ARTICLE
Journal of Cellular Biochemistry 113:650–657 (2012)

Journal of Cellular Biochemistry

# Inhibition by Zinc of Deoxycholate-Induced Apoptosis in HCT-116 Cells

Abigail F. Smith, Jennifer Longpre, and George Loo*

*Cellular and Molecular Nutrition Research Laboratory, Department of Nutrition, University of North Carolina at Greensboro, Greensboro, North Carolina 27412,*

## ABSTRACT

The bile acid, deoxycholate, can induce apoptosis although the effect of trace elements on such cell death is unknown. The aim of this study was to determine if deoxycholate-induced apoptosis is influenced by zinc. HCT-116 colon epithelial cells were pre-treated with zinc and then exposed to deoxycholate. Membrane blebbing, formation of apoptotic bodies, and greater overall production of reactive oxygen species (ROS) occurred in cells exposed to deoxycholate, but zinc inhibited the occurrence of these three events caused by deoxycholate. Upon finer analysis, stimulation of mitochondrial superoxide production, mitochondrial dysfunction, and cytochrome c release were detected in cells exposed to deoxycholate, but zinc did not inhibit any of these three effects caused by deoxycholate. Additionally, caspase-3 activation, plasma membrane phospholipid translocation, and also chromatin condensation and fragmentation were observed in cells exposed to deoxycholate, but all of these effects of deoxycholate, including the greater overall ROS production, were all inhibited by zinc. Because zinc did not prevent the three mitochondrial effects caused by deoxycholate, the last set of findings suggested that zinc hampered activation of an initiator caspase upstream of effector caspase-3, in inhibiting deoxycholate-induced HCT-116 cell death. In examining this possibility, it was found that caspase-8 activation caused by deoxycholate was blocked by zinc. Collectively, the results suggest that zinc can inhibit deoxycholate-induced apoptotic cell death mediated by caspases. J. Cell. Biochem. 113: 650–657, 2012. © 2011 Wiley Periodicals, Inc.

**KEY WORDS:** APOPTOSIS; DEOXYCHOLATE; ZINC

Deoxycholate (deoxycholic acid) is a potentially toxic secondary bile acid. Through the enzymatic action of resident bacteria in the large intestine, deoxycholate is produced from the cholate that escapes from the small intestine as an aftermath of dietary fat digestion and absorption. It is thought that high concentrations of deoxycholate in the large intestine can have detrimental effects on colon epithelial cells in particular. This notion is supported by previous cell culture studies. For example, deoxycholate induces DNA damage in HT29 cells [Booth et al., 1997]. Additionally, deoxycholate induces DNA damage but also apoptosis in HCT-116 cells [Powolny et al., 2001]. Because of its genotoxicity, deoxycholate is suspected to be mutagenic and carcinogenic [Bernstein et al., 2005]. Thus, there is considerable interest in ascertaining factors that have the potential to influence the capacity of deoxycholate to produce its cellular effects.

Zinc is an essential trace element or micronutrient found in all cells. It has multiple roles in the functioning of different types of cellular proteins, such as numerous enzymes that require zinc as a cofactor and numerous transcription factors having zinc fingers [Coleman, 1992]. Whereas iron and copper are redox-active in biological systems and can promote the formation of reactive oxygen species (ROS) [Halliwell and Gutteridge, 1990], this is not the case with zinc. In fact, it has been known for some time that zinc can act indirectly as an antioxidant [Powell, 2000]. Because deoxycholate is known to stimulate ROS production [Payne et al., 2007; Longpre and Loo, 2008] and ROS are involved in initiating apoptosis [Rollet-Labelle et al., 1998; Dumont et al., 1999], we were prompted to test the hypothesis that zinc can inhibit the apoptotic effects of deoxycholate. Accordingly, the purpose of the present study was to initially determine if zinc affects HCT-116 cell death caused by deoxycholate. Having found that zinc inhibited deoxycholate-induced apoptosis, the next purpose was to determine the apoptotic step where zinc acted in exerting its inhibitory effect.

## MATERIALS AND METHODS

### MATERIALS

HCT-116 and HT-29 human colon adenocarcinoma cells were obtained from American Type Culture Collection (Manassas, VA). All reagents were purchased from Sigma Chemical Co. (St. Louis,

Grant sponsor: North Carolina Agricultural Research Service; Grant number: NC02282.
*Correspondence to: George Loo, Cellular and Molecular Nutrition Research Laboratory, Department of Nutrition, University of North Carolina at Greensboro, Greensboro, NC 27412. E-mail: g_loo@uncg.edu
Received 27 September 2011; Accepted 28 September 2011 • DOI 10.1002/jcb.23394 • © 2011 Wiley Periodicals, Inc.
Published online 4 October 2011 in Wiley Online Library (wileyonlinelibrary.com).

MO), unless otherwise stated. Mouse anti-cytochrome c antibody was obtained from BD Pharmingen Inc. (San Diego, CA). Rabbit polyclonal antibodies against cleaved caspase-3 (product #9661) and against cleaved caspase-8 (product #9496) were obtained from Cell Signaling Technology (Danvers, MA). Mouse β-actin monoclonal antibody was from Sigma Chemical Co. (St. Louis, MO). Goat anti-rabbit IgG-HRP and goat anti-mouse IgG-HRP were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Goat anti-rabbit IgG conjugated to Alexa Fluor 488 was obtained from Molecular Probes (Eugene, OR).

### CELL CULTURE AND TREATMENT

HCT-116 and HT-29 cells were propagated in McCoy's 5A medium supplemented with 100 ml/L fetal bovine serum, 0.54 μmol/L amphotericin B, 100,000 units/L penicillin, and 100 mg/L streptomycin. Upon reaching 70–80% confluency, cell samples (i.e., cells grown in petri dishes, multi-well microplates, or chamber slides) were exposed to deoxycholate (sodium salt). Also, cells were pre-treated with zinc (zinc sulfate), and then without changing the culture medium, exposed to deoxycholate (sodium salt).

