their protein complex with BSA or FBS in the region of 200–500 nm. As described in Section 2, the absorptive spectra of indicated metal protoporphyrins with or without 0.2% BSA (right panel) or 5% FBS (left panel) was described. (B) A decrease in the BSA content in FBS by gel filtration kit. As described in Section 2, depletion of BSA from FBS was performed, and an equal amount (10 μl) of each sample was applied to SDS-PAGE, followed by Coomassie blue staining. (C) Removing BSA from FBS inhibits FBS protection of C6 cells from FePP or CoPP-induced cell death. Cells were treated with FePP or CoPP (20 μM) in the condition of SF, 5% FBS, and 5% BSA-depleted FBS for 12 h, and the viability of cells was analyzed by MTT assay.

is observed at the dose of 20 mM of NAC treatment (Fig. 4E). Additionally, the expression of two apoptosis-related proteins caspase 3 and PARP was examined in FePP and CoPP-treated C6 cells in the SF, FBS, BSA, and NAC-treated conditions. Data of Fig. 4F indicate the cleavage of caspase 3 and its downstream substrate PARP protein was detected in FePP and CoPP-treated C6 cells, and that was inhibited by adding FBS and BSA. NAC addition inhibits FePP, but not CoPP, -induced caspase 3 and PARP protein cleavage. Morphological observations in consistent with cytotoxic data indicate that NAC addition protects C6 cells from FePP, but not CoPP, -induced chromatin-condensed cells via Giemsa staining (Fig. 4G).

3.5. Induction of ERKs and JNKs protein phosphorylation in FePP or CoPP-treated C6 cells

Examination of MAPKs and AKT activation in FePP or CoPP-treated C6 cells was performed through detecting the phosphorylated and total form of indicated proteins by Western blotting using specific antibodies. As illustrated in Fig. 5A, FePP dose-dependently induces the expression of phosphory-

lated ERKs and JNKs, but not p38 and AKT, protein, in the SF condition. FePP-induced phosphorylated ERKs and JNKs protein was significantly abolished by FBS or BSA addition (Fig. 5B). As the same part of experiment, CoPP induces a time-dependent increase of phosphorylated ERKs and JNKs protein in SF condition (Fig. 5C). The phosphorylated ERKs and JNKs induced by CoPP were inhibited by FBS or BSA addition (Fig. 5D). In order to examine if ERKs and JNKs activation is involved in FePP and CoPP-induced cell death, both ERKs inhibitor PD98059 and JNKs inhibitor SP600125 were used in the present study. As described in Fig. 5E, neither PD98059 nor SP600125 affects the cytotoxic effect elicited by FePP and CoPP in glioma cells C6 (Fig. 5E). NAC inhibition of FePP, not CoPP, induced ERKs protein phosphorylation in according with decreasing HO-1 protein expression was identified in glioma cells C6 (Fig. 5F and G, and data not shown).

3.6. FePP and CoPP-induced cell death is independent on HO-1 protein induction

FePP and CoPP have been shown to induce HO-1 protein expression, therefore we investigate if HO-1 protein induc-

