

Metalloporphyrins inactivate caspase-3 and -8

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ABSTRACT Activation of caspases represents one of the earliest biochemical indicators for apoptotic cell death. Therefore, measurement of caspase activity is a widely used and generally accepted method to determine apoptosis in a wide range of *in vivo* and *in vitro* settings. Numerous publications characterize the role of the heme-catabolizing enzyme heme oxygenase-1 (HO-1) in regulating apoptotic processes. Different metalloporphyrins representing inducers and inhibitors of this enzyme are often used, followed by assessment of apoptotic cell death. In the present work, we found that caspase-3-like activity, as well as activity of caspase-8 measured in either Fas (CD95) ligand-treated Jurkat T-lymphocytes or by the use of recombinant caspase-3 or -8, was inhibited by different metalloporphyrins (cobalt(III) protoporphyrin IX, tin and zinc(II) protoporphyrin-IX). Moreover, employing the mouse model of Fas-induced liver apoptosis these properties of porphyrins could also be demonstrated *in vivo*. The metalloporphyrins were shown to inhibit caspase-3-mediated PARP cleavage. Molecular modeling studies demonstrated that porphyrins can occupy the active site of caspase-3 in an energetically favorable manner and in a binding mode similar to that of known inhibitors. The data shown here introduce metalloporphyrins as direct inhibitors of caspase activity. This finding points to the need for careful employment of metalloporphyrins as modulators of HO-1.—Blumenthal, S. B., Kiemer, A. K., Tiegs, G., Seyfried, S., Höltje, M., Brandt, B., Höltje, H.-D., Zahler, S., Vollmar, A. M. Metalloporphyrins inactivate caspase-3 and -8. *FASEB J.* 19, 1272–1279 (2005)

Key Words: porphyrin ring • HO-1 expression • heme-iron

The caspase family comprises at least 10 cysteine proteases that play a critical role during apoptotic cell death. Their name phrases the active cysteine group and the characteristic cleavage of their targets at aspartate residues. Caspases are expressed as inactive zymogens in the cytoplasm. The pro-caspases become activated during apoptosis by proteolytic processing at specific sites, followed by assembly of the active form

(1–3). The apoptotic caspases are generally divided into two classes: the initiator caspases, which include caspase-8, -9, and -10; and the effector caspases, which include caspase-3, -6, and -7. The active enzyme can in turn cleave a number of defined substrates involved in the biochemical and morphological feature of apoptosis. One example is poly-ADP ribose polymerase (PARP), which is cleaved at a specific Asp-Glu-Val-Asp (DEVD) sequence. Caspase-3 has been identified as the caspase responsible for much of this activity (4). These findings led to the measurement of caspase-3-like activity as a reliable, simple, and generally accepted method to quantify apoptotic cell death (5).

Heme oxygenase (HO) is the rate-limiting enzyme in the degradation of heme (6). It catalyzes the oxidative cleavage of heme to yield equimolar amounts of free iron, carbon monoxide (CO), and biliverdin, which is subsequently converted to bilirubin by biliverdin reductase. As known so far, the HO system includes three isoforms of the enzyme. Of these, the HO-1 isoform is distributed ubiquitously and is strongly induced by a variety of physiological and pathophysiological stimuli, including heme, heavy metals, endotoxins, oxidants, and several inflammatory cytokines (7). The substrate of HO-1, Fe protoporphyrin IX (FePP, heme, hemin), is a multifunctional molecule in nature. The porphyrin ring is a tetra dentate ligand that binds metals through two imino nitrogen atoms capable of accepting protons and two pyrrole nitrogens capable of either losing or accepting protons. Several metals such as Fe, Cu, Zn, Sn, and Co can form a complex with the porphyrin ring (8). The binding pocket of HO has specificity toward the side chains of the porphyrin ring and does not recognize the metal moiety of the molecule. In consequence, metalloporphyrins, in which the heme-iron has been replaced by other metals like Zn, Sn, and Co, can compete for heme and inhibit the activity of the enzyme because they cannot be degraded to bile pigments (9). On the other hand, heme and other metal-

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doi: 10.1096/fj.04-3259com

loporphyrins are able to induce HO-1 expression, to various degrees though (10–12). In fact, cobalt(III) protoporphyrin IX (CoPP) is often used as a strong HO-1 inducer in different experimental settings because its inducing effect on HO synthesis overweighs its inhibitory effect on the enzyme. In contrast to CoPP, SnPP and ZnPP are only weak inducers of HO-1 expression but markedly decrease its activity, which explains their usage as potent HO-1 inhibitors.

Several studies in the recent past have led to the hypothesis that HO-1 confers cellular protection against oxidant insults and serves a vital function in maintaining cellular homeostasis through anti-oxidative, anti-inflammatory, and anti-apoptotic actions (13, 14). In studies concerning the anti-apoptotic features of HO-1 (15–19), different metalloporphyrins are often used to either stimulate HO-1 expression or inhibit its activity, in combination with various assay methods such as the measurement of caspase-3-like activity.

