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Increased level of exogenous zinc induces cytotoxicity and up-regulates the expression of the ZnT-1 zinc transporter gene in pancreatic cancer cells☆

Arathi K. Jayaraman, Sundararajan Jayaraman*

Department of Surgery, College of Medicine, University of Illinois at Chicago, Chicago, IL 60612 USA

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Abstract

A balance between zinc uptake by ZIP (SLC39) and efflux of zinc from the cytoplasm into subcellular organelles and out of the cell by ZnT (SLC30) transporters is crucial for zinc homeostasis. It is not clear whether normal and cancerous pancreatic cells respond differently to increased extracellular zinc concentrations. Use of flow cytometry-based methods revealed that treatment with as little as 0.01 mM zinc induced significant cytotoxicity in two human ductal adenocarcinoma cell lines. In contrast, normal human pancreatic islet cells tolerated as high as 0.5 mM zinc. Insulinoma cell lines of mouse and rat origin also succumbed to high concentrations of zinc. Exposure to elevated zinc concentrations enhanced the numbers of carcinoma but not primary islet cells staining with the cell-permeable zinc-specific fluorescent dye, FluoZin-3, indicating increased zinc influx in transformed cells. Mitochondrial membrane depolarization, superoxide generation, decreased antioxidant thiols, intracellular acidosis and activation of intracellular caspases characterized zinc-induced carcinoma cell death. Only the antioxidant glutathione but not inhibitors of enzymes implicated in apoptosis or necrosis prevented zinc-induced cytotoxicity in insulinoma cells. Immunoblotting revealed that zinc treatment increased the ubiquitination of proteins in cancer cells. Importantly, zinc treatment up-regulated the expression of ZnT-1 gene in a rat insulinoma cell line and in two human ductal adenocarcinoma cell lines. These results indicate that the exposure of pancreatic cancer cells to elevated extracellular zinc concentrations can lead to cytotoxic cell death characterized by increased protein ubiquitination and up-regulation of the zinc transporter ZnT-1 gene expression.
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1. Introduction

Pancreatic cancer is the fifth common most cause of cancer-related deaths in western countries and accounts for about 3% of all cancers [1]. In some cases, pancreatic cancer is also associated with pancreatic insufficiency-diabetes [2]. Only 15% to 20% of pancreatic cancers can be surgically removed when diagnosed at advanced or metastatic stage, and chemotherapeutic agents gemcitabine and 5-fluorouracil provide benefits only to some patients [3]. A number of clinical trials including the use of signal transduction inhibitors and ablation of the hormonal influence produced marginal effects when combined with the standard cytostatic drugs [1,3]. However, some of these combinations displayed severe and sometimes fatal toxicity. Thus, pancreatic carcinomas still remain a great challenge for the oncologists, and the continuous search for better treatment modalities is highly warranted.

Zinc, normally found in cells, is important for the regulation of gene expression, protein synthesis and intracellular protein trafficking [4]. In insulin-producing β cells, large amounts of zinc are required for insulin synthesis and storage [5], and zinc is dissociated from insulin after exocytosis [6]. High concentrations of zinc appear to modulate the function of neighboring glucagon-producing α cells [7]. Although exposure to extracellular zinc has been shown to induce necrosis in the mouse insulinoma cell line, Min6 [8,9], the underlying mechanisms have not been fully elucidated. Zinc homeostasis is achieved by two groups of proteins with opposing functions, solute-linked carrier genes, SLC39 (ZIP- Zrt- and Irt-like proteins) and SLC30 (ZnT-Zinc transporters) [10–12]. ZIP family consists of 14 members that allow the influx of extracellular zinc and transport of zinc from the lumen of subcellular organelles into the cytoplasm. In insulinoma cell lines, zinc uptake is predominantly mediated by the L-type Ca^{2+} channels [13,14]. The cation diffusion facilitators have been termed as ZnTs and consist of 10 members implicated in reducing cytosolic zinc concentrations, either through efflux across the plasma membrane or through sequestration into intracellular compartments such as Golgi and endoplasmic reticulum [10–12]. ZnT-1 is ubiquitously expressed on the plasma membrane of a variety of cells including rat pancreatic islets [10–12,15]. In the mouse, ZnT-1 was reported to be present in pancreatic α but not β cells [16]. ZnT-2 has a vesicular localization in pancreatic acinar cells but not in pancreatic β cells [16]. Although ZnT-4 is abundantly expressed in the mammary gland and important for zinc transport into milk, it is also expressed in the brain, small intestine and pancreatic islets [10,15]. While ZnT-5 is present in a

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* Corresponding author. Tel.: +1 312 355 5133; fax: +1 312 355 1497.
E-mail address: anue2468@uic.edu (S. Jayaraman).

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number of tissues, it is abundantly expressed in human pancreatic β cells but not in glucagon-producing α cells or the exocrine acinar cells [17]. ZnT-8 was first described to be located in insulin secretory granules of pancreatic β cells [18]. ZnT-8 was also suggested to be an autoantigen in type 1 diabetes [19]. However, recent studies indicated the presence of ZnT-8 in α cells and other secretory cell types [20], as well as in peripheral blood lymphocytes [21]. The expression of ZnT-3, ZnT-6 and ZnT-7 has not been reported in the pancreas [10–12].

In contrast to the distribution of ZnTs in normal pancreatic tissues, little is known about their expression in pancreatic cancers. Importantly, it is not known whether pancreatic cancer cells display aberrant zinc homeostasis unlike normal pancreatic cells, such as β cells. A previous report indicated that clinical pancreatic adenocarcinoma specimens overexpressed the zinc influx transporter, ZIP-4, suggesting that this may lead to enhanced zinc uptake in cancer cells and possibly contribute to carcinogenesis [22]. However, the consequences of altered zinc homeostasis in pancreatic cancer cells with reference to the modulation of ZnTs that facilitate the efflux of zinc out of the cell and sequestration into subcellular organelles have not been reported. Our study reveals previously undocumented characteristics of pancreatic cancer cells, up-regulation of selected ZnT genes and induction of cell death by cytotoxic mechanisms in response to a challenge with zinc. These results suggest that the ZnT transporter(s) may serve as a molecular target for pancreatic cancer therapy.