### ASSESSMENT OF CELL VIABILITY

After harvesting and washing the cells with phosphate-buffered saline (pH 7.4), the cells were stained with propiduim iodide (PI) [Altman et al., 1993]. Viable cells exclude PI, whereas non-viable cells take up PI.

### ASSESSMENT OF OVERALL ROS PRODUCTION

Overall ROS production was assessed with an Image-iT LIVE Green Reactive Oxygen Species Detection Kit (Molecular Probes, Eugene, OR), containing 5(and 6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate. The manufacturer's set of instructions was followed.

### ASSESSMENT OF MITOCHONDRIAL SUPEROXIDE PRODUCTION

Mitochondrial superoxide production was assessed with the MitoSOX Red fluorogenic probe (Molecular Probes, Inc.), which is based on dihydroethidium reagent. The manufacturer's set of instructions was followed.

### ASSESSMENT OF MITOCHONDRIAL FUNCTION

Mitochondrial function in the cells was assessed with MitoTracker Red CMXRos (Invitrogen, Carlsbad, CA), following the protocol of the supplier. This mitochondria-specific molecular probe fluoresces when it undergoes oxidation and sequestration by mitochondria but only in actively respiring cells [Poot et al., 1996].

### DETERMINATION OF CYTOCHROME C RELEASE INTO CYTOSOL

Using a Dounce homogenizer, the harvested cells were processed for homogenization and centrifugation to isolate the cytosolic fraction, as fully described in a standard procedure [Bossy-Wetzel and Green, 2000]. After protein determination, aliquots were subjected to electrophoresis and Western-immunoblotting, as detailed in the next section below. The membrane was probed with mouse anti-cytochrome c antibody and goat anti-mouse IgG-HRP before chemiluminescence imaging (Kodak 440 CF image station). Afterwards, the membrane was stripped and reprobed with mouse β-actin monoclonal antibody and anti-mouse IgG-HRP for re-imaging.

### DETECTING CLEAVAGE FORMS OF CASPASE-8 AND CASPASE-3

For detection by Western-immunoblotting analysis, harvested cells were sonicated in lysis buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate and Roche Complete Protease Inhibitor Cocktail, pH 7.4) and placed in ice for 30 min. The lysed samples were centrifuged at 16,000*g* for 15 min at 4°C. After protein determination, sample aliquots were electrophoresed using 4–12% Bis-Tris NuPAGE mini-gels (Invitrogen) before electroblotting to nitrocellulose membrane. After blocking with 5% skim milk powder in TBST (20 mM Tris–HCl, 150 mM sodium chloride, 0.05% Tween 20, pH 7.4), the blocked membrane was incubated overnight at 4°C with rabbit polyclonal antibodies against cleaved caspase-3 or cleaved caspase-8, followed by goat anti-rabbit IgG-HRP. A SuperSignal WestFemto Maximum Sensitivity Kit (Pierce, Rockford, IL) was used to generate the chemiluminescence signal that was detected and captured (Kodak 440 CF image station).

For detection by immunocytofluorescence microscopy, cells in chamber slides were fixed with 4% formaldehyde and permeabilized with 0.2% Triton X-100. After blocking with 3% bovine serum albumin, the cells were incubated overnight with rabbit polyclonal antibodies against the cleaved caspases, followed by goat anti-rabbit IgG conjugated to Alexa Fluor 488. Counterstaining was done with the DNA stain, 4′,6-diamidine-2-phenylindole (DAPI), in order to visualize nuclei. After extensive washing, the slides were viewed and images captured.

### DETECTION OF PLASMA MEMBRANE PHOSPHOLIPID TRANSLOCATION

HCT-116 cells that had been grown and treated with the test agents in chamber slides were washed twice with phosphate-buffered saline. The washed cells were equilibrated for 10 min in binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4), before being incubated with fresh binding buffer containing annexin V-Alexa Fluor 488 conjugate (Molecular Probes, Eugene, OR) for 15 min in the dark. After washing with binding buffer, cells on the slides were examined.

### ASSESSMENT OF CHROMATIN CONDENSATION AND FRAGMENTATION

After washing and fixation of the cells, they were stained with DAPI and then examined by fluorescence microscopy for subsequent scoring, as detailed previously [Powolny et al., 2001].

## RESULTS

### CHANGES IN CELL MORPHOLOGY CAUSED BY DEOXYCHOLATE AND EFFECT OF ZINC

Exposing HCT-116 cells to deoxycholate produced morphological evidence of apoptosis, namely, membrane blebbing and apoptotic body formation (Fig. 1A). Such morphological changes was not produced by zinc (Fig. 1B), but instead, zinc inhibited the development of apoptotic morphology caused by deoxycholate (Fig. 1C). Cellular uptake of exogenously-added zinc has been