Here we can report an important finding: the HO-1 inhibitors tin and zinc(II) protoporphyrin IX (SnPP, ZnPP) and the strong HO-1 inducer cobalt(III) protoporphyrin IX (CoPP) inhibit caspase activity unrelated to HO-1 expression and activity *in vitro* and *in vivo*. In fact, by using recombinant caspase-3 and -8 and by performing molecular modeling studies for caspase-3, a direct inhibition of caspase activity by the porphyrins could be demonstrated.

MATERIALS AND METHODS

Reagents

Cell culture medium (RPMI 1640) and penicillin/streptomycin were from PAN (Aidenbach, Germany). FCS was from PAA Laboratories (Cölbe, Germany) and human, recombinant Fas ligand, cobalt(III), tin, and zinc(II) protoporphyrin were from Alexis (Grünberg, Germany). Substrates for caspase-3 and -8, Ac-DEVD-AFC and Ac-IETD-AFC, were from Biosource (Solingen, Germany). The human recombinant caspases-3 and -8 as well as the anti-PARP monoclonal mouse antibody were purchased from Calbiochem (Schwalbach, Germany), the ECL Plus Western Blot Detection Reagent from Amersham Biosciences (Freiburg, Germany), and Complete[®] from Roche (Mannheim, Germany). Anti-hsp32 (HO-1) antibody, peroxidase-conjugated anti-caspase-3 (CPP32) antibody, and anti-CD95 (Fas) antibody were from BD/PharMingen (Heidelberg, Germany), and peroxidase-conjugated goat anti-mouse antibody was from Cell Signaling (Frankfurt/M, Germany). Protoporphyrin IX disodium salt was from Frontier Scientific Porphyrin Products (Carnforth/Lancashire, UK). All other materials were purchased from either Sigma (Taufkirchen, Germany), Carl-Roth GmbH (Karlsruhe, Germany) or Merck-Eurolab (Munich, Germany).

Cell culture

Human leukemia Jurkat T cells (clone J16) were cultured (37°C and 5% CO₂) in RPMI 1640 supplemented with 10% FCS, penicillin (100 U/mL)/streptomycin (100 ng/mL), and 0.1 M pyruvate. For experiments, cells of passage numbers 3 to 15 were used at a density of 0.6 to 0.7 × 10⁶ cells/mL.

Animal model

BALB/c-mice (age: 6–8 wk; weight range: 18–22 g) were obtained from the animal facilities of the Institute of Experimental and Clinical Pharmacology and Toxicology of the University of Erlangen-Nuremberg, Erlangen, Germany. All mice received human care according to the guidelines of the National Institute of Health as well as to the legal requirements in Germany. They were maintained under controlled conditions (22°C, 55% humidity and 12 h day/night rhythm) and fed a standard laboratory chow. Activating anti-CD95 antibody (Ab) was administered intravenously at 125 µg/kg in 200 µL pyrogen-free saline. Animals were killed after 6 h and livers were frozen in liquid nitrogen. Cobalt(III) protoporphyrin IX (CoPP, *i.p.*, 10 mg/kg) and tin protoporphyrin IX (SnPP, *i.p.*, 25 mg/kg) were dissolved in sterile 0.2 M NaOH and pH was adjusted to a neutral pH level. CoPP or SnPP were administered 2 h after induction of liver injury by anti-CD95 Ab.

Western blot analysis

Jurkat T cells were either left untreated or stimulated with Fas ligand (FasL, 100 ng/mL) in the absence or presence of the different porphyrins (CoPP, SnPP, ZnPP, 10 µM each) or treated with the porphyrins alone for the indicated times. Western blot was performed as published previously (20). Lysis buffer contained 150 mM NaCl, 50 mM Tris-HCl, 1% Nonidet P40, 0.25% deoxycholate, and 0.1% SDS, supplemented with a protease inhibitor cocktail (Complete[®]) and 1 mM PMSF. For electrophoresis gels were loaded with 10 µg protein/sample. ECL Plus Western Blot Detection Reagent (Amersham Biosciences) and an AGFA Developer were used for visualization of the bands.

Caspase activity measurement

Cells were treated with FasL (100 ng/mL) for the indicated times in the presence or absence of the different porphyrins (CoPP, SnPP, ZnPP, 10 µM each) for the indicated times. Caspase-3-like activity was measured in cell lysates and tissue homogenates as described previously (21). Release of free 7-amino-4-trifluoromethyl coumarin (AFC) from the synthetic substrate Ac-DEVD-AFC (50 µM) at 37°C was determined by fluorescence measurement in a SpectraFluor Plus plate reader (Tecan, Crailsheim, Germany) at an excitation wavelength of 390 nm and an emission wavelength of 535 nm. Activity of caspase-8 was determined similarly using Ac-IETD-AFC as substrate.