2. Methods and materials

2.1. Cells

NIT-1 cells (ATCC) were grown in F-12K medium, while RINm5F (provided by Louis Philipson, University of Chicago), CD18 and S2013 (obtained from David Benstrem, Northwestern University) and L929 cells were cultured in RPMI medium (Invitrogen) substituted with 10% fetal bovine serum [23]. Islets from cadaveric human pancreata (n=8) were isolated at the University of Pennsylvania, Washington University in St. Louis and the City of Hope, Duarte. Isolated islets were made available for this study as a part of the ICR Basic Science Islet Distribution Program. Islets were cultured at 37°C in CMRL 1066 medium (Mediatech) supplemented with antibiotics, nicotinamide, insulin and 0.25% human serum albumin as described earlier [24].

2.2. Cell treatment

Adherent cell lines were dislodged from the culture flasks by treating with 0.05% trypsin–EDTA for 5 min at 37°C [24], and 1×10^6 cells/ml were resuspended in complete medium and treated with zinc chloride (Sigma) that was dissolved in plain RPMI medium to minimize sequestration of zinc by serum proteins. Islets were treated with zinc chloride overnight and then dispersed by trypsin treatment and vice versa in some experiments. L-Glutathione (GSH, Sigma) and L-glutathione monoethyl ester, GSH-MEE (Calbiochem), were dissolved, respectively, in plain RPMI medium and dimethyl sulfoxide (DMSO). The following inhibitors were dissolved at 10 mM concentrations in DMSO: Q-VD-OPH (R&D Systems), necrostatin-1, SB203580, SP600125 and U0126 (all from Biomol International). NIT-1 cells were incubated with 10 μM final concentrations of inhibitors for 45 min prior to treatment with zinc chloride. *N*-Acetyl cysteine (NAC, Sigma) was dissolved in plain RPMI medium and used at concentrations indicated in the text. In some experiments, cells were incubated with GSH or GSH-MEE for 45 min at 37°C, centrifuged and resuspended in complete media and incubated further with or without zinc chloride overnight.

2.3. Flow cytometry

Viability was determined by incubating with 50 nM tetramethylrhodamine ethyl ester (TMRE, Invitrogen) and 100 μM monochlorobimane (mBcl, Invitrogen) at 37°C for 30 min as described [23,24]. Islet cells were also incubated with 1 μM FluoZin-3-AM (Invitrogen) and analyzed by flow cytometry [24]. Activation of caspases was determined by staining with 10 μM FITC-VAD-FMK (Promega, Madison, WI) and propidium iodide (PI) as described before [25]. Cells were also incubated with 10 μM MitoSox Red (Invitrogen) for 30 min at 37°C or with 5 μM SNARF-1 (carboxy-seminaphthorhodafluor-1, Invitrogen) for 20 min at 37°C [26]. Cells were analyzed on a BD LSR (Becton-Dickinson) or a CyAn ADP (DakoCytomation) flow cytometer, and 10,000 events were collected and analyzed using FlowJo 6.3.4 software (Treestar).

2.4. Western blotting

Human pancreatic cancer cells were cultured in media or treated with 0.25 mM zinc chloride for 4 h at 37°C. Total cell lysates were prepared and 10 μg of proteins were separated on a 12% sodium dodecyl sulfate (SDS) polyacrylamide gel, transferred to a polyvinylidene fluoride (PVDF) membrane and probed with rabbit antisera against Bcl-XL, total ERK-1/2, caspase-9 poly ADP ribose polymerase (PARP) (all from Santa Cruz) or phosphorylated ERK-1/2 (Cell Signaling). Monoclonal antibodies against C-Abl (Oncogene Research Products), XIAP (MBL), Cbl-b and ubiquitin (Santa Cruz) as well as goat antiserum against β actin (Santa Cruz) were used for blotting. Immunoblots were developed using the ECL Plus system (GE Health Care).

2.5. Real-time real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated using TRIzol (Invitrogen) and treated with DNase either using SV Total RNA Isolation System (Promega) or TURBO DNA-free kit (Applied Biosystems). Total RNA isolated from rat pancreas was obtained from BioChain Institute (Hayward, CA). DNase-treated RNA (5–10 μg) was reverse transcribed into cDNA using High-Capacity cDNA Reverse Transcription kit (Applied Biosytems). Real-time quantitative RT-PCR was performed on an ABA Prism 7500 Real-Time polymerase chain reaction (PCR) system (Applied Biosystems) using 1 μl of cDNA equivalent to 250 to 500 ng of RNA using 2× SYBR Premix Ex Taq (Perfect Real Time) reagent (Takara-Clontech). All rat *znt* and human *GAPDH* primers were designed using PRIMER EXPRESS version 2.0 software (Applied Biosciences). The primers for human *ZNT1*, *ZNT5* and *ZNT7* were synthesized as described earlier [27]. All primers were synthesized at Integrated DNA Technologies (Coralville, IA) except that the rat *znt4* primers were synthesized at Invitrogen and used at 0.4-μM concentrations. Primer sequences are shown in Table 1. Relative quantitation for all assays used *GAPDH* as the normalizer. Quantitation of gene expression in comparison to *GAPDH* and fold change in gene expression due to zinc treatment were calculated using the comparative threshold cycle method [28].

2.6. Statistical analysis

The statistical analysis was performed by unpaired, two-tailed Student's t test between the groups using GraphPad Prism 4.0c software.

3. Results

3.1. Pancreatic cancer cells are vulnerable to extracellular zinc

Previous studies indicated that the mouse insulinoma cell line, Min6, underwent necrosis when exposed to high concentrations of zinc [8–9]. However, it is not clear whether this is unique to mouse insulinoma or other pancreatic cancer cells from different phylogenic origins can also undergo cell death in response to increases in extracellular zinc. To address this possibility, pancreatic cancer cells derived from mouse, rat and human were treated with 0.01 to 0.5 mM zinc concentrations, considered to be physiological in mammalian cells [11]. After overnight culture, viability was determined by incubating cells with the mitochondrial membrane potential indicator, TMRE [23] and mBcl, which binds to intracellular antioxidant thiols [24]. Dead cells and debris were excluded based on forward