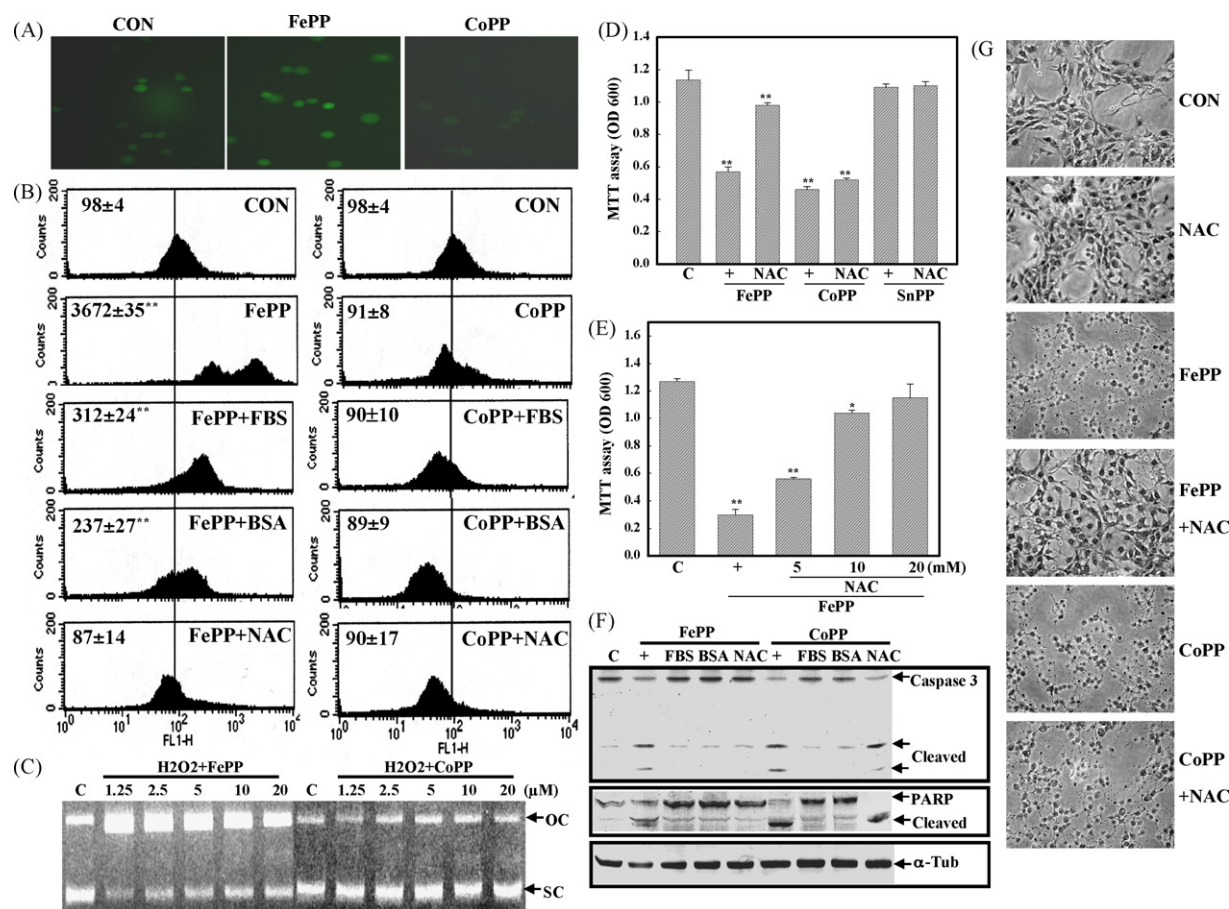


Fig. 4. ROS generation in FePP and CoPP-treated glioma cells C6. (A) FePP, but not CoPP, treatment increases intracellular DCF fluorescent intensity under fluorescent microscopic observations. Cells were treated with FePP or CoPP (20 μM) in the SF condition for 1 h, followed by adding peroxide-sensitive fluorescent dye DCFH-DA for an additional 30 min. The DCF fluorescence in cells was observed under fluorescent microscope. (B) FBS, BSA, and NAC attenuate FePP-induced DCF intensity by flow cytometric analysis. As described in (A), cells were treated with FePP or CoPP (20 μM) in the SF, 5% FBS (FBS), or 0.2% BSA (BSA) condition for 1 h, followed by adding peroxide-sensitive fluorescent dye DCFH-DA for an additional 30 min. In the NAC (10 mM)-treated group, the cells were treated with NAC (10 mM) for 30 min followed by FePP or CoPP treatment for additional 1 h. The fluorescent intensity of DCF was measured, and expressed as a mean ± S.E. from data of three independent experiments. (C) FePP, not CoPP, catalyzes the production of hydroxyl radical to convert the conformation of pBR322 plasmid from supercoiled (SC) to open circle (OC) form. As described in Section 2, pBR322 plasmid (1 μg) was incubated with different doses (1.25, 2.5, 5, 10, and 20 μM) of FePP or CoPP in the presence of H₂O₂ (40 μM) for 30 min. The conformational change of pBR322 plasmid under different treatments was analyzed by agarose electrophoresis. (D) NAC addition protects C6 cells from FePP, but not CoPP, -induced cytotoxicity. Cells were treated with NAC (10 mM) for 30 min followed by FePP, CoPP or SnPP (20 μM) for a further 12 h. The viability of cells under different treatments was measured by MTT assay. (E) NAC dose-dependently prevents FePP-induced cell death. As described in (D), C6 cells were treated with different doses (5, 10, and 20 mM) of NAC, followed by FePP (20 μM) treatment for 12 h. (F) FBS and BSA inhibition of FePP and CoPP-induced caspase 3 and PARP protein cleavage, however NAC protects C6 cells from FePP, but not CoPP, -induced caspase 3 and PARP protein cleavage. As described before, the expression of caspase 3, PARP, and α-tubulin (α-Tub) protein was detected by Western blotting using specific antibodies. (G) NAC addition inhibits FePP, but not CoPP, -induced chromatin-condensed cells. As described in (A), the condensed cells under different treatments were observed microscopically via Giemsa staining. Data derived from three independent experiments are expressed as the mean ± S.E., and **p* < 0.05, ***p* < 0.01 indicates a significant difference from FePP or CoPP-treated group (B), and control group (D and E).