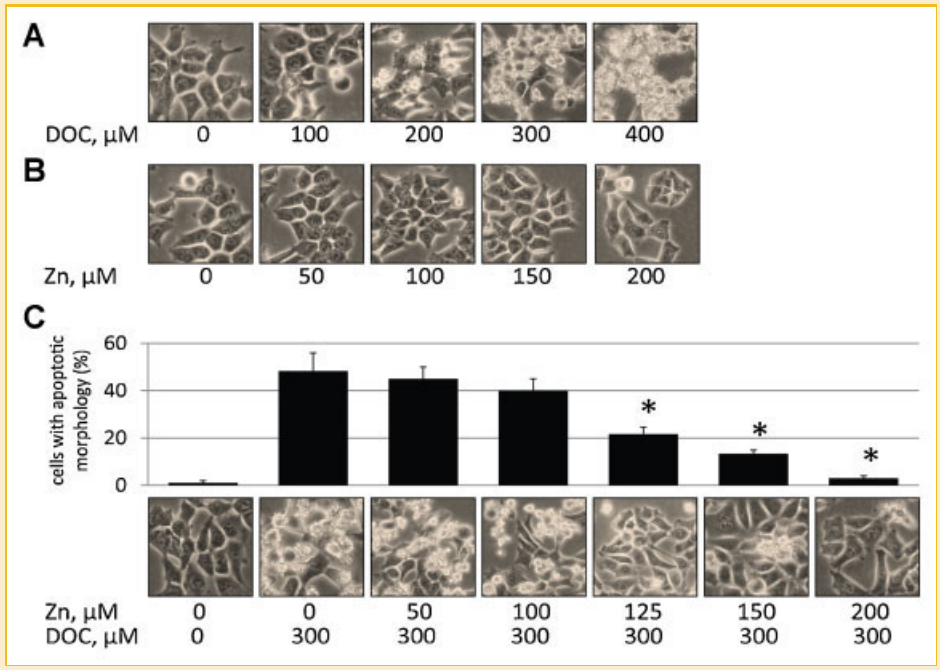


Fig. 1. Effect of deoxycholate and zinc on cell morphology. HCT-116 cells were incubated with deoxycholate (DOC) alone (**A**) and zinc sulfate (Zn) alone (**B**) for 3 h. Additionally, cells were pre-treated with Zn for 1 h, and then without changing the culture medium, exposed to DOC for 2 h (**C**). Cells were examined for the presence of apoptotic morphology (membrane blebbing and apoptotic body formation). The results are representative of three different experiments. $^{*}P < 0.05$ versus 0 μM Zn/300 μM DOC.

shown to occur in 0.25 h [Cortese et al., 2008] most likely via ZIP zinc-uptake proteins [Liuzzi and Cousins, 2004] that permit cells to acquire zinc apparently by non-saturable passive electrodiffusion rather than by active transport [Lin et al., 2010].

Zinc also had such an inhibitory effect against deoxycholate in HT-29 cells (Fig. 2A), in comparison to HCT-116 cells (Fig. 2B). Regarding the fate of the latter after 21 more hours, the HCT-116 cells had detached from the petri dishes and rounded up (Fig. 2C, upper images), but no noticeable membrane blebbing and/or apoptotic bodies were evident. However, in assessing cell viability, most of the cells were non-viable or dead based on the results seen after propidium iodide staining (Fig. 2C, lower images).

The remaining experiments were conducted using only HCT-116 cells in order to keep the study more manageable.

## STIMULATION OF ROS AND MITOCHONDRIAL SUPEROXIDE PRODUCTION BY DEOXYCHOLATE AND EFFECT OF ZINC

Initiation of apoptosis involves ROS [Rollet-Labelle et al., 1998; Dumont et al., 1999]. To determine if deoxycholate induces oxidative stress and whether such induction might be affected by zinc, ROS production in live cells was examined by fluorescence microscopy based on ROS-mediated oxidation of 5(and 6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate reagent to a product emitting green fluorescence. This reagent does not detect a specific ROS, but instead detects a broad range of oxidizing reactions associated with intracellular oxidant stress that may be downstream

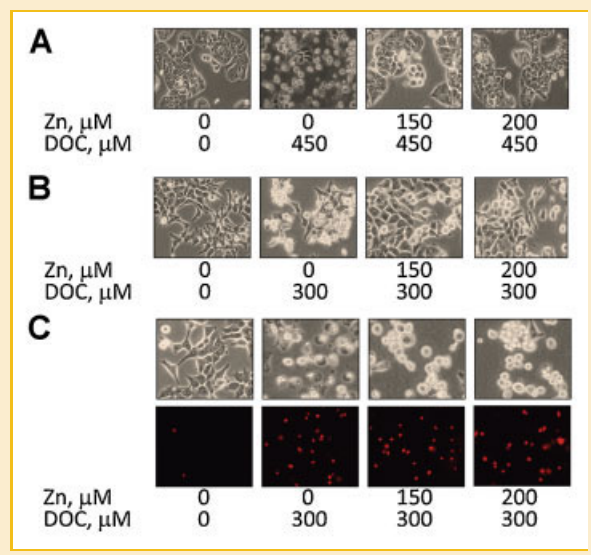


Fig. 2. Comparative inhibition by zinc of deoxycholate-induced apoptotic morphology in HT-29 and HCT-116 cells. HT-29 cells (**A**) and HCT-116 cells (**B**) were pre-treated with zinc sulfate (Zn) for 1 h, and then without changing the culture medium, exposed to deoxycholate (DOC) for 2 h. Additionally, after the Zn pre-treatment, HCT-116 cells were exposed to DOC for 24 h (**C**). These cells were then stained with propidium iodide (bottom row of images). The results are representative of three to four different experiments. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

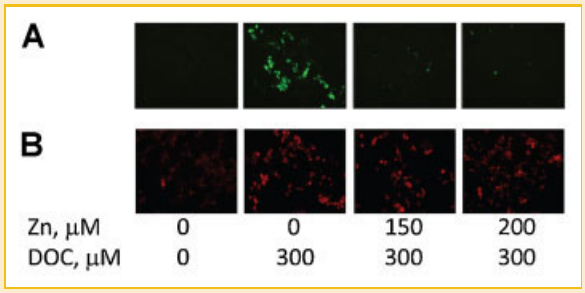


Fig. 3. Deoxycholate induces oxidative stress and impact of zinc. HCT-116 cells were pre-treated with zinc sulfate (Zn) for 1 h, and then without changing the culture medium, exposed to deoxycholate (DOC) for 1 h. The production of cellular ROS **(A)** and mitochondrial superoxide **(B)** was assessed by detecting the oxidation of inserted carboxydichlorodihydrofluorescein diacetate probe (green fluorescence) and dihydroethidium probe (red fluorescence), respectively. The images are representative of three different experiments. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