Table 1
Sequences of the primers used in quantitative real-time RT-PCR assays

Gene name	Sequence
Rat *znt1*	Forward 5′-ATC CAG CCT GAA TTC GCT AGC-3′ Reverse 5′-CGT GTC CCA CAG CAC TGC T-3′
Rat *znt2*	Forward 5′-GAC CCC ATC TGC ACC TTC CT-3′ Reverse 5′-CCT TTG GGA GTC CCT TCC AT-3′
Rat *znt4*	Forward 5′-GAT CGG AGA GCT TGT AGG TGG ATA C-3′ Reverse 5′-AAC ATG GTG TCC CCT TTG ATC TC-3′
Rat *znt5*	Forward 5′-TGG CTA AGA TGG CCG AAC AC-3′ Reverse 5′-CCA GGA AGG CGA TAG CTG TAT AAA-3′
Rat *znt8*	Forward 5′-CTT GAG AGG ACT TAC CTT GTG-3′ Reverse 5′-GTG AAG GCA TAC ATC TTG GTG-3′
Rat *gapdh*	Forward 5′-TGA TGC TGG TGC TGA GTA TGT CGT-3′ Reverse 5′-TTG TCA TTG AGA GCA ATG CCA GCC-3′
Human *ZNT1*	Forward 5′-GAG ATG CCT TGG GTT CAG TGA TTG-3′ Reverse 5′-GGT CAG GGA AAC ATG GAT TCA CAC-3′
Human *ZNT5*	Forward 5′-GGA GGC ATG AAT GCT AAC ATG AGG-3′ Reverse 5′-GTG GAT ACG ATC ACA CCA ATG CTG-3′
Human *ZNT7*	Forward 5′-TTT CTT CCT GTG CCT GAA CCT CTC-3′ Reverse 5′-GAG TCG GAA ATC AAG CCT AAG CAG-3′
Human *GAPDH*	Forward 5′-TTG CCA TCA ATG ACC CCT TCA-3′ Reverse 5′-CGC CCC ACT TGA TTT TGG A-3′

angle and side scatter light properties, and intact cells were electronically gated (Fig. 1A) and analyzed for the retention of both TMRE and mBcl (Fig. 1B). When the commonly used mouse insulinoma cell line NIT-1 was treated with 0.25 mM zinc chloride, absolute numbers of viable cells were reduced from 59% in controls to 21.5% after zinc treatment (Fig. 1A). Substantial reduction in viable cells retaining both TMRE and mBcl was also noted after zinc treatment (from 96.7% to 22.7%, Fig. 1B, upper right quadrants). Significant cytotoxicity was detectable after treatment of NIT-1 cells with as little as 0.1 mM zinc, and enhanced cell death was observed with increasing zinc concentrations (Fig. 1C). The rat insulinoma cell line, RINm5F, also displayed similar levels of sensitivity to zinc (Fig. 1C). In addition, the mouse insulinoma cell line, Min6, and the rat insulinoma cell line, βTC3, exhibited comparable levels of sensitivity to zinc (data not shown). Thus, rodent insulinomas succumbed to concentrations of extracellular zinc considered to be physiological in mammalian cells [11].

It was next examined whether human pancreatic ductal adenocarcinomas, in comparison to normal human islet cells, could also display enhanced sensitivity to increased concentrations of extracellular zinc, like rodent insulinomas. To this end, freshly isolated normal human islets were treated overnight with various concentrations of zinc. Islets were mildly dispersed using trypsin, and β cells were identified by staining with FluoZin-3-AM [24], a cell-permeant dye with higher affinity to Zn^{2+} (K_D=15 nM) and higher quantum yield than other zinc-sensitive dyes [29]. Data in Fig. 1D show that normal human β (FluoZin-3$^+$) cells were resistant to treatment with 0.1- to 0.5 mM zinc concentrations, as indicated by normal levels of mitochondrial membrane potential and intracellular thiols among FluoZin-3$^+$ (β) cells. Resistance of β cells to these zinc concentrations was consistently observed in eight different islet preparations cultured either in CMRL 1066 medium supplemented with human serum albumin (medium used for islet transplantation) or in RPMI medium supplemented with fetal bovine serum (data not shown). In some experiments, islets were first dispersed by trypsin treatment and then treated overnight with 0.1 to 0.5 mM zinc. Islet cells were again treated with trypsin just before staining to avoid clumping. Under these conditions also, no increase in cytotoxicity was observed in β (FluoZin-3$^+$) cells treated with 0.5 mM zinc (% viability: control − 48% ± 8 vs. treated − 45% ± 9; n=3), indicating that trypsin treatment did not simply render β cells more sensitive to high concentrations of zinc. In sharp contrast to normal β cells, human pancreatic ductal adenocarcinoma cell lines, CD18 and S2013 [30] underwent significant cell death when treated with as little as 0.01 mM zinc (Fig. 1D). Although the underlying mechanisms are not clear, the human pancreatic carcinoma cells were more sensitive to extracellular zinc concentrations than rodent insulinomas.

Kinetic experiments revealed that zinc treatment induced mitochondrial membrane depolarization in both CD18 and S2013 cell lines within 1 h, as indicated by the reduction in TMRE retention (Fig. 1E). Concurrent reduction in intracellular thiol levels as detected by staining with mBcl also occurred within 1 h of zinc treatment in both CD18 and S2013 cancer cells. A concomitant increase in the plasma membrane permeability was seen, as indicated by the uptake of PI in zinc-treated cells. Taken together, these results indicate that transformed but not normal human pancreatic cells are highly susceptible to physiological concentrations of zinc, 0.01 to 0.5 mM [11], leading to cell death characterized by rapid induction of mitochondrial dysfunction, intracellular oxidation and plasma membrane permeability.

3.2. Events during zinc-induced cell death

We next examined whether differential influx of Zn^{2+} could explain the various outcomes of exposure to extracellular zinc in normal and cancerous pancreatic cells. To test this possibility, we stained cells treated with various concentrations of zinc with the cell-permeable FluoZin-3-AM, which binds to labile Zn^{2+} following the cleavage of the acetomethyl ester (AM) moiety by nonspecific esterases present in viable cells [24,29]. As can be seen in Fig. 2A, incubation of CD18 and S2013 carcinoma cells with low concentrations of zinc (0.01–0.1 mM) resulted in significantly increased numbers of FluoZin-3$^+$ cells. In contrast, treatment of primary islet cells with higher concentrations of zinc (0.1–0.5 mM) increased the frequency of FluoZin-3$^+$ cells slightly but not significantly (Fig. 2B). In data not shown, we observed that zinc treatment resulted in comparable levels of zinc influx into primary β cells and pancreatic cancer cells, as indicated by similar mean fluorescence intensities in these cells. Taken together, these results indicate that exposure to high concentrations of zinc can lead to the increase in free intracellular zinc levels in more malignant cells than in normal pancreatic cells.