tion participates in FePP and CoPP-induced cell death. As illustrated in Fig. 6A, FePP dose-dependently induces HO-1 protein expression in C6 cells in the SF condition, and HO-1 induction by FePP was blocked by FBS addition (Fig. 6A). Similarly, a dose-dependent increase in HO-1 protein expression was examined in CoPP-treated C6 cells without FBS or BSA addition (Fig. 6B). HO-1 protein induced by FePP or CoPP in the SF condition was significantly suppressed by FBS or BSA addition (Fig. 6C). We further examine the role of ERKs and JNKs activation in HO-1 protein expression stimulated by FePP or CoPP. As illustrated in Fig. 6D, incubation of C6 cells with ERKs inhibitor PD98059 dose-dependently

blocks FePP or CoPP-induced phosphorylated ERKs protein, but PD98059 shows no effect on HO-1 protein expression stimulated by FePP or CoPP. Interestingly, application of JNKs inhibitor SP600125 dose-dependently inhibits FePP-induced or CoPP-induced HO-1 protein expression with reducing JNKs protein phosphorylation in C6 cells (Fig. 6E). Inhibition of HO-1 protein expression by a transfection of antisense HO-1 oligonucleotide has been described in our previous study (Lin et al., 2007). In the present study, HO-1 protein induced by FePP or CoPP was reduced by transfection of C6 cells with antisense HO-1 oligonucleotide, however the cytotoxic effect elicited by FePP or CoPP was not affected (Fig. 6F).