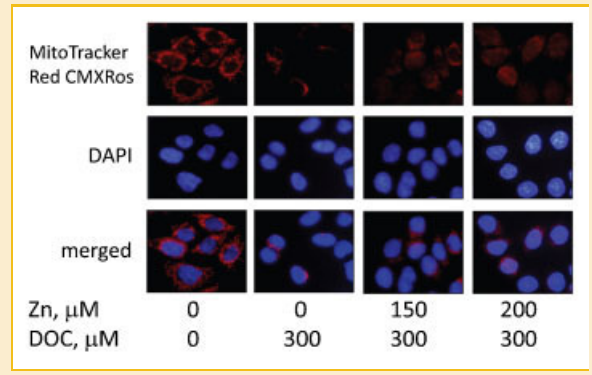


Fig. 4. Deoxycholate causes mitochondrial dysfunction that is not inhibited by zinc. HCT-116 cells were pre-treated with zinc sulfate (Zn) for 1 h, and then without changing the culture medium, exposed to deoxycholate (DOC) for 0.5 h. Mitochondrial function in the cells was visually assessed by detecting the mitochondrial oxidation of inserted MitoTracker Red CMXRos probe (red fluorescence). Counterstaining was with the nuclear stain, 4′,6-diamidine-2-phenylindole or DAPI (blue fluorescence). The results are representative of 3 different experiments. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

of superoxide production [Hempel et al., 1999]. As seen in Figure 3A, the production of ROS was quite evident in HCT-116 cells exposed to deoxycholate. This ROS production was absent in cells pre-treated with zinc and then exposed to deoxycholate.

To determine if deoxycholate induces compartmentalized ROS production in mitochondria of HCT-116 cells, oxidation of the specific MitoSOX fluorogenic probe (dihydroethidium) by superoxide to a product emitting red fluorescence was evaluated (Fig. 3B). As can be seen in comparing the images, deoxycholate stimulated mitochondrial superoxide production, but zinc did not inhibit this effect.

## SUPPRESSION OF MITOCHONDRIAL FUNCTION BY DEOXYCHOLATE AND EFFECT OF ZINC

It is well known that mitochondrial dysfunction can occur during the early stages of apoptosis. To assess by fluorescence detection whether deoxycholate disrupts mitochondrial function in HCT-116 cells, a mitochondria-specific probe (MitoTracker Red CMXRos) was used for the assessment. When inserted in live cells, the probe stains active mitochondria. Less red fluorescence indicates respiratory dysfunction, an early event in apoptosis. As shown in Figure 4, the red fluorescence intensity emanating from mitochondria of cells exposed to deoxycholate was weak in comparison to control cells. Hence, mitochondrial function was disrupted in cells exposed to deoxycholate. As further shown, this effect of deoxycholate was not prevented by pre-treatment of the cells with zinc.

## STIMULATION OF CYTOCHROME C RELEASE BY DEOXYCHOLATE AND EFFECT OF ZINC

A common characteristic of apoptosis is the release of cytochrome c into cytosol. Immunocytofluorescence microscopy of HCT-116 cells (Fig. 5A) revealed that cytochrome c (green fluorescence) was localized in mitochondria which appear visually to surround the nuclei (blue fluorescence). However, in cells exposed to deoxycholate, the green fluorescence was diffuse rather than being localized in mitochondria, indicating that noticeable amounts of cytochrome c was released into cytosol. As further shown, this effect of deoxycholate was not prevented by pre-treatment of the cells with zinc. These immunocytofluorescence microscopic observations were essentially confirmed by Western-immunoblotting analysis of isolated cytosolic fractions (Fig. 5B).

## STIMULATION OF CASPASE-8 AND CASPASE-3 CLEAVAGES BY DEOXYCHOLATE AND EFFECT OF ZINC

Apoptosis is also typically characterized by the activation of initiator and effector caspases via protelolytic cleavage. To determine if initiator caspase-8 and effector caspase-3 underwent cleavage and thus activation, a cleaved (at Asp391) caspase-8 antibody that recognizes the activated p18 fragment and a cleaved (at Asp175) caspase-3 antibody that recognizes the activated p17/p19 fragment were used for detection. As seen in Figure 6A, Western-immunoblotting analysis revealed that deoxycholate caused the appearance of both cleaved caspase-8 and cleaved caspase-3 in HCT-116 cells, but this effect was not produced in zinc-pretreated cells exposed to deoxycholate. These results were confirmed by immunocytofluorescence analysis (Fig. 6B). After exposure to deoxycholate, cleaved caspase-8 and cleaved caspase-3 were present in many of the examined cells as indicated by the presence of green fluorescence. Less cleaved caspase-8 and cleaved caspase-3 were present in zinc-pretreated cells exposed to deoxycholate, indicating that zinc inhibited activation of caspase-8 and caspase-3 caused by deoxycholate.

## CHANGES TO CELL SURFACE PHOSPHOLIPID AND CHROMATIN CAUSED BY DEOXYCHOLATE AND EFFECT OF ZINC

To determine if two later stages of apoptosis occurred in HCT-116 cells exposed to deoxycholate, the cells were examined for changes in plasma membrane phospholipid localization (detectable