Activity of recombinant caspase-3 and-8

Human recombinant caspase-3 and -8 were supplied as stock solution in a buffer containing 50 mM HEPES (pH 7.4), 100 mM NaCl, 0.1% CHAPS, 1 mM EDTA, 10% glycerol and 10 mM dithiothreitol (DTT). Before each experiment, the stock solutions were diluted to 2 U/µL with buffer A (50 mM HEPES pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM EDTA, 10% glycerol) plus 400 µM DTT. Activity of the enzymes (final concentration: 40 U/mL) was measured at 37°C in 100 µL buffer A (without addition of DTT) following the generation of free AFC from the synthetic substrate Ac-DEVD-AFC for caspase-3 or Ac-IETD-AFC for caspase-8 respectively (50 µM each) with a SpectraFluor Plus plate reader. Metalloporphyrins were used in a concentration range between 0.001 and 100 µM.

Molecular modeling

Automated docking analysis was carried out by using AutoDock 3.0 (22). The crystal structure of caspase-3 was obtained from the Brookhaven Protein Databank (code: 1QX3). To identify potential ligand locations, a protoporphyrin molecule (without metal ligand) was docked in the active site of the enzyme. Protoporphyrin was taken due to the fact that standard force field methods cannot calculate physicochemical properties of metal ions adequately. The ligand was kept flexible so that it could adjust to the positions of the protein side chains. Fifty independent docking runs were carried out. For each calculation, similar ligand geometries were clustered and represented by the one with the most favorable interaction energy. The obtained complexes were energetically minimized by using a conjugate gradient algorithm permitting the ligand side chain atoms to relax. Energy minimizations were carried out by using the PRGEN program (23). To validate the results obtained by the docking procedure, a series of GRID calculations was performed. The program predicts favorable interactions between a molecule of known 3-dimensional structure (i.e., caspase-3) and different probes representing characteristic chemical features of a ligand molecule. Unlike the docking studies, the complete structure of caspase-3 was investigated here, in order to search for possible protoporphyrin binding sites in addition to the known active site.

Statistical analysis

Unless stated otherwise at least three independent experiments were performed in triplicates. Data are expressed as mean \pm SE. Values with $P < 0.05$ were considered statistically different compared with control cells (one-sample *t* test). Statistical analysis was performed with Graph Pad Prism (version 3.02).

RESULTS

Porphyrins inhibit FasL-induced caspase-3-like activity in vitro

Inhibition of caspase-3-like activity by different metalloporphyrins was determined using Jurkat T-lymphocytes treated with FasL (100 ng/mL) for various times. Enzyme activity is recorded as an increase in fluorescence due to cleavage of the caspase-3 substrate Ac-DEVD-AFC. As shown in Fig. 1A, the HO-1 inducer cobalt(III) protoporphyrin-IX (CoPP, 10 μ M) as well as the HO-1 inhibitors tin and zinc(II) protoporphyrin-IX (SnPP, ZnPP, 10 μ M each) were able to inhibit enzyme activity induced by FasL.

The different metalloporphyrins, as well as FasL, have been demonstrated to affect HO-1 expression in different cell types including Jurkat cells (12, 24). However, Western blot analysis assured that in our experimental setting HO-1 was neither induced by FasL nor by porphyrins (Fig. 1B). Therefore, the effect on caspase activity by the metalloporphyrins seems to be HO-1-independent.