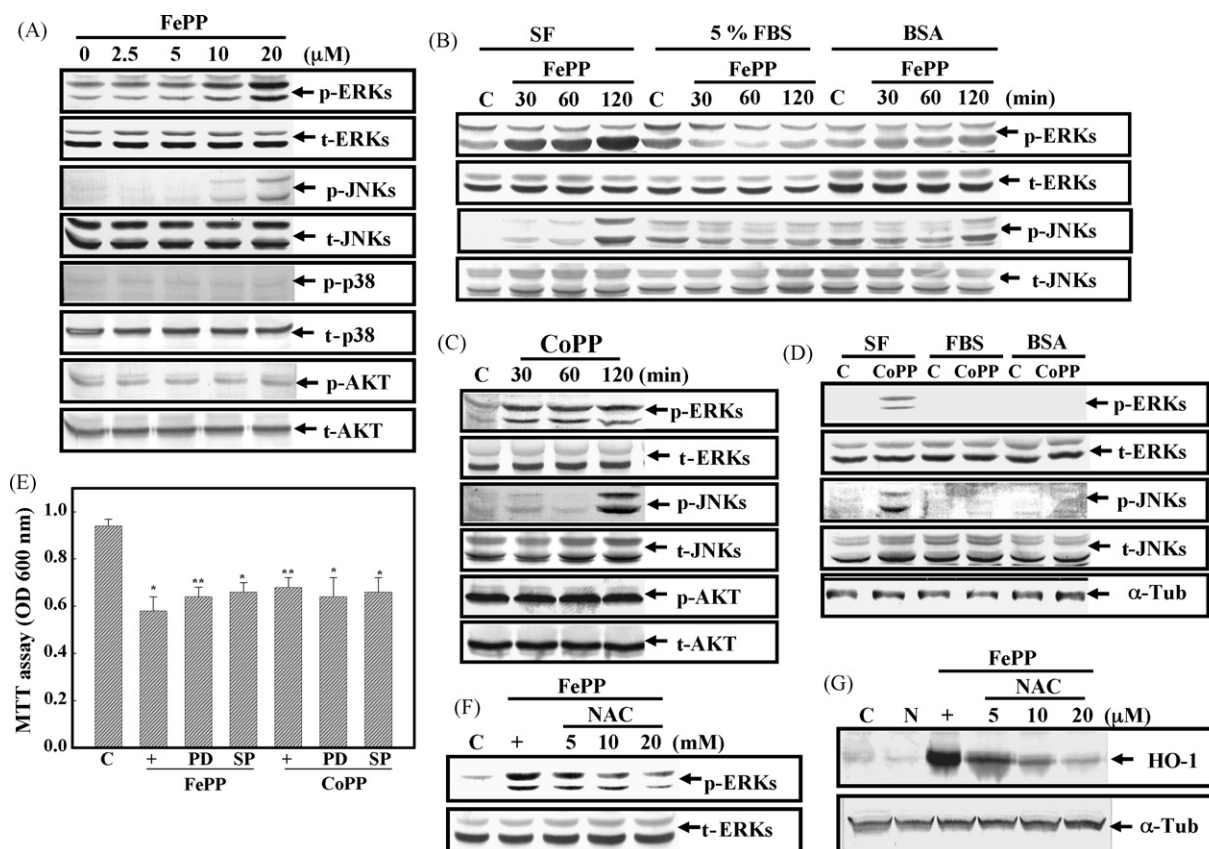


Fig. 5. Induction of ERKs and JNKs protein phosphorylation in FePP and CoPP-treated C6 cells. (A) FePP dose-dependently induces ERKs and JNKs, but not AKT and p38, protein phosphorylation in C6 cells. Cells were treated with different doses (2.5, 5, 10, and 20 μ M) of FePP for 120 min, and the expression of indicated proteins was performed by Western blotting using specific antibodies. (B) FePP induction of ERKs and JNKs protein phosphorylation in the SF, but not 5% FBS and 0.2% BSA, condition. Cells were treated with FePP (20 μ M) for different times (30, 60, and 120 min) in SF, 5% FBS, and 0.2% BSA condition, and the expression of total and phosphorylated form of ERKs and JNKs protein was performed by Western blotting using specific antibodies. (C) CoPP time-dependently induces ERKs and JNKs protein phosphorylation in the SF condition. Cells were treated with CoPP (20 μ M) for different times (30, 60, and 120 min) in the SF condition, and the expression of indicated protein was performed. (D) FBS and BSA attenuate CoPP-induced ERKs and JNKs protein phosphorylation. Cells were treated with CoPP (20 μ M) in the presence or absence of 5% FBS or 0.2% BSA for 120 min, and the expression of indicated proteins was performed by Western blotting. (E) ERKs inhibitor PD98059 (PD) or JNKs inhibitor SP600125 (SP) shows no effect on FePP-induced or CoPP-induced cell death. Cells were incubated with PD or SP (10 μ M) for 30 min, followed by FePP or CoPP (20 μ M) treatment for a further 12 h. The viability of cells under different treatments was analyzed by MTT assay. (F) NAC inhibits FePP-induced ERKs protein phosphorylation in glioma cells C6. As described previously, the expression of indicated ERKs protein was examined. (G) NAC addition dose-dependently reduces the HO-1 protein induced by FePP (20 μ M). Cells were treated with different doses of NAC (5, 10, and 20 mM) for 30 min, followed by FePP treatment for an additional 12 h, and the expression of HO-1 and α -tubulin protein was detected by Western blotting. Data derived from three independent experiments are expressed as the mean $\pm$ S.E., and * p < 0.05, ** p < 0.01 indicates a significant difference from control (C) group.