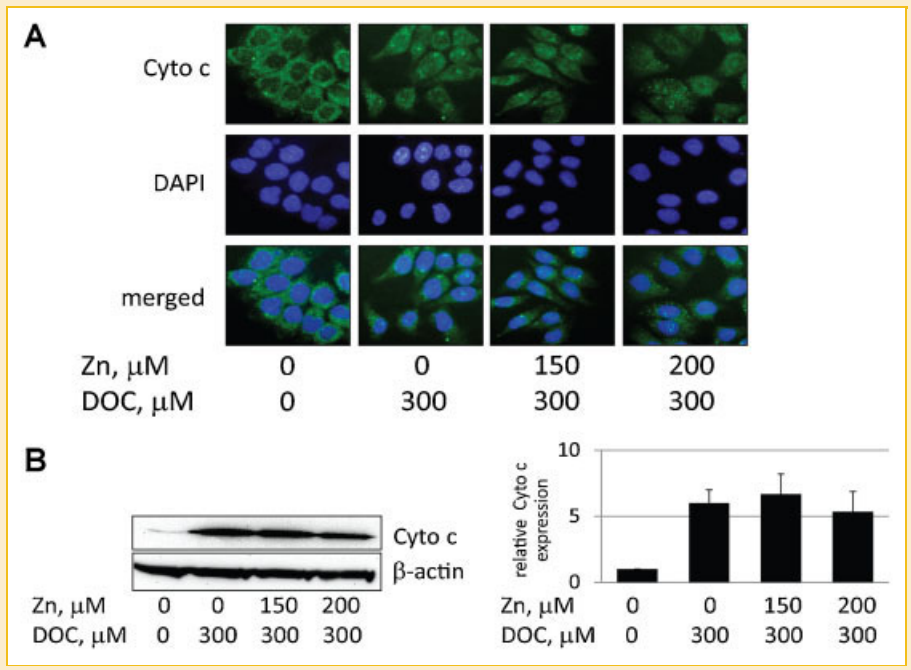


Fig. 5. Deoxycholate causes cytochrome c release that is not inhibited by zinc. HCT-116 cells were pre-treated with zinc sulfate (Zn) for 1 h, and then without changing the culture medium, exposed to deoxycholate (DOC) for 1 h. Cytochrome c release was determined by localized immunocytofluorescence detection of cytochrome c (green fluorescence) and visualization of nuclei with DAPI (blue fluorescence) (**A**) and also by Western-immunoblotting analysis of isolated cytosolic fractions (**B**). The results are representative of three different experiments. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

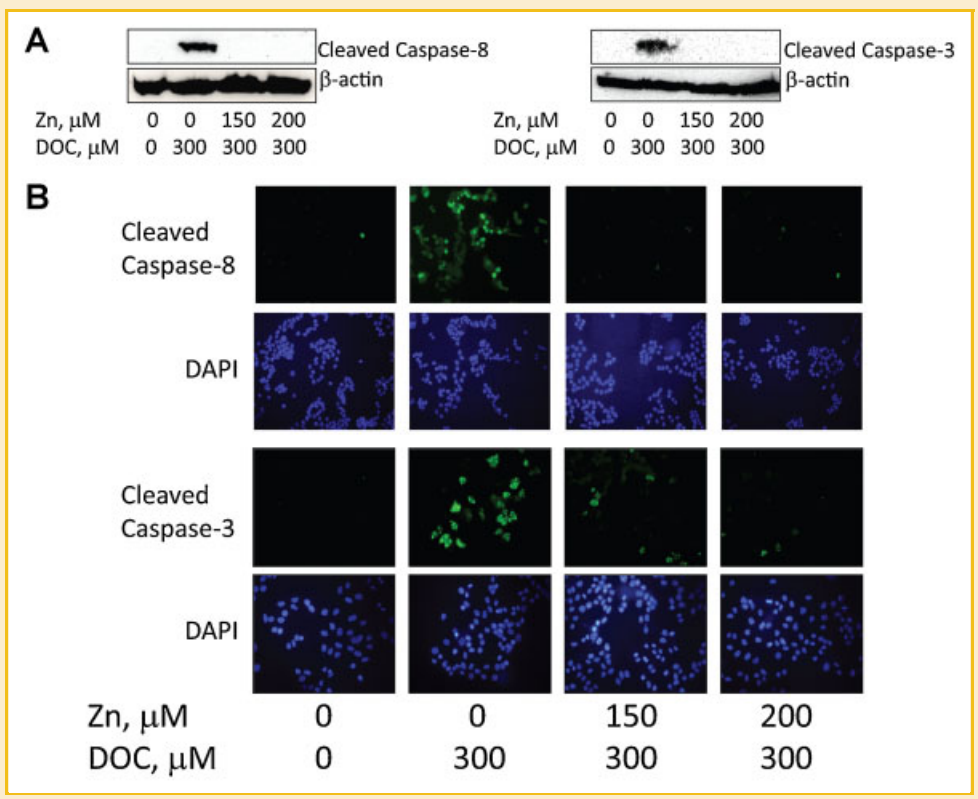


Fig. 6. Cleavage forms of caspase-8 and caspase-3 are produced upon exposure to deoxycholate, but zinc inhibits this effect of deoxycholate. HCT-116 cells were pre-treated with zinc sulfate (Zn) for 1 h, and then without changing the culture medium, exposed to deoxycholate (DOC) for 1 h. The caspase cleavage forms were determined by Western-immunoblotting analysis of whole cell lysates (**A**) and immunocytofluorescence detection (**B**), using antibodies that recognize the activated fragments of caspase-8 and caspase-3. The results are representative of three to four different experiments. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

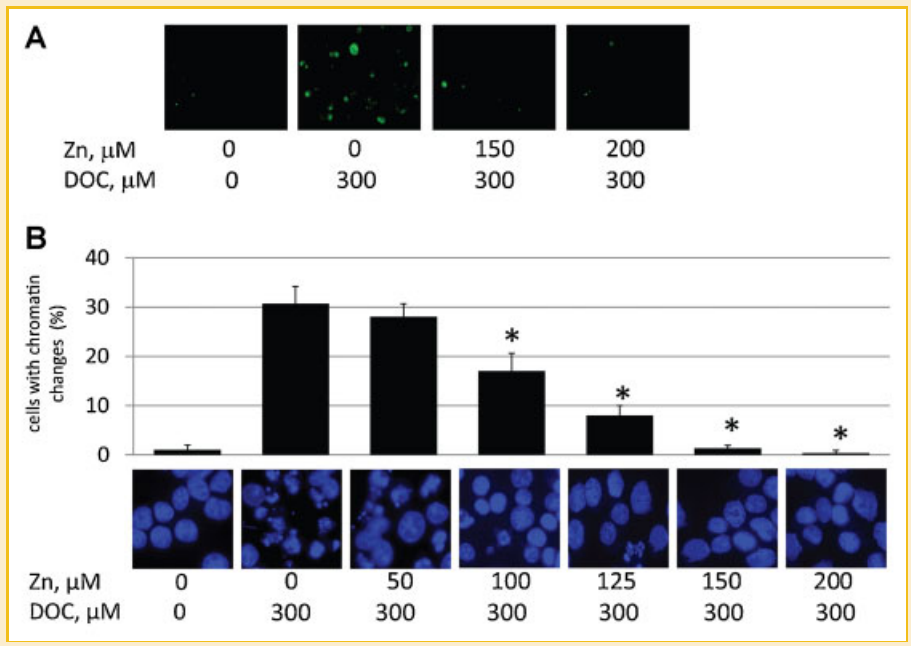


Fig. 7. Deoxycholate increases annexin-V cell surface binding and changes chromatin structure, but zinc inhibits these effects of deoxycholate. HCT-116 cells were pre-treated with zinc sulfate (Zn) for 1 h, and then without changing the culture medium, exposed to deoxycholate (DOC) for 1 h. The cells were stained with annexin V-Alexa Fluor 488 conjugate to visually detect plasma membrane phospholipid translocation (A). Additionally, HCT-116 cells were pre-treated with Zn for 1 h, and then without changing the culture medium, exposed to DOC for 3 h (B). The cells were stained with DAPI to visually assess chromatin condensation and fragmentation. The results are representative of three different experiments. $^{*}P < 0.05$ versus 0 μM Zn/300 μM DOC. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