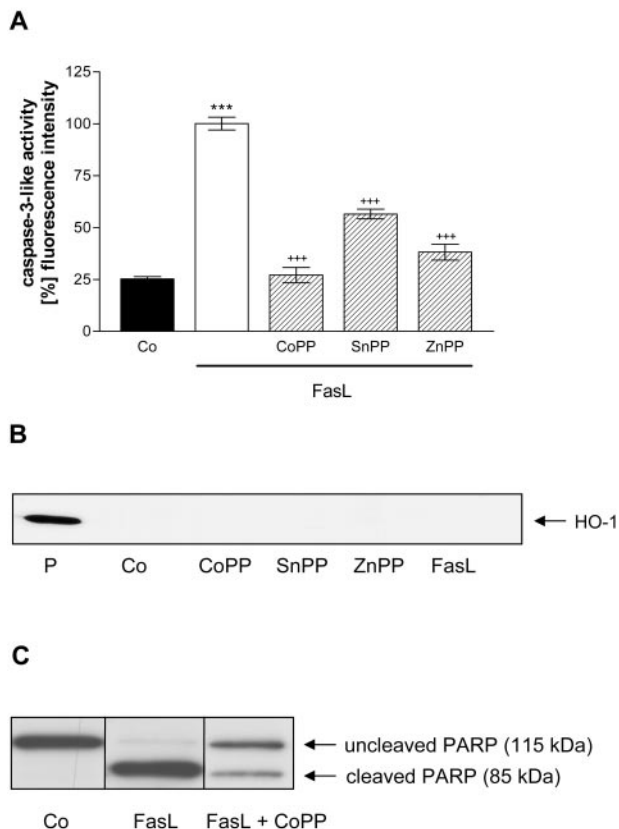


Figure 1. The influence of metalloporphyrins on caspase-3-like activity, HO-1 protein expression, and PARP cleavage in intact Jurkat T-lymphocytes. *A*) Cells were either left untreated (Co) or treated with FasL (100 ng/mL, 16 h). The HO-1 inducer cobalt(III) protoporphyrin-IX (CoPP, 10 μ M) and the HO-1 inhibitors tin and zinc(II) protoporphyrin-IX (SnPP, ZnPP, 10 μ M each) were added 30 min before the cells were lysed. Caspase-3-like activity was determined. Bars represent means \pm SE of 3 independent experiments performed in triplicates and values of untreated cells were set as 1. *** $P < 0.001$ represents significant differences compared with Co. +++ $P < 0.001$ represents significant differences compared with cells treated with FasL alone. *B*) To detect HO-1 protein expression by Western blot, cells were either left untreated (Co) or treated with FasL (100 ng/mL, 16 h) or with the different porphyrins (CoPP, SnPP, ZnPP, 10 μ M each, 30 min). Human umbilical vein endothelial cells (HUVEC) treated with CoPP (10 μ M, 30 min) served as a positive control (P) for induction of HO-1 protein expression. One representative blot of three is shown. *C*) To detect PARP cleavage by Western blot, cells were either left untreated (Co) or treated with FasL (100 ng/mL) alone or together with CoPP (10 μ M) for 6 h. Experiments were performed 3 times with consistent results and 1 representative blot is shown.

Metalloporphyrins inhibit caspase-3-dependent PARP cleavage

Poly-ADP ribose polymerase (PARP) is known to be a prominent substrate of caspase-3 during apoptosis. In fact, Western blot analysis of Jurkat T-lymphocytes revealed a reduction of the 85 kDa cleavage product of PARP in CoPP-treated cells compared with Jurkat cells treated with FasL alone (Fig. 1C).

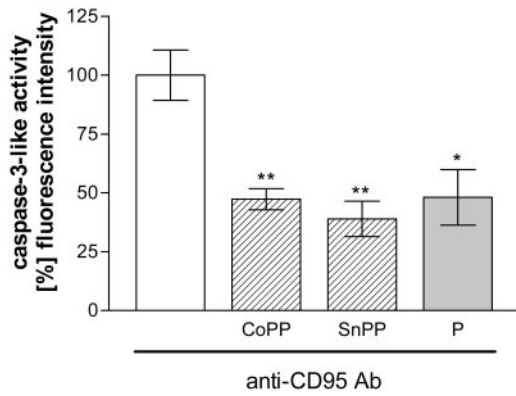


Figure 2. Porphyrins influence caspase-3-like activity in vivo. Mice were treated with CoPP (10 mg/kg) or SnPP (25 mg/kg) 2 h after induction of caspase activity by activating anti-CD95 Abs (anti-CD95 Ab, 10 mg/kg). Caspase-3-like activity was measured at 6 h after challenge. SnPP was added to liver homogenates of mice treated with anti-CD95 Ab immediately before the measurement of caspase activity serving as a positive control (P). Bars represent means \pm SE of 4 mice and values of anti-CD95 Ab-treated mice were set as 100%. ** $P < 0.01$, * $P < 0.05$ represent significant differences compared with anti-CD95 Ab-treated animals.

Porphyrin treatment inhibits caspase-3-like activity in vivo

Mice were treated with activating anti-CD95-antibody for 6 h resulting in high caspase-3 activity. CoPP and SnPP were administered 2 h after anti-CD95-antibody in order to make sure that no considerable HO-1 protein expression occurred yet (data not shown). Measurement of caspase-3-like activity in liver homogenates clearly showed that metalloporphyrins are able to inhibit this enzyme in vivo (Fig. 2).

Porphyrins are direct caspase-3 inhibitors

To elucidate the mechanism of caspase inhibition, porphyrins were added directly to lysates of FasL-treated Jurkat cells containing activated caspases. Activ-