4. Discussion

This study accomplishes several aims. First, we have characterized that FePP and CoPP induce the cytotoxic effect in glioblastoma cells that may be relevant to hemorrhagic CNS injury. Second, the cytotoxic effect of FePP and CoPP is prevented by albumin or FBS addition. Third, FePP and CoPP-induced apoptosis of glioblastoma cells may respectively attribute to ROS-dependency and -independency. Four, the effect of ERKs and JNKs activation and HO-1 protein induction on the vulnerability of glioma cells to FePP and CoPP was investigated. An increase in the expression phosphorylated ERKs and JNKs protein, and HO-1 protein was detected in FePP and CoPP-treated glioma cells C6 in the absence of FBS or BSA. However, none of ERKs inhibitor PD98059, JNKs inhibitor SP600125, and HO-1 antisense oligonucleotide was able to reduce the cytotoxicity elicited by FePP or CoPP. It suggests that FePP and

CoPP-induced cytotoxicity is irrelevant to ERKs, JNKs, and HO-1 induction.

FePP is an oxidant in several model systems. Goldstein et al. (2003) indicated that hemin induced the oxidative injury to human neuron-like cells through an iron-dependency. Huffman et al. (2000) delineated that hemoglobin may potentate the production of ROS in alveolar macrophages. Additionally, FePP may stimulate the ROS production via depletion of glutathione. Ohashi et al. (2002) indicate that FePP is able to oxidize DCFH-DA, therefore, oxidation of DCFH is not consistently related to the generation of ROS (Ohashi et al., 2002). The effect of ROS in CoPP-induced cytotoxicity is poorly understood. There are three-independent methods including DCFH-DA assay, plasmid digestion, and antioxidant prevention for analyzing the ROS generation of FePP and CoPP in glioblastoma cells. Data of the present study show that FePP, but not CoPP, treatment induces intracellular peroxide level by DCHF-DA assay,