Zinc treatment increases the level of reactive oxygen species (ROS) in neuronal cells [31,32] and Hep-2 tumor cells [33]. Therefore, it was next examined whether zinc-induced cytotoxicity in pancreatic cancer cells was also associated with the generation of ROS. To this end, cells were stained with MitoSox Red, a cell-permeant dye that becomes highly fluorescent when oxidized by superoxide and subsequent binding to nucleic acids [29]. Flow cytometric analysis revealed that zinc treatment substantially increased the frequency of cells stained with MitoSox Red (Fig. 3). Since MitoSox Red is oxidized by superoxide but not by other reactive oxygen or nitrogen species [29], the data indicate that zinc causes a significant increase in mitochondrial superoxide production in insulinoma cells.

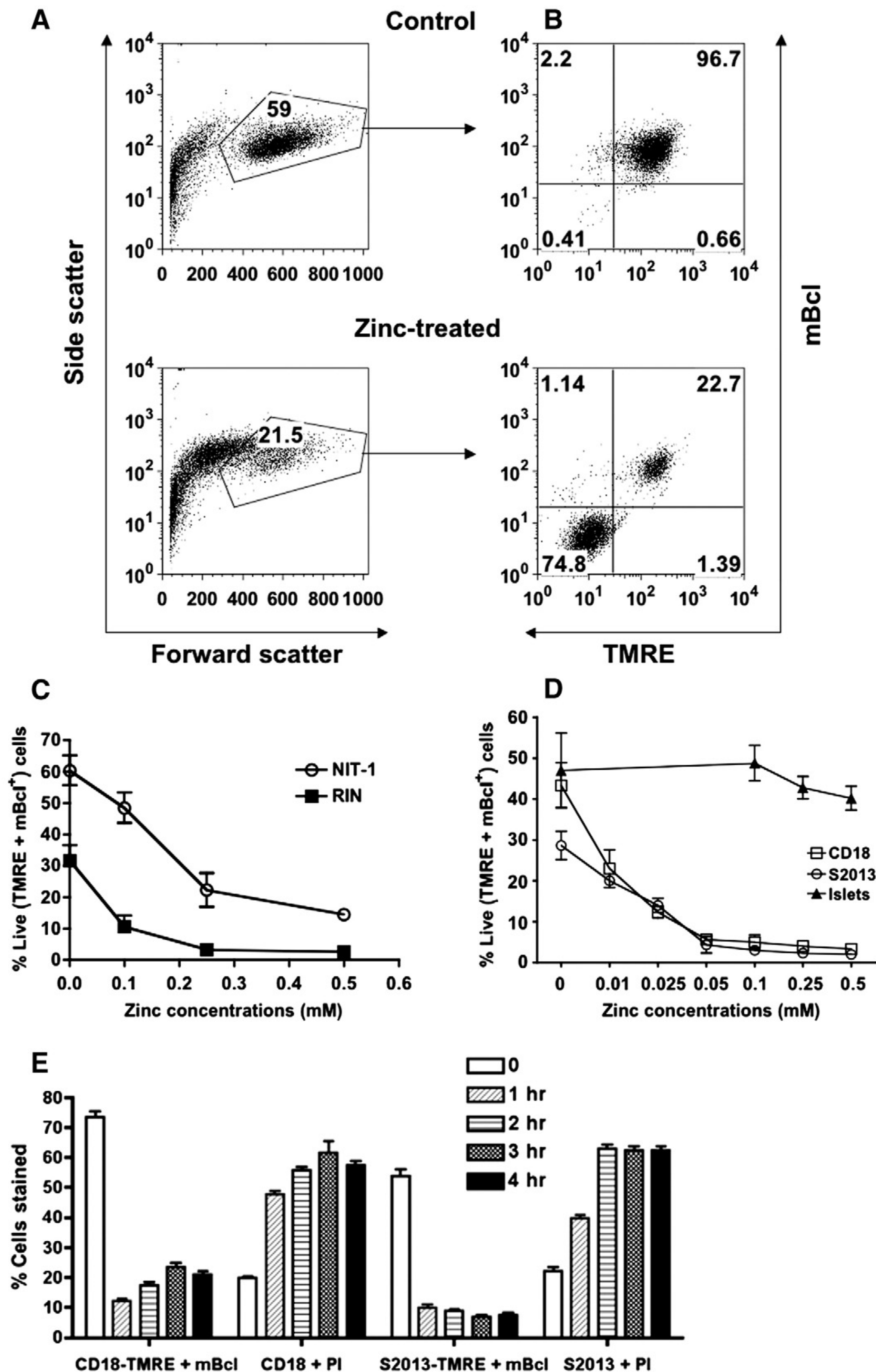
Both caspase-dependent and -independent cell death are associated with intracellular acidosis [34]. Therefore, intracellular pH was analyzed by staining cells with SNARF-1, an emission-rationing dye that shows a clear pH-dependent shift in its absorbance and emission spectra [26]. Data shown in Fig. 3 also indicate that zinc treatment significantly induced intracellular acidification as shown by the decrease in the frequency of NIT-1 cells exhibiting pH-sensitive SNARF-1 fluorescence. Collectively, the data indicate that zinc treatment resulted in zinc influx, mitochondrial superoxide generation and intracellular acidification in mouse insulinoma cells.

3.3. Protection against zinc-induced insulinoma cell death by antioxidants

Since extracellular [35,36] and intracellular [36] presence of reduced GSH prevents zinc-induced cell death in astrocytes, it was next examined whether they could similarly prevent zinc-induced cytotoxicity in insulinomas. Data shown in Fig. 4A indicate that continuous incubation of NIT-1 cells with 10 mM GSH or GSH-MEE, a cell permeable form of GSH, could prevent zinc-induced cell death significantly. However, removal of cell-impermeable GSH as well as the cell-permeant GSH-MEE antioxidants after 45 min of incubation abrogated protection against zinc-induced cytotoxicity. Similar results were obtained using rat insulinomas, RINm5F and βTC3 (data not shown). Protection was efficient at 10 mM but not at 5 or 1 mM concentrations of either of these antioxidants (data shown), indicating a requirement for certain stoichiometry between zinc and the antioxidants for effective neutralization of toxicity [37].