with annexin-V staining) and nuclear morphology (detectable with DAPI staining). Compared to control cells, greater cell surface annexin-V binding (Fig. 7A) and also chromatin condensation and fragmentation (Fig. 7B) were observed in cells exposed to deoxycholate. These apoptotic features were less pronounced or absent in cells pre-treated with zinc and then exposed to deoxycholate.

## DISCUSSION

Previous studies have shown that ROS generation [Payne et al., 2007; Longpre and Loo, 2008] and apoptosis [Powolny et al., 2001] are induced in HCT-116 cells upon incubation with deoxycholate. Thus, these past findings are largely corroborated by the results of the present study but are broadened from a different perspective by the major new finding that the above effects of deoxycholate, particularly the induction of apoptosis, can be inhibited with zinc. Inhibition of cell death by zinc has also been shown in U937 monocytes exposed to ricin [Tamura et al., 2002], HepG2 hepatocytes exposed to ethanol [Szuster-Ciesielska et al., 2008], and Caco-2 colonocytes exposed to an enzymatic hydrogen peroxide-generating system [Kilari et al., 2010]. Thus, the present study adds to this existing pool of knowledge.

It is reasonable to think that zinc inhibited the effects of deoxycholate in HCT-116 cells by working indirectly to scavenge ROS, since zinc is regarded as an antioxidant [Powell, 2000]. In support of this possibility, there are reports showing that antioxidants inhibit the effects of bile acids. α-tocopherol and β-carotene inhibited oxidative DNA damage caused by deoxycholate [Rosignoli et al., 2008], and these two antioxidants attenuated ROS generation and protected against cell death caused by glycochenodeoxycholate [Gumpricht et al., 2004]. However, not all antioxidants or ROS scavengers are protective. *N*-acetylcysteine, superoxide dismutase, and catalase were ineffective in inhibiting glycochenodeoxycholate-induced cell death [Woudenberg-Vrenken et al., 2010]. One possible mechanism of zinc in inhibiting the ROS production and apoptosis caused by deoxycholate is that it induced a cytoprotective protein. Zinc induces metallothionein [Pérez and Cederbaum, 2003; John et al., 2011], which can scavenge ROS [Kumari et al., 1998] and suppress apoptosis [Wang et al., 2001; Shimoda et al., 2003]. Zinc is able to increase metallothionein protein expression in HCT-116 cells [John et al., 2011]. However, the cells were incubated with 250 μM zinc for at least 24–72 h before levels of metallothionein increased. Therefore, considering these much longer incubation times compared to that in the present study, it is unlikely that zinc at relatively high concentrations worked via metallothionein to inhibit the apoptosis in HCT-116 cells caused by deoxycholate. It is unknown what the zinc concentrations are in the colonic lumen or colon epithelial cells. However, it has been stated that the normal range of plasma zinc concentration is 15–25 μmol/L, and that in the gut lumen could be much higher [Reeves et al., 1998].

A more plausible explanation for how zinc inhibits deoxycholate-induced apoptosis in HCT-116 cells would need to consider certain apoptotic events, as portrayed in the "intrinsic" and "extrinsic" cell death pathways [Brenner and Mak, 2009]. Apparently, deoxycholate

initiates a few apoptotic events common to both pathways. Regarding the intrinsic pathway, it was observed in the present study that deoxycholate stimulated mitochondrial superoxide production and caused mitochondrial dysfunction, and cytochrome c release. Also, deoxycholate is known to decrease the mitochondrial membrane potential [Payne et al., 2007; Longpre and Loo, 2008] and cause formation of cleaved caspase-9 [Yui et al., 2005; Lawson et al., 2011] that is known to be capable of activating caspase-3. Regarding the extrinsic pathway, cleaved caspase-8 was detected in HCT-116 cells exposed to deoxcyholate in the present study, which is consistent with another study [Yui et al., 2005]. Caspase-8 is another activator of caspase-3 but can also cleave BH3-interacting domain death agonist (BID) so that truncated BID translocates to mitochondria to cause cytochrome c release. Thus, it is possible that caspase-8 and caspase-9 converged on caspase-3, whose cleavage form was clearly detected, in mediating HCT-116 cell death caused by deoxycholate. In any event, enzymatic activity of caspase-3 increased 8- and 18-fold in HCT-116 cells exposed to 250 and 500 μM deoxycholate, respectively, for 0.75–1 h [Glinghammar et al., 2002].

In light of the above, it is conceivable that zinc directly interferes with deoxycholate-induced HCT-116 cell death at the level of caspases. The apoptosis induced by deoxycholate in HCT-116 cells is dependent on caspases since a pan-caspase inhibitor blocked cell death [Yui et al., 2005]. However, caspase-3 is perhaps the most pivotal because of its effector or executioner role in dismantling cellular components. Similar to the present finding that deoxycholate causes caspase-3 activation that can be inhibited with zinc, it has been found that pre-treating HCT-116 cells with zinc prevented caspase-3 activation caused by the DNA-alkylating agent, methylmethane sulfonate [Jaiswal and Narayan, 2004].