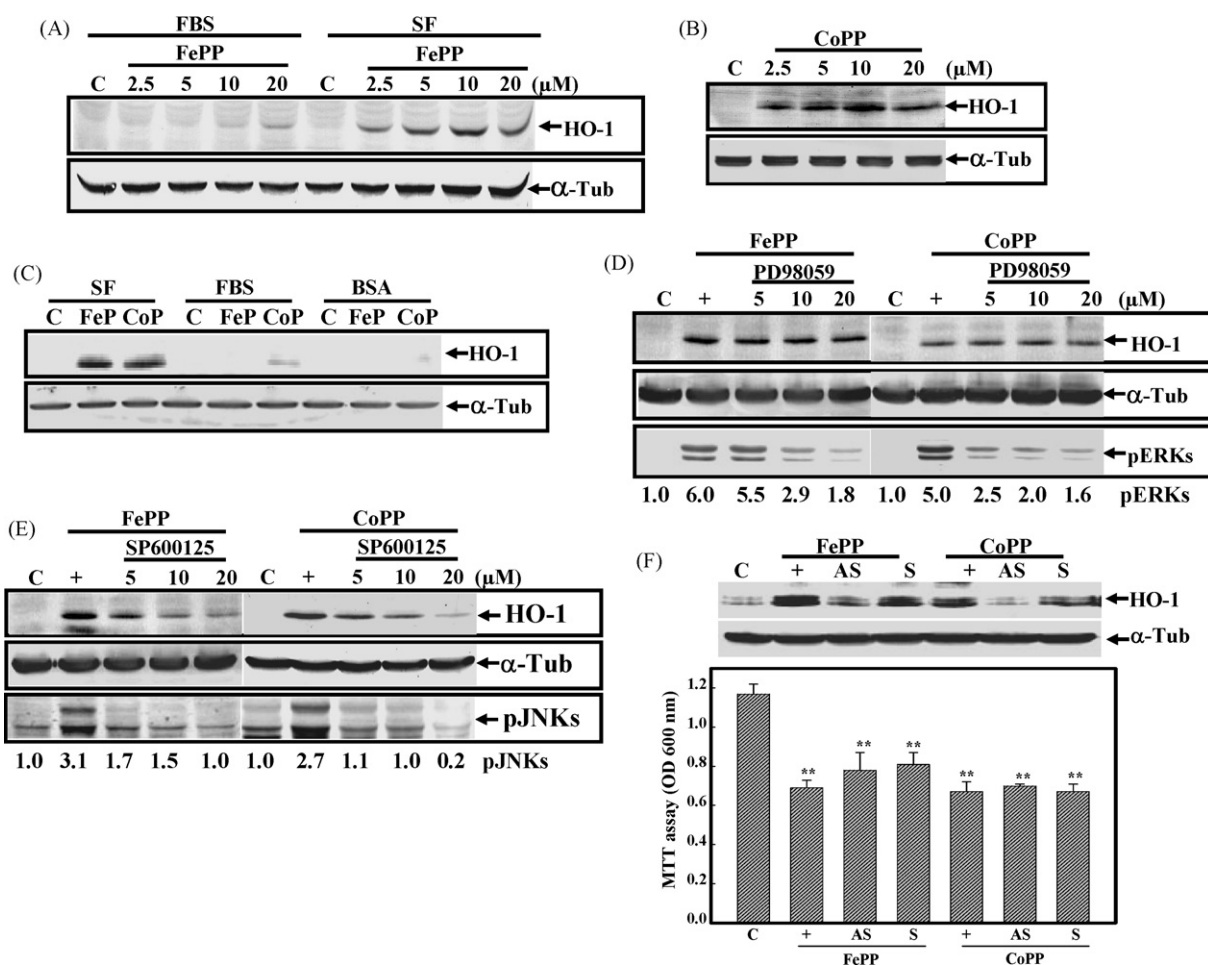


Fig. 6. Induction of HO-1 protein may not be involved in FePP-induced or CoPP-induced cell death of glioma cells C6. (A) FePP dose-dependently induces HO-1 protein expression in the SF, not 5% FBS, condition. Cells were treated with different doses (2.5, 5, 10, and 20 μ M) of FePP in the SF or 5% FBS (FBS) condition for 12 h, and the expression of HO-1 and α -tubulin (α -Tub) protein was detected by Western blotting. (B) CoPP dose-dependently elevates HO-1 protein expression in the SF condition. Cells were treated with different doses (2.5, 5, 10, and 20 μ M) of CoPP in the SF condition for 12 h, and the expression of HO-1 and α -tubulin (α -Tub) protein was detected by Western blotting. (C) FBS and BSA inhibit FePP and CoPP-stimulated HO-1 protein expression in the SF condition. As described before, the expression of HO-1 and α -tubulin (α -Tub) protein was analyzed. (D) PD98059 dose-dependently inhibits FePP-induced or CoPP-induced phosphorylated ERKs, but not HO-1, protein expression. Cells were treated with different doses (5, 10, and 20 μ M) of PD98059 for 30 min, followed by FePP or CoPP (20 μ M) treatment for 12 h (for detecting HO-1 and α -tubulin) or 120 min (for detecting phosphorylated ERKs protein). (E) SP600125 dose-dependently inhibits FePP-induced or CoPP-induced phosphorylated JNKs and HO-1 protein expression. Cells were treated with different doses (5, 10, and 20 μ M) of SP600125 for 30 min, followed by FePP or CoPP (20 μ M) treatment for 12 h (for detecting HO-1 and α -tubulin) or 120 min (for detecting phosphorylated and total JNKs protein). (F) Neither antisense (AS) nor sense (S) HO-1 oligonucleotides affects the cytotoxicity of FePP and CoPP (20 μ M) in glioma cells C6. As described in Section 2, the effect of AS and S transfection on FePP-induced or CoPP-induced HO-1/ α -tubulin protein (upper panel) and cytotoxicity (lower panel) was detected by Western blotting and MTT assay, respectively. Data derived from three independent experiments are expressed as the mean $\pm$ S.E., and ** p < 0.01 indicates a significant difference from control (C) group.

and incubation of glioma cells C6 with a chemical antioxidant NAC significantly protects cells from FePP, not CoPP, -induced cell death. Additionally, FePP, not CoPP, can catalyze the conversion of pBR322 plasmid conformation from supercoiled (SC) to open circle (OC) in the presence of H_2O_2 . In relation to why CoPP shows no effect on ROS production in glioblastoma cells is still unclear. Di Noia et al. (2006) recently reported that an increase in carnitine, citrate, and ADP/ATP carriers, and carnitine has been shown to reduce oxidative stress. It suggests that CoPP appears to reduce oxidative stress.