Coincubation of NIT-1 cells with GSH and zinc also significantly prevented mitochondrial production of ROS, as indicated by the reduction in MitoSox Red fluorescence significantly (Fig. 4B). In addition, zinc-induced activation of intracellular caspases was substantially reduced by GSH treatment, as indicated by decreased staining of cells with the FITC-conjugated polycaspase inhibitor, VAD-FMK, that binds to all intracellular activated caspases [25]. Activation of caspases by zinc in NIT-1 cells is consistent with previous studies showing zinc-induced activation of caspase-3 and -9 in HL-60 [38] and

Ramos B and Jurkat cells [39]. However, zinc-induced death could not be blocked by pretreating NIT-1 cells with the potent polycaspase inhibitor, Q-VD-OPH (Fig. 4C) [40]. In addition, the inhibitor of necrosis, necrostatin [41], failed to prevent zinc-induced cell death. In data not shown, we observed that the necrostatin inhibited tumor necrosis factor (TNF)-α-induced necrosis of L929 fibroblasts, as reported [41]. In addition, the p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580 and the selective ERK-1/2 inhibitor U0126 [42] failed to block zinc-induced apoptosis in NIT-1 cells. SP600125, inhibitor of JNK-1, -2 and -3 as well as 13 other protein kinases [43] failed to prevent zinc-mediated cytotoxicity. Although the precursor of GSH, NAC [37], has been shown to prevent zinc-induced cell death in



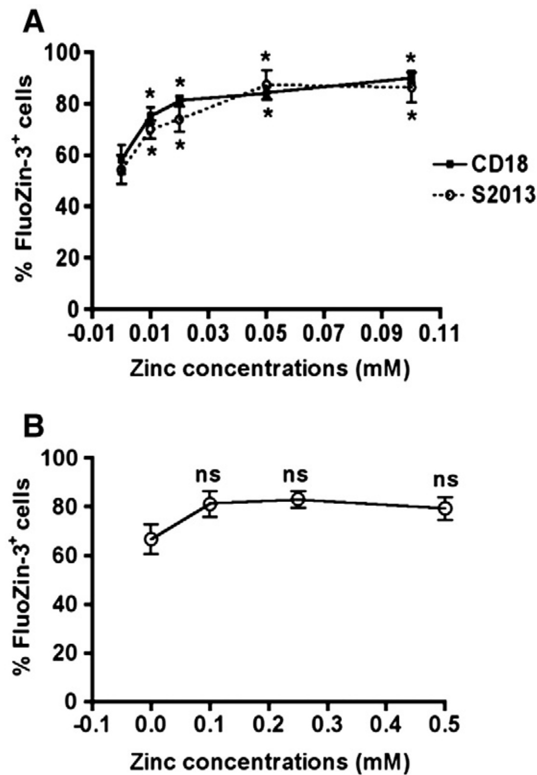


Fig. 2. Increased zinc influx in pancreatic carcinoma cells. (A) Human ductal adenocarcinoma cell lines CD18 and S2013 were treated overnight with indicated concentrations of zinc. Cells were stained with the zinc-specific dye FluoZin-3 and assessed by flow cytometry. The increase in FluoZin-3^+ cells after treatment with 0.025, 0.05 and 0.1 mM zinc was significant (P<.01) in both of the cell lines, as indicated by asterisks (n=3). (B) Normal human islets were treated overnight with various concentrations of zinc and analyzed for intracellular free Zn^{2+} as detected by the binding of FluoZin-3. The numbers of FluoZin-3^+ cells increased slightly but not significantly after zinc treatment. Data shown are pooled from eight separate islet preparations.

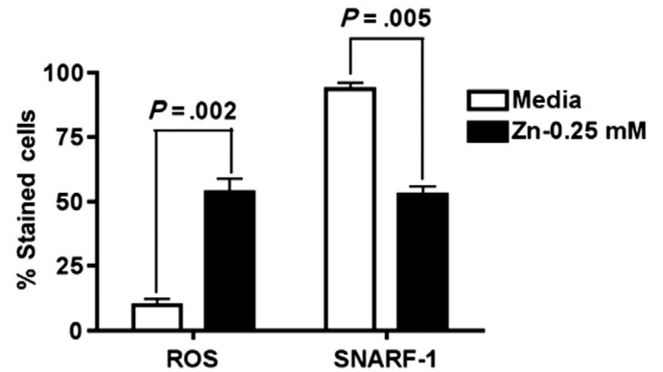


Fig. 3. Zinc treatment induced ROS generation and intracellular acidosis in insulinoma cells. NIT-1 cells were treated with 0.25 mM zinc chloride overnight and then stained with MitoSox Red and analyzed for the generation of mitochondrial ROS by flow cytometry (n=3). Cells were stained with SNARF-1 for the determination of intracellular pH and analyzed by flow cytometry (n=3). P values are shown.

retinal pigment epithelial cells [44], addition of NAC failed to protect NIT-1 cells from cell death, as reported in epithelial type II cells [45]. In addition, incubation with melatonin, which can detoxify oxidants [37], also failed to protect NIT-1 cells from zinc-induced cell death. Collectively, these data demonstrate that zinc treatment rapidly induced irreversible damage to mitochondria and activated caspases, and only the sequestration of extracellular zinc with the antioxidant GSH can protect the insulinoma cells from cytotoxicity.

3.4. Zinc-induced carcinoma cell death involves increased protein ubiquitination

To gain insights into the mechanisms of zinc-induced cell death, whole cell lysates were prepared from human pancreatic carcinomas treated with zinc and immunoblotted with well-characterized antibodies. Data shown in Fig. 5 indicate that zinc treatment did not alter the levels of the survival factors, Bcl-X_L expressed in certain pancreatic carcinomas [46], XIAP that directly prevents the activation of caspases 3 and 7 [47] and c-Abl [48]. Interestingly, the prototype survival factor, Bcl-2, expressed in some but not all pancreatic carcinomas [49], could not be detected in CD18 and S2013 cells but detected in the T cell leukemia, Jurkat (data not shown). Signaling via the MAPK family member, ERK-1/2 has been implicated in the survival of pancreatic carcinomas [50]. However, zinc treatment neither prevented the signaling event, phosphorylation of ERK1/2, nor reduced the absolute amounts of ERK1/2 in these carcinomas. Zinc treatment also failed to reduce the level of pro-caspase-9, an apical caspase involved in mitochondria-dependent intrinsic apoptotic pathway, and activates effector caspase -3 and -7, leading to the proteolytic cleavage of PARP [51]. Consistently, zinc treatment did not result in the cleavage of PARP, implicated in DNA damage and apoptosis [51]. The level of Cbl-b, an E3 ubiquitin ligase, involved in the ubiquitination of proteins [52], was also not altered by zinc treatment. Notably, enhanced ubiquitination of proteins, critical for cell death [53], was evident in both of the carcinoma cell lines treated with zinc. Collectively, these data indicate that zinc treatment induced cytotoxicity and not caspase-dependent apoptosis in pancreatic carcinomas. Although cytotoxicity was not associated with proteolytic cleavage of the prosurvival factors analyzed, substantial increase in protein ubiquitination was evident.