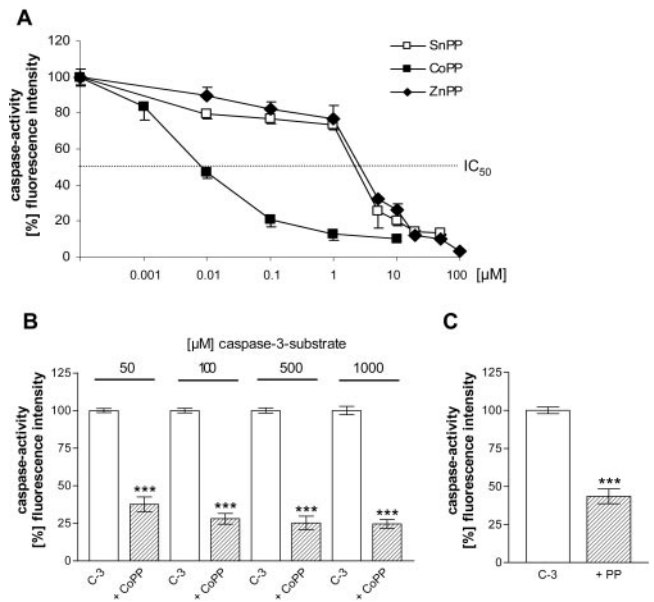
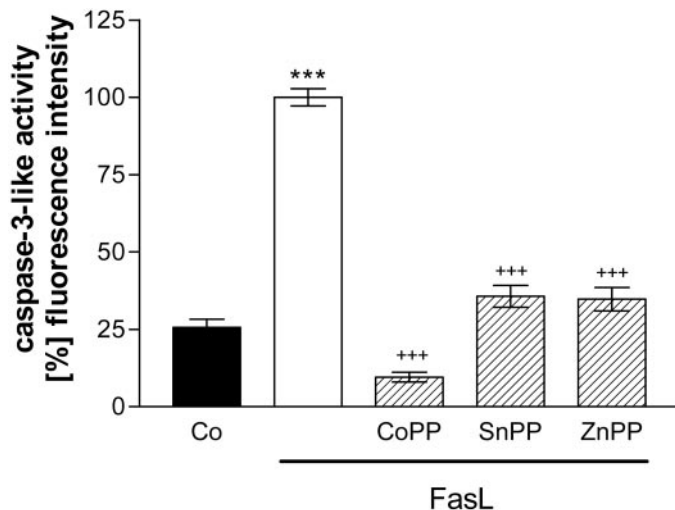


Figure 4. Metalloporphyrins and protoporphyrin sodium salt influence the activity of recombinant caspase-3. CoPP (0.001–10 μ M), SnPP (0.001–100 μ M), ZnPP (0.001–100 μ M) (A), or protoporphyrin-IX disodium salt (PP, 10 μ M) (C) were added to recombinant caspases-3 (C-3) immediately before measurement of caspase activity. In further experiments, CoPP (10 μ M) was added to recombinant caspase-3 with increasing amounts of the caspase-3 substrate (B). Data represent means \pm SE of 3 independent experiments performed in triplicates and activity of caspase-3 in the absence of the porphyrins was taken as 100%. *** $P < 0.001$ represents significant differences compared with the respective C-3 group.

ities, as shown in Fig. 3, gave a first hint that the mode of inhibition may occur in a direct manner. The three metalloporphyrins showed an even stronger inhibitory effect on caspase-3-like activity than in whole cells.

To further confirm a direct inhibition of caspase-3-like activity by porphyrins, recombinant caspase-3 was used. As can be seen in Fig. 4A, cobalt, tin, and zinc protoporphyrin were able to inhibit the isolated enzyme. The IC_{50} values of the three metalloporphyrins

Figure 3. The effect of metalloporphyrins on caspase-3-like activity in cell lysates of Jurkat T-lymphocytes. Cells were either left untreated (Co) or treated with FasL (100 ng/mL, 16 h). CoPP, SnPP, and ZnPP (10 μ M each) were added to the cell lysates immediately before the measurement of caspase activity. Bars represent means \pm SE of 3 independent experiments performed in triplicates and values of FasL-treated cells were set as 100%. *** $P < 0.001$ represents significant differences compared with Co. +++ $P < 0.001$ represents significant differences compared with cells treated with FasL.

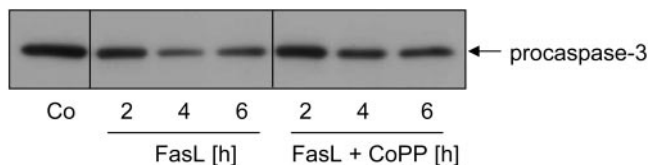


Figure 5. Procaspase-3-processing is affected by protoporphyrins. Jurkat T-lymphocytes were either left untreated (Co) or treated with FasL (100 ng/mL) in the presence or absence of CoPP (10 μ M) for the indicated times. Cellular levels of uncleaved procaspase-3 were examined by Western blot. The experiment was performed 3 times and 1 representative blot is shown.

demonstrate that the inhibitory potency of CoPP ($IC_{50} \sim 10$ nM) clearly exceeds that of the two other porphyrins ($IC_{50} \sim 2.5$ μ M).

Using increasing amounts of the caspase-3-substrate revealed that the inhibitory effect of the porphyrins is not reversible at substrate concentrations of up to 1 mM, as shown in Fig. 4B.

The fact that protoporphyrin IX lacking the metal moiety, was able to inhibit caspase-3 activity (Fig. 4C) points to the protoporphyrin ring as responsible structural element for the caspase-3 inhibition. Molecular modeling data confirmed this assumption.