In inhibiting apoptosis caused by factors apart from deoxycholate, zinc is thought to inhibit transformation of the inactivated (uncleaved) form of caspase-3 into its activated (cleaved) form [Perry et al., 1997; Aiuchi et al., 1998]. Hence, in inhibiting deoxycholate-induced HCT-116 cell death, zinc may have hampered cleavage or activation of initiator caspases, such as was found with caspase-8 although caspase-9 cannot be ruled out. Zinc did not block the stimulation of mitochondrial superoxide production, mitochondrial dysfunction, and cytochrome c release. But, zinc did block production of the cleavage forms of caspase-3 and caspase-8, as well as the ensuing plasma membrane phospholipid translocation, plasma membrane blebbing, apoptotic body formation, and chromatin changes. Foremost, the blockage of cleaved caspase-3 formation would seem most critical in view of the multiple actions of the activated enzyme. Because it is involved in regulating phospholipid scramblase activity, caspase-3 is required for plasma membrane phospholipid translocation [Frasch et al., 2000]. It is known that caspases such as caspase-3 are involved in dismembering cytoskeletal proteins [Caulin et al., 1997], which consequently permits plasma membrane blebbing and apoptotic body formation to take place. Caspase-3 also plays the major role in allowing chromatin condensation and fragmentation to happen during apoptosis [Slee et al., 2001]. Lastly, caspase-3 disrupts the function of complexes I and II of the mitochondrial electron transport chain, which permits sustained generation of ROS during apoptosis [Ricci et al., 2003]. Hence, blockage of caspase-3 cleavage by zinc would not permit these cell death events to occur.

The fundamental basis for the notion that zinc inhibits apoptosis by affecting caspases has to do largely with modification of a certain structural feature of caspases. The thiol group of cysteinyl residues in proteins has an affinity for zinc ion, as exemplified by the large capacity of cysteine-rich metallothionein to bind zinc ions in protecting against zinc toxicity [Palmiter, 2004]. Theoretically, this type of chemical bonding can be extended to caspases that are known to be cysteine proteases [Thornberry, 1997] in which cysteinyl residues are found in their active sites. These sites could bind zinc, which would inhibit caspase catalysis. To the contrary, however, zinc apparently does not inhibit the activity of the activated caspase-3 enzyme, but instead blocks the process whereby pro-caspase-3 becomes activated [Truong-Tran et al., 2000]. In regards to upstream caspases, caspase-9 has been suggested as a potential target of zinc in explaining the ability of zinc to inhibit apoptosis induced by other factors outside of deoxycholate [Chai et al., 1999]. In HepG2 cells exposed to ethanol that caused apoptosis, caspase-8 was activated but zinc pre-treatment inhibited such activation [Szuster-Ciesielska et al., 2008], which is consistent with the corresponding data in the present study. In conclusion, the results suggest that zinc can inhibit deoxycholate-induced apoptotic cell death mediated by caspases.

## REFERENCES


Aiuchi T, Mihara S, Nakaya M, Masuda Y, Nakajo S, Nakaya K. 1998. Zinc ions prevent processing of caspase-3 during apoptosis induced by geranylgeraniol in HL-60cells. J Biochem 124:300–303.

Altman SA, Randers L, Rao G. 1993. Comparison of trypan blue dye exclusion and fluorometric assays for mammalian cell viability determinations. Biotechnol Prog 9:671–674.

Bernstein H, Bernstein C, Payne CM, Dvorakova K, Garewal H. 2005. Bile acids as carcinogens in human gastrointestinal cancers. Mutat Res 589: 47–65.

Booth LA, Gilmore IT, Bilton RF. 1997. Secondary bile acid induced DNA damage in HT29cells: Are free radicals involved? Free Radic Res 26:135–144.

Bossy-Wetzel E, Green DR. 2000. Assays for cytochrome c release from mitochondria during apoptosis. Methods Enzymol 322:235–242.

Brenner D, Mak TW. 2009. Mitochondrial cell death effectors. Curr Opin Cell Biol 21:871–877.

Caulin C, Salvesen GS, Oshima RG. 1997. Caspase cleavage of keratin 18 and reorganization of intermediate filaments during epithelial cell apoptosis. J Cell Biol 138:1379–1394.

Chai F, Truong-Tran AQ, Ho LH, Zalewski PD. 1999. Regulation of caspase activation and apoptosis by cellular zinc fluxes and zinc deprivation: A review. Immunol Cell Biol 77:272–278.

Coleman JE. 1992. Zinc proteins: Enzymes, storage proteins, transcription factors, and replication proteins. Annu Rev Biochem 61:897–946.

Cortese MM, Suschek CV, Wetzel W, Kröncke KD, Kolb-Bachofen V. 2008. Zinc protects endothelial cells from hydrogen peroxide via Nrf2-dependent stimulation of glutathione biosynthesis. Free Radic Biol Med 44:2002–2012.

Dumont A, Hehner SP, Hofmann TG, Ueffing M, Dröge W, Schmitz ML. 1999. Hydrogen peroxide-induced apoptosis is CD95-independent, requires the release of mitochondria-derived reactive oxygen species and the activation of NF-kappaB. Oncogene 18:747–757.

Frasch SC, Henson PM, Kailey JM, Richter DA, Janes MS, Fadok VA, Bratton DL. 2000. Regulation of phospholipid scramblase activity during apoptosis and cell activation by protein kinase Cδ. J Biol Chem 275:23065–23073.

Glinghammar B, Inoue H, Rafter JJ. 2002. Deoxycholic acid causes DNA damage in colonic cells with subsequent induction of caspases, COX-2 promoter activity and the transcription factors NF-kB and AP-1. Carcinogenesis 23:839–845.

Gumpricht E, Dahl R, Devereaux MW, Sokol RJ. 2004. Beta-carotene prevents bile acid-induced cytotoxicity in the rat hepatocyte: Evidence for an antioxidant and anti-apoptotic role of beta-carotene in vitro. Pediatr Res 55:814–821.

Halliwell B, Gutteridge JM. 1990. Role of free radicals and catalytic metal ions in human disease: An overview. Methods Enzymol 186: 1–85.

Hempel SL, Buettner GR, O'Malley YQ, Wessels DA, Flaherty DM. 1999. Dihydrofluorescein diacetate is superior for detecting intracellular oxidants: Comparison with 2′,7′-dichlorodihydrofluorescein diacetate, 5(and 6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, and dihydrorhodamine 123. Free Radic Biol Med 27:146–159.