3.5. Zinc-induced cytotoxicity is associated with the up-regulation of ZnTs

Since the retention of zinc was considerably increased in pancreatic cancer cells but not in primary islet cells treated with

Fig. 1. Pancreatic cancer cell lines were sensitive to zinc treatment than normal β cells. (A) The mouse insulinoma cell line, NIT-1 treated with 0.25 mM $ZnCl_2$, was analyzed by flow cytometry after overnight incubation. Note that the intact cells gated based on forward angle light scatter are reduced after zinc treatment. (B) Intact cells were electronically gated and analyzed for viability after staining with TMRE and mBcl. The frequency of viable cells is shown in upper right quadrants. Representative data from 12 experiments are shown. (C) Dose-dependent toxicity of mouse insulinoma, NIT-1 (n=6) and rat insulinoma, RINm5F (n=6) cells are shown. The differences between untreated vs. cells treated with 0.25 and 0.5 mM zinc were significant in both NIT-1 and RINm5F cell lines (P<.05). (D) Freshly isolated human islets (n=8) and human ductal adenocarcinoma cell lines CD18 and S2013 (n=5) were treated with indicated concentrations of $ZnCl_2$ and analyzed for viability. Insulin-producing β cells were gated based on staining with FluoZin-3 and analyzed for the retention of TMRE and mBcl. The viability of cells treated with zinc was not significantly altered (control vs. 0.1 mM Zn, P=.2, control vs. 0.25 mM Zn, P=.09, control vs. 0.5 mM Zn, P=.06). Viability of carcinoma cells was based on retention of TMRE and mBcl. The differences between untreated and zinc-treated CD18 and S2013 cells were significant (>0.01 mM: P<.05). (E) CD18 and S2013 carcinoma cell lines were treated with 0.25 mM $ZnCl_2$ and harvested at indicated time points and analyzed for viability and plasma membrane integrity, respectively, by staining with TMRE + mBcl and PI. Data are pooled from three experiments. Zinc treatment significantly (P<.05) reduced the viability and concomitantly increased the plasma membrane permeability within 1 h of treatment in both CD18 and S2013 cells.