Caspase-3-processing is inhibited by protoporphyrins

Active caspase-3 is cleaved from its inactive zymogen procaspase-3 by initiator caspases such as caspase-8. As shown in Fig. 5, treatment with FasL leads to a decrease of the uncleaved procaspase-3. In the presence of CoPP the processing of procaspase-3 is blocked. The same effect could be seen for SnPP (data not shown). This

suggests that porphyrins also interfere with caspases upstream of caspase-3.

Metalloporphyrins inhibit caspase-8 activity

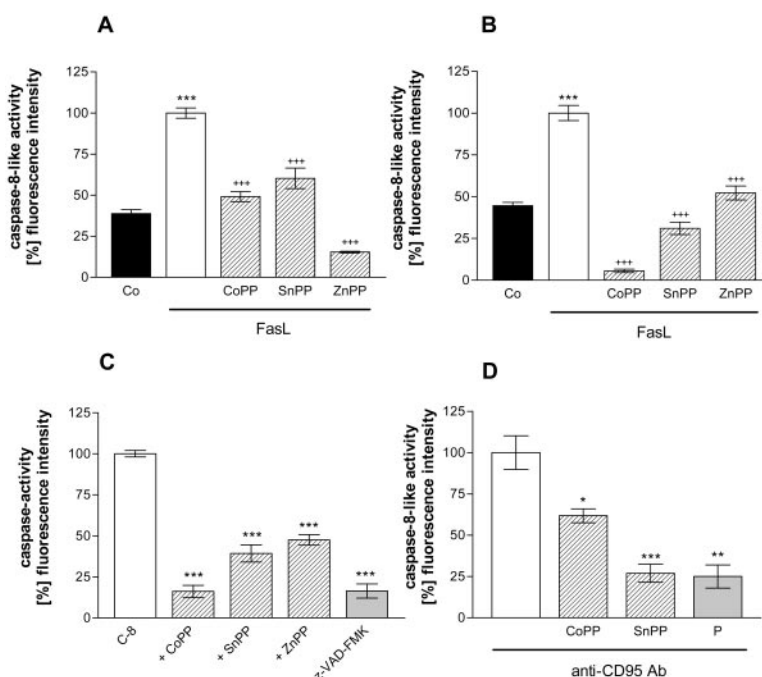
To clarify, if the inhibitory effect of the porphyrins is specific for caspase-3 or if other caspases are equally affected, we looked at the activity of caspase-8, as a representative of the initiator caspases upstream of the executive caspase-3. Application of porphyrins to whole cells (Fig. 6A), cell lysates of FasL-activated Jurkat cells (Fig. 6B) or directly to recombinant caspase-8 (Fig. 6C) showed similar inhibitory action on caspase-8 activity as on caspase-3 activity. Caspase-8 was inhibited by the porphyrins in the in vivo model (Fig. 6D).

Molecular modeling studies

Molecular modeling studies confirmed the biochemical data. The protoporphyrin fits well into the active site of caspase-3, as can be seen from the molecular superposition of the binding conformations extracted from the AutoDock results (Fig. 7A).

In Fig. 7B, the energetically most favorable interaction complex can be viewed in detail: one carboxylate group of the protoporphyrin forms a bidentate salt bridge to the guanidinium group of Arg-341. Moreover, one of the carboxylate oxygens forms a hydrogen bond with the backbone $-NH$ of Ser-180. The second carboxylate group acts as a hydrogen bond acceptor for the backbone NH of Arg-341 and for the $-OH$ of Tyr-338. The $-OH$ group of Ser-343 points in the direction of the N/NH atoms of the protoporphyrin in a way that either hydrogen bonds or interactions with a central metal cation can occur. The protoporphyrin carbon

Figure 6. Effect of metalloporphyrins on caspase-8-like activity. Cells were either left untreated (Co) or treated with FasL (100 ng/mL, 5 h). CoPP, SnPP or ZnPP (10 μ M each) were added either 30 min before the cells were lysed (A) or immediately before measurement of caspase-8-like activity (B). The metalloporphyrins (10 μ M each) were added to recombinant caspases-8 (C-8) immediately before the measurement of caspase activity (C). Bars represent means \pm SE of 2 independent experiments performed in triplicates and values of untreated cells were set as 1. *** $P < 0.001$ represents significant differences compared with Co. +++ $P < 0.001$ represents significant differences compared with cells treated with FasL alone. Mice were treated with CoPP (10 mg/kg) or SnPP (25 mg/kg) 2 h after induction of caspase activity by activating anti-CD95 Abs (anti-CD95 Ab, 10 mg/kg) (D). Activity of caspase-8 was detected at 6 h after challenge. SnPP was added to liver homogenates of mice treated with anti-CD95 Ab immediately before the measurement of caspase activity serving as a positive control (P). Bars represent means \pm SE of 4 mice and values of anti-CD95 Ab-treated mice were set as 100%. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ represent significant differences compared with anti-CD95 Ab-treated animals.