Jaiswal AS, Narayan S. 2004. Zinc stabilizes adenomatous polyposis coli (APC) protein levels and induces cell cycle arrest in colon cancer cells. J Cell Biochem 93:345–357.

John S, Briatka T, Rudolf E. 2011. Diverse sensitivity of cells representing various stages of colon carcinogenesis to increased extracellular zinc: Implications for zinc chemoprevention. Oncol Rep 25:769–780.

Kilari S, Pullakhandam R, Nair KM. 2010. Zinc inhibits oxidative stress-induced iron signaling and apoptosis in Caco-2cells. Free Radic Biol Med 48:961–968.

Kumari MV, Hiramatsu M, Ebadi M. 1998. Free radical scavenging actions of metallothionein isoforms I and II. Free Radic Res 29:93–101.

Lawson TE, Redlak MJ, Yager DR. 2011. Preconditioning with cobalt protoporphyrin protects human gastric mucosal cells from deoxycholate-induced apoptosis. Wound Repair Regen 19:241–249.

Lin W, Chai J, Love J, Fu D. 2010. Selective electrodiffusion of zinc ions in a Zrt-, Irt-like protein, ZIPB J Biol Chem 285:39013–39020.

Liuzzi JP, Cousins RJ. 2004. Mammalian zinc transporters. Annu Rev Nutr 24:151–172.

Longpre JM, Loo G. 2008. Protection of human colon epithelial cells against deoxycholate by rottlerin. Apoptosis 13:1162–1171.

Palmiter RD. 2004. Protection against zinc toxicity by metallothionein and zinc transporter 1. Proc Natl Acad Sci USA 101:4918–4923.

Payne CM, Weber C, Crowley-Skillicorn C, Dvorak K, Bernstein H, Bernstein C, Holubec H, Dvorakova B, Garewal H. 2007. Deoxycholate induces mitochondrial oxidative stress and activates NF-kappaB through multiple mechanisms in HCT-116 colon epithelial cells. Carcinogenesis 28:215–222.

Pérez MJ, Cederbaum AI. 2003. Metallothionein 2A induction by zinc protects HEPG2cells against CYP2E1-dependent toxicity. Free Radic Biol Med 34:443–455.

Perry DK, Smyth MJ, Stennicke HR, Salvesen GS, Duriez P, Poirier GG, Hannun YA. 1997. Zinc is a potent inhibitor of the apoptotic protease, caspase-3. A novel target for zinc in the inhibition of apoptosis. J Biol Chem 272:18530–18533.

Poot M, Zhang YZ, Krämer JA, Wells KS, Jones LJ, Hanzel DK, Lugade AG, Singer VL, Haugland RP. 1996. Analysis of mitochondrial morphology and function with novel fixable fluorescent stains. J Histochem Cytochem 44:1363–1372.

Powell SR. 2000. The antioxidant properties of zinc. J Nutr 130:1447S–1454S.

Powolny A, Xu J, Loo G. 2001. Deoxycholate induces DNA damage and apoptosis in human colon epithelial cells expressing either mutant or wild-type p53. Int J Biochem Cell Biol 33:193–203.

Reeves PG, Briske-Anderson M, Johnson L. 1998. Physiologic concentrations of zinc affect the kinetics of copper uptake and transport in the human intestinal cell model, Caco-2. J Nutr 128:1794–1801.

Ricci JE, Gottlieb RA, Green DR. 2003. Caspase-mediated loss of mitochondrial function and generation of reactive oxygen species during apoptosis. J Cell Biol 160:65–75.

Rollet-Labelle E, Grange MJ, Elbim C, Marquetty C, Gougerot-Pocidalo MA, Pasquier C. 1998. Hydroxyl radical as a potential intracellular mediator of polymorphonuclear neutrophil apoptosis. Free Radic Biol Med 24:563–572.

Rosignoli P, Fabiani R, De Bartolomeo A, Fuccelli R, Pelli MA, Morozzi G. 2008. Genotoxic effect of bile acids on human normal and tumour colon cells and protection by dietary antioxidants and butyrate. Eur J Nutr 47:301–309.

Shimoda R, Achanzar WE, Qu W, Nagamine T, Takagi H, Mori M, Waalkes MP. 2003. Metallothionein is a potential negative regulator of apoptosis. Toxicol Sci 73:294–300.

Slee EA, Adrain C, Martin SJ. 2001. Executioner caspase-3, -6, and -7 perform distinct, non-redundant roles during the demolition phase of apoptosis. J Biol Chem 276:7320–7326.

Szuster-Ciesielska A, Plewka K, Daniluk J, Kandefer-Szerszeń M. 2008. Zinc inhibits ethanol-induced HepG2 cell apoptosis. Toxicol Appl Pharmacol 229:1–9.

Tamura T, Sadakata N, Oda T, Muramatsu T. 2002. Role of zinc ions in ricin-induced apoptosis in U937cells. Toxicol Lett 132:141–151.

Thornberry NA. 1997. The caspase family of cysteine proteases. Br Med Bull 53:478–490.

Truong-Tran AQ, Ho LH, Chai F, Zalewski PD. 2000. Cellular zinc fluxes and the regulation of apoptosis/gene-directed cell death. J Nutr 130:1459S–1466S.

Wang GW, Klein JB, Kang YJ. 2001. Metallothionein inhibits doxorubicin-induced mitochondrial cytochrome c release and caspase-3 activation in cardiomyocytes. J Pharmacol Exp Ther 298:461–468.

Woudenberg-Vrenken TE, Buist-Homan M, Conde de la Rosa L, Faber KN, Moshage FH. 2010. Anti-oxidants do not prevent bile acid-induced cell death in rat hepatocytes. Liver Int 30:1511–1521.

Yui S, Saeki T, Kanamoto R, Iwami K. 2005. Characteristics of apoptosis in HCT116 colon cancer cells induced by deoxycholic acid. J Biochem 138:151–157.