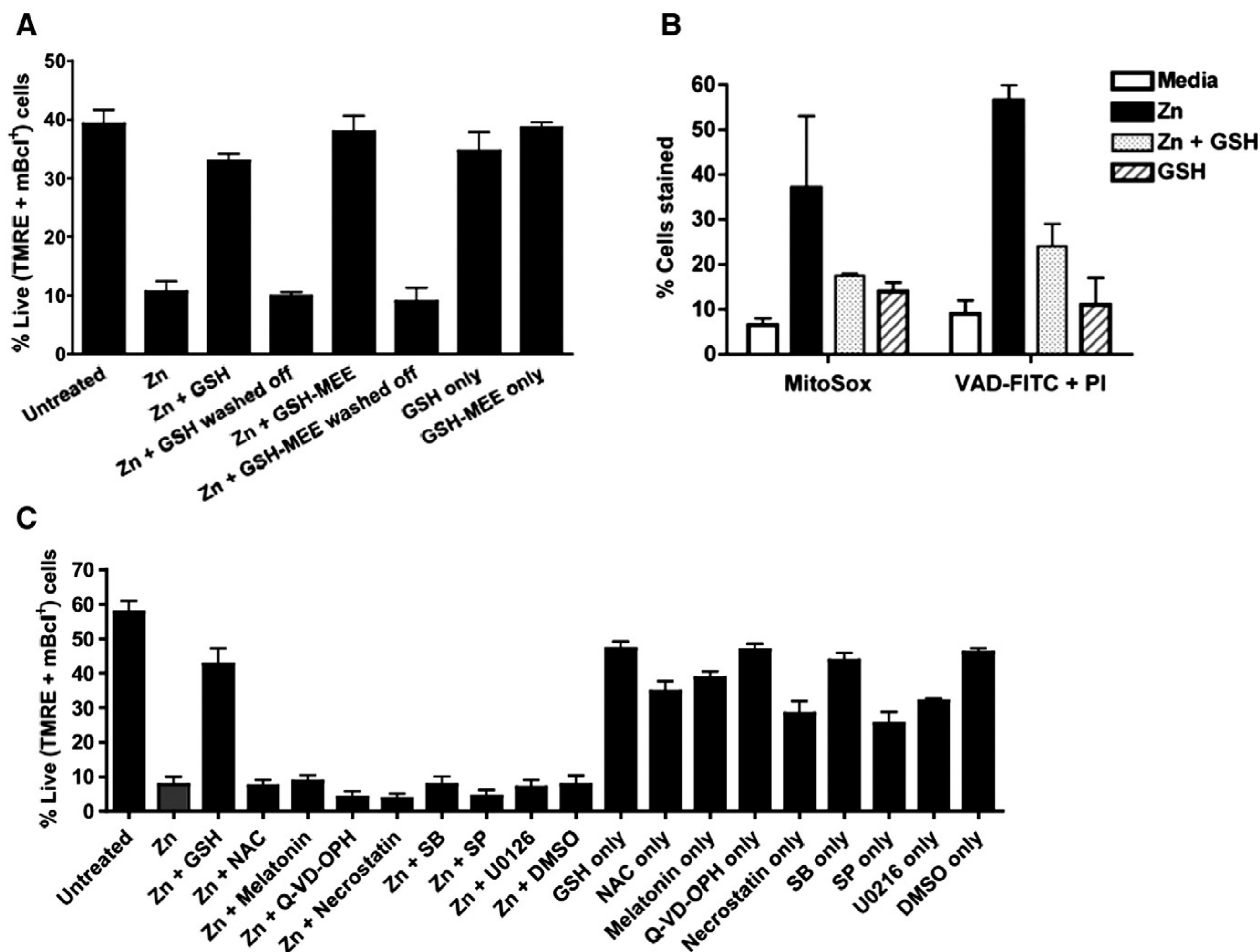


Fig. 4. Antioxidants protected insulinoma cells from zinc-induced cytotoxicity. (A) NIT-1 cells were incubated with 10 mM GSH or the cell-permeable GSH-MEE for 45 min and then removed by centrifugation. Cells were then incubated with 0.25 mM zinc. Aliquots of cells were also simultaneously incubated with GSH/GSH-MEE plus zinc overnight. Viability of cells was assessed by flow cytometry (n=4). Addition of GSH or GSH-MEE significantly prevented zinc-induced cell death (P<.05). However, removal of GSH and GSH-MEE after a brief period of incubation did not restore viability. (B) Intracellular activated caspases were determined by incubating NIT-1 cells with FITC-VAD-FMK, and PI was added to assess plasma membrane permeability. GSH was added simultaneously with zinc (n=3). The addition of GSH to zinc decreased ROS production and activation of caspases significantly (P<.05). (C) NIT-1 cells were incubated with 10 mM GSH, with 200 μM NAC or melatonin or with 10 μM concentrations of the cell-permeable inhibitors of caspases, Q-VD-OPH, necrostatin or MAPK inhibitors for 45 min prior to the addition of 0.25 mM zinc. Controls included treatment of cells only with GSH or the inhibitors or DMSO. After overnight incubation, cells were stained with TMRE and mBcl, and analyzed by flow cytometry (n=4).

zinc, cytotoxicity in cancer cells could be due to perturbed zinc homeostasis. Altered expression of ZnTs, implicated in the efflux of zinc ions out of the cell and sequestration into intracellular vesicles [10–12], may contribute to aberrant zinc homeostasis in cancer cells. To test this possibility, total RNA was extracted from zinc-treated rat insulinoma cell line, RINm5F and cDNA was synthesized and quantitative real-time RT-PCR was performed using selected primer sets and SYBR Green dye. As shown in Fig. 6A, both rat whole pancreas and the RINm5F insulinoma expressed comparable levels of *znt1*, *znt2*, *znt4*, *znt5* and *znt8* mRNA. Treatment of RINm5F cells with 0.25 mM zinc chloride increased the expression of *znt1* and *znt2* significantly when compared to the level of *znt8* mRNA (Fig. 6B). However, the abundance of ubiquitously expressed *znt4* [10–12] and the β cell selective *znt5* [17] was not significantly altered when compared to *znt8* in RINm5F insulinoma cells following zinc treatment.

We next determined whether human pancreatic cancer cells could also differentially regulate the expression of selected *ZNT* genes in response to zinc treatment. A comparison was made with similarly treated normal human islets (n=3) containing 60% to 80% viable β cells, as determined by staining with a combination of FluoZin-3, TMRE and mBcl [24]. The levels of the ubiquitously expressed *ZNT1* were not different among untreated islets and pancreatic ductal carcinoma cells (Fig. 6C), while the expression of the β cell-selective *ZNT5* was lower in CD18 (P<.05) but not in S2013 carcinoma in comparison to islets. The expression of *ZNT7* mRNA was several-fold lower in both of the carcinomas than in islets (P<.05). After overnight culture of human islets with 0.25 mM zinc, the expression of *ZNT5* and *ZNT7* mRNA was significantly reduced, but the level of *ZNT1* remained unaltered. In sharp contrast, zinc treatment increased the abundance of *ZNT1* mRNA in both CD18 and S2013 cell lines significantly. Interestingly, the level of *ZNT7* mRNA was increased by zinc treatment in S2013 but not in CD18 carcinoma, indicating variations in *ZNT* expression among different pancreatic cancer cells. Collectively, these results indicate that zinc-induced cytotoxicity is associated with consistent up-regulation of *ZNT1* in pancreatic cancer cells, and other *ZNT* genes may also be up-regulated in a cell type-specific manner.

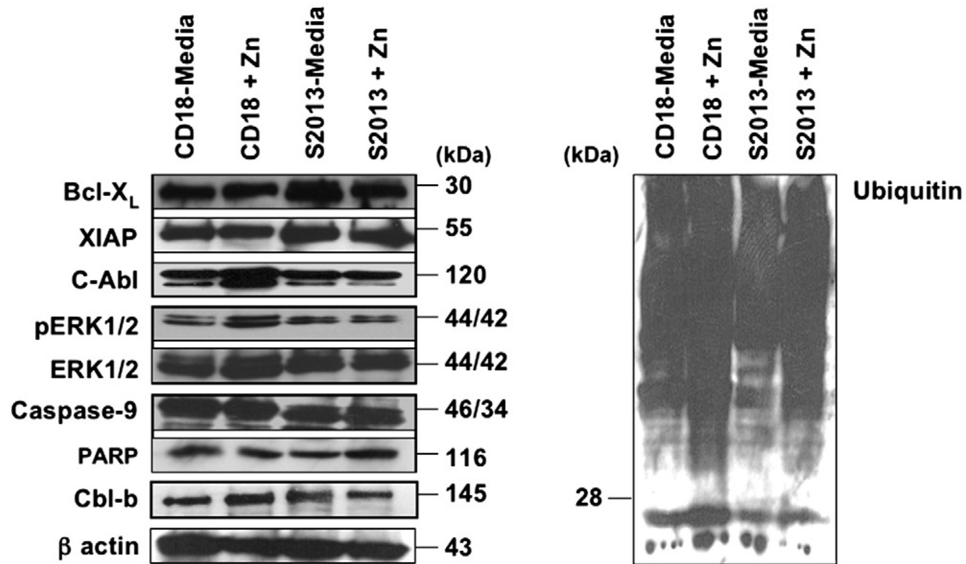


Fig. 5. Zinc-induced protein ubiquitination in carcinoma cells. Human pancreatic ductal adenocarcinoma cells were treated with 0.025 mM zinc for 4 h, and cell lysates were subjected to electrophoresis and the separated proteins were blotted using indicated antibodies. Apparent molecular weights of proteins are shown. Equal protein loading was verified by immunoblotting with an antibody against actin. Note that zinc treatment increased the amounts of ubiquitinated proteins. Representative data from three to five experiments are shown.