A



B

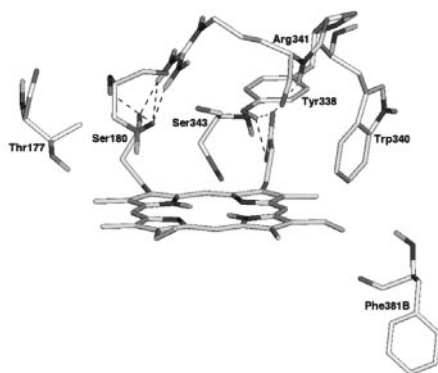


Figure 7. Auto-Dock results. Caspase-3 together with 10 putative binding conformations of protoporphyrin (A). Representation of the energetically most favorable caspase-3/protoporphyrin complex showing the major protein-ligand interactions (B).

skeleton makes some hydrophobic contacts with Thr-177, Trp-340, and Phe-381B.

The program "GRID" was used to test the complete protein for areas that offered energetically favorable interactions with a ligand molecule. Since the protoporphyrin molecule exhibits mainly carbons and carboxylate groups, which may act as binding partners for a protein, we selected a hydrophobic probe and a CO⁻ probe as representatives. The calculations suggest that the most favored region of interactions for the probes is located in the active site of the enzyme, which is in agreement with our docking procedure. However, from the GRID results one further potential binding region was detected. To evaluate its suitability to act as a specific binding site for the protoporphyrin, subsequent AutoDock investigations were performed (data not shown). No energetically favorable binding mode was detected by AutoDock since the protoporphyrin molecule is too bulky to fit well into this pocket. As mentioned above, metal ion properties and influences cannot be estimated reliably with force field methods. To investigate the differences seen in the various metalloporphyrins on caspase-3 activity, extensive quantum chemical computations have to be performed.

DISCUSSION

In the present study, we show that different metalloporphyrins can inactivate caspases *in vitro* as well as *in vivo*. This inactivation could be seen when adding the porphyrins to cells or tissue where the caspases had been activated during apoptosis, to cell lysates containing the activated enzymes, and to recombinant caspase-3 and -8. This strongly suggests that the observed inhibitory effect is a direct one.

Most important, the data indicate that metalloporphyrins seem to have additional effects to their HO-dependent actions. It has been shown that different metalloporphyrins have a substantial effect on other heme-dependent enzymes, such as guanylate cyclase and nitric oxide synthase (25–27). However, the caspases do not contain heme. Thus, the outcome of this study may show a novel mode of action for the metalloporphyrins and introduces them as a new class of caspase inhibitors.

The metalloporphyrin cobalt(III) protoporphyrin IX is often used as a strong HO-1 inducer in experimental settings concerning the functions of HO-1. In fact, CoPP acts as inhibitor of HO-1 activity, like the other metalloporphyrins, as it cannot be converted to the heme degradation products but is able to bind to the catalytic site of the enzyme. On the other hand, CoPP is able to induce the HO-1 expression by a mechanism fundamentally different from that of sodium arsenite and other stress inducers, possibly via a newly identified regulatory region of the HO-1 gene named metalloporphyrin-responsive element (MPRE) (10). Those different effects on HO-1 activity and expression by CoPP result in total in an increased HO-1 activity.

Based on our findings, attention should be paid to the usage of porphyrins as HO-1 modulators in experimental settings, which include the measurement of caspase-3-like activity. As shown by the presented data, direct effects of porphyrins on caspases could occur time dependently, independent of HO-1 expression. Certainly, our data do not argue against several studies using other HO-1 inducers than CoPP, such as sodium arsenite (28), tetracycline-regulated expression systems (29), adenoviral transfer (15), or doxorubicin (30), and reporting cytoprotective features of HO-1.

In contrast to CoPP, SnPP and ZnPP are generally viewed as being only weak inducers of HO-1 expression (11). However, there are contradictory results that claim ZnPP is the most potent inducer of HO-1 expression (31). Nevertheless, it has been proved that tin- and zinc-containing porphyrins decrease HO-1 activity, justifying their usage as potent HO-1 inhibitors. Based on our findings, using SnPP and ZnPP as HO-1 inhibitors in order to prove anti-apoptotic functions of HO-1 could also lead to contradictory results depending on the time frame.

Metalloporphyrins as inhibitors of caspase-3 and -8 raise the question of the physiological function of these compounds. A possible *in vivo* relevance may be discussed using zinc protoporphyrin as an example. Re-

search concerning ZnPP as a naturally occurring metabolite of heme biosynthesis has dramatically increased in the past decade. So far, proposed physiological functions are the control of heme catabolism until bilirubin conjugation becomes activated in neonates and a possible modulation of CO production in brain metabolism (32). In addition, the therapeutic potential of ZnPP and other metalloporphyrins has attracted interest in the treatment of hyperbilirubinemia (33, 34). As yet, all of these suggested functions have been connected to the inhibitory effect of ZnPP on the heme oxygenase system. Our data introduce a direct inhibitory effect on caspase activity as an additional mechanism of action of ZnPP. This knowledge may provide a better understanding of the role of this metabolite in physiology as well as in pathophysiology.