The precise molecular mechanism by which FePP and CoPP activate ERKs and JNKs in glioblastoma cells has not been

defined. FePP is a reactive moiety in regulating numerous metabolic pathways including cell growth, differentiation, and cell death. Zhu et al. (1999) indicate that activation of Ras signaling cascade is involved in heme-induced erythroid differentiation. In astrocyte cultures, activation of ERKs may potentiate hemin toxicity via a ROS-independent mechanism (Regan et al., 2001). Few evidences related to ERKs and JNKs activation in CoPP-treated glioblastoma cells are delineated. In the present study, activation of ERKs and JNKs via inducing protein phosphorylation was examined in FePP and CoPP-treated glioma cells C6 in the condition without BSA or FBS. Application of ERKs inhibitor PD98059 or JNKs inhibitor SP600125 dose-dependently inhibits FePP-stimulated or CoPP-stimulated

ERKs and JNKs protein phosphorylation, but does not affect the cytotoxicity elicited by FePP and CoPP. It suggests that inhibition of ERKs and JNKs is insufficient to prevent cell death induced by FePP and CoPP.

FePP and CoPP are known to be potent and effective inducers of HO-1 activities in many cells and tissues. Administration of FePP results in induction of HO activity, however CoPP is not a substrate for HO-1 and unable to catalyze ROS production in cells. Previous studies have shown that CoPP may be a more promising HO-1 inducer than FePP without increasing oxidative stress. The cytoprotective function of HO-1 activity against cell injury induced by several stimuli such as H₂O₂, chemical anoxia, and ischemia/reperfusion has been reported (Abraham and Kappas, 2005; Ockaili et al., 2005). Although induction of HO-1 during FePP and CoPP-mediated cellular injury has been verified in several studies, the mechanisms by which activation of HO-1 gene in the cytotoxic actions elicited by FePP and CoPP are less well understood. Data of the present study show that both FePP and CoPP induce HO-1 protein expression without FBS or BSA addition, and HO-1 protein expression stimulated by FePP or CoPP is attenuated by JNKs inhibitor SP600125. Addition of SP600125 or transfection of HO-1 antisense oligonucleotide does not appear effective on FePP-induced or CoPP-induced cell death. It suggests that HO-1 induction is not involved in FePP-induced or CoPP-induced cell death of glioblastoma cells.

The plasma protein albumin is known to bind heme with medium affinity, and is thought to prevent the toxic effects of heme on blood cells and vascular endothelium. However, if albumin can bind with other metal protoporphyrins such as CoPP, and SnPP, and the effect of albumin binding on the cytotoxic actions of metal protoporphyrins against the viability of glioma cells are still unclear. Our data support that metal protoporphyrins possesses ability to bind with albumin, and albumin binding may attenuate the cytotoxic effect of FePP and CoPP in glioblastoma cells. The physiologic concentration of BSA in FCS is about 0.2 g/l, and the ratio of serum to BSA is about 150:1, and the hemin concentration may reach hundred micromolar after CNS hemorrhage. Therefore, the concentrations of BSA and FBS used are much higher, and the FePP concentrations used were lower than would be commonly encountered after CNS hemorrhage in the presence study. An additional experiment using low doses of BSA (0.02% and 0.04%) or FBS (0.7% and 14%) with hemin (100 μ M) has been performed, and BSA and FBS addition slightly but significantly reduced the cytotoxic effect of hemin (100 μ M) by MTT assay (data not shown). Abolishment of FePP and CoPP-induced intracellular signal cascade including ERKs and JNKs activation and HO-1 protein induction by albumin is firstly identified in the present study.

Acknowledgements

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