Our work identifies metalloporphyrins as a novel class of irreversible caspase inhibitors. So far, known inhibitors are members of the family of inhibitors of apoptosis protein (IAP) and the two virus-derived proteins cowpox serpin CrmA and p35 from baculovirus (35). Besides these naturally occurring compounds, well established and widely used caspase inhibitors have been generated by synthetically coupling caspase-specific peptide-sequences to certain aldehyde (CHO), chloromethylketone (CMK), fluoroacyloxymethylketone (FAOM), or fluoromethylketone (FMK) compounds (36).

Several X-ray crystal structures reveal how specific peptidic and non-peptidic inhibitors bind to caspase-3 (37–40). The ligands occupy the active site of the caspase in the S1-S4 regions. The interactions between the ligands and the protein mainly result from hydrophobic contacts and hydrogen bonds. A free carboxyl acid seems to play an important role in the ligand structure since it is critical for the binding of the inhibitors to the caspase enzyme. Our investigations demonstrate that the protoporphyrin can occupy the active site of caspase-3 energetically favorable and in a binding mode similar to that of known inhibitors. This postulated binding mode is consistent with crystal data of caspase-3, in which the inhibitors occupy similar parts of the active site. The very potent peptidic inhibitor XIAP-BIR2 exhibits an Asp-148 residue that is essential for the inhibiting effect (mutations of Asp-148 lead to a complete loss of inhibitory activity). Asp-148 forms hydrogen bonds via its backbone and its side chain atoms to Arg-341 and Ser-343. The protoporphyrin seems to meet the structural requirements for caspase inhibitors well since it is able to form (among hydrophobic contacts) the important interactions between its free carboxylic acids and the corresponding amino acids in the catalytic center of the enzyme.

To clarify the possible molecular mechanism of inhibition, we examined which part of the metalloporphyrin complex is responsible for the inhibitory effect on caspase-3-like activity. In fact, we saw a similar effect by using protoporphyrin IX disodium salt on the isolated caspase-3. Thus, it may be that the inhibition of the

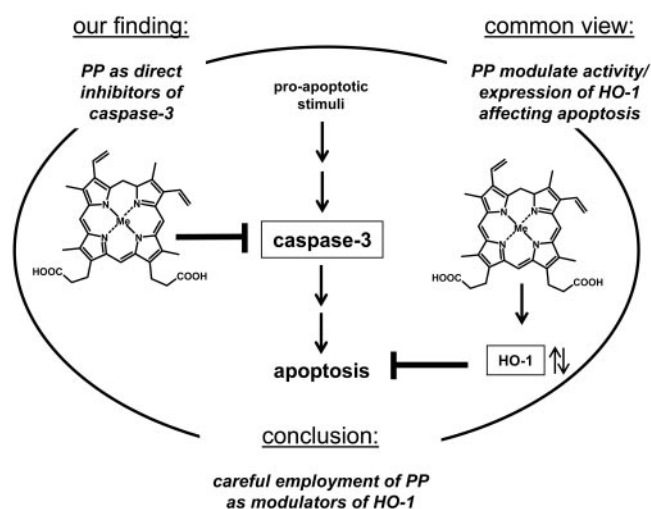


Figure 8. Schematic model of caspase inhibition by the porphyrins. Besides the known mechanism of metalloporphyrins to interfere with apoptosis via HO-1 modulation, porphyrins can directly inhibit caspases after activation by proapoptotic stimuli.

caspase activity by the metalloporphyrins is mainly accountable to the porphyrin ring. However, CoPP is the most efficient inhibitor of the three metalloporphyrins. It has been shown that heavy metals like zinc at micromolar concentrations are able to influence activity of the isolated caspase-3 (41–43), possibly by coordinating with one or two amino acids of the active site of the enzyme. However, considering the complex stabilities of typical metalloporphyrins that allow free metal to occur in the attomolar (10^{-18} M) range (44), it is most unlikely that enough free metal is available to account for the observed effect. Detailed molecular modeling studies are under way to investigate effects on other caspases as well as the molecular basis for the differences seen in the various metalloporphyrins.

In summary, the finding that metalloporphyrins directly inhibit caspase activity points to a novel class of caspase inhibitors, but also requests their careful use as modulators of the HO system (Fig. 8). EJ

The excellent technical support of Cornelia Niemann and Johanna Stöckl is gratefully acknowledged. This work was supported by the Deutsche Forschungsgemeinschaft (DFG FOR440/1 TP 2; Vo 376/10-1). A.K.K. is supported by the Alexander von Humboldt foundation.

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Received for publication October 14, 2004.
Accepted for publication November 1, 2004.