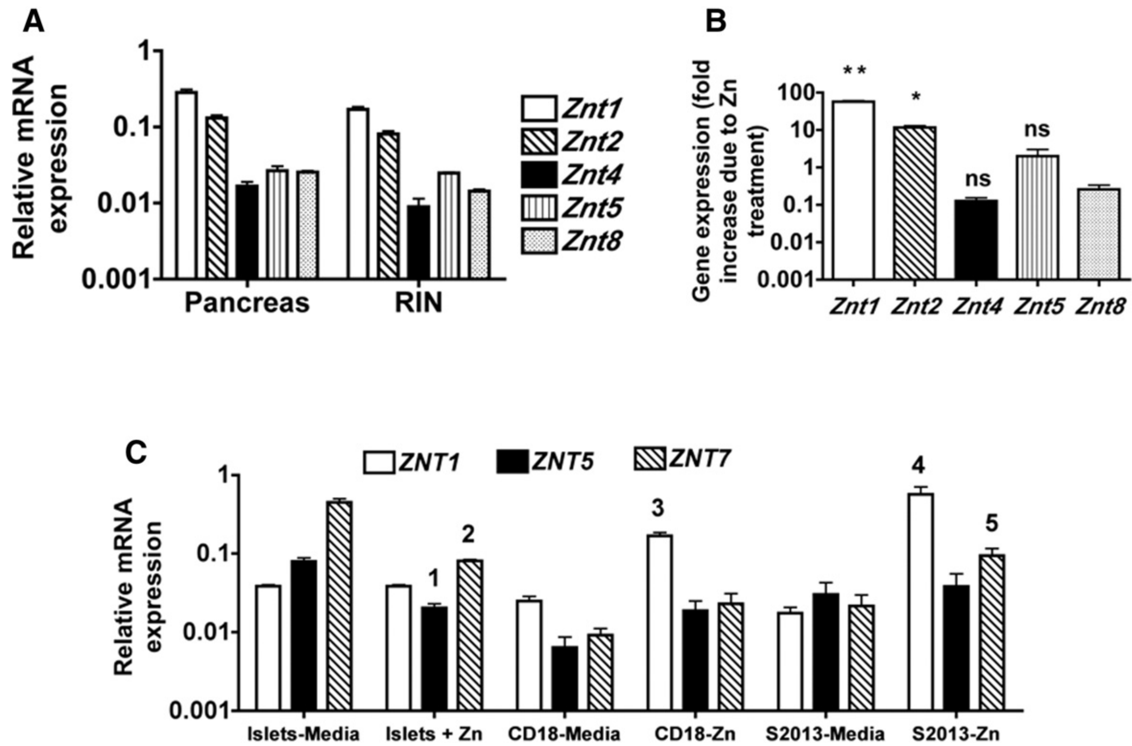


Fig. 6. Zinc treatment induced the up-regulation of selected ZnT genes in pancreatic cancer lines. (A) Quantitative real-time RT-PCR was performed with cDNA synthesized using RNA extracted from rat whole pancreas and the rat insulinoma cell line, RINm5F. Relative expression of indicated *znt* genes was determined using *gapdh* as the normalizer. Determinations were performed in triplicates in each experiment, and the data are the mean±SD of all three experiments. The levels of expression of indicated genes were not significantly different between rat pancreas and rat insulinoma cells. (B) The expression levels of indicated *znt* genes were determined separately in untreated RINm5F cells and those treated overnight with zinc (0.25 mM), using *gapdh* as the normalizer. Fold change in the expression of indicated genes due to zinc treatment was then calculated and expressed as described [28]. Assays were done in triplicates, and the data were pooled from three to four experiments. The expression levels of *znt1*, *znt2*, *znt4* and *znt5* were compared with that of *znt8*. ***P*=.0001, **P*=.008 and ns, not significant. (C) Changes in relative mRNA expression levels of selected ZnT transporters after zinc treatment were determined using *GAPDH* as the normalizer in normal human islet preparations and in human pancreatic cancer cell lines, CD18 and S2013. Data are expressed as the means ± SD of triplicate values of a representative of three separate islet preparations. Data for CD18 and S2013 were pooled from four independent experiments. The *P* values between untreated and zinc-treated cells are as follows: 1, *P*=.0038; 2, *P*=.0016; 3, *P*<.0001; 4, *P*=.0007; and 5, *P*=.005.

4. Discussion

The data presented herein demonstrate that treatment with physiological concentrations of zinc (0.01–0.5 mM) can induce cytotoxicity in human ductal adenocarcinoma cell lines but not in normal human insulin-producing β cells. Insulinoma cell lines derived from rat and mouse also displayed enhanced sensitivity to higher concentrations of extracellular zinc, indicating the conservation of this characteristic in transformed pancreatic cells through phylogeny. Zinc-induced cytotoxicity in pancreatic cancer cells was associated with rapid mitochondrial dysfunction, generation of ROS, intracellular acidification, compromise in plasma membrane integrity and increased protein ubiquitination, without apparent reduction in prosurvival factors. Notably, zinc treatment consistently increased the abundance of ZnT-1 mRNA in a rat insulinoma and two human ductal adenocarcinoma cell lines but not in normal human islet cells.

Zinc treatment has been shown to induce cell death in a number of transformed cell lines [21,27,54] including insulinomas [8,9]. Our data demonstrate for the first time that ZnT-1 gene is up-regulated in both rat insulinoma and human pancreatic ductal adenocarcinomas but not in normal islet cells challenged with physiological concentrations of zinc. Thus, a correlation between *ZNT1* gene up-regulation and induction of cytotoxicity is evident in zinc-treated pancreatic cancer cells. Our data are consistent with the observation that ZnT-1, ubiquitously expressed in a variety of transformed cells including THP-1, Molt-4 and Raji [54], HeLa cells [27] and Epstein-Barr (EB) virus transformed human lymphoblastoid cells [21], is the most up-regulated zinc transporter gene in response to enhanced extracellular zinc concentrations. Interestingly, rat mammary tumor [55] and human breast cancer grown in nude mice [56] also responded to zinc supplementation by increasing the level of ZnT-1 mRNA. However, it is not known whether zinc supplementation can lead to cytotoxic death of these tumor cells without affecting normal cells in vivo. Our data indicate that unlike the primary islet cells, pancreatic ductal adenocarcinomas succumbed to very low concentrations of exogenous zinc accompanied by ZnT-1 mRNA up-regulation in vitro. This finding may have important implications to the treatment of cancers including pancreatic cancers with dietary zinc supplementation or via pharmacological maneuvers to enhance ZnT-1 expression preferentially in transformed cells.

Exposure to higher concentrations of zinc increased the frequency of transformed but not primary islet cells binding to the zinc-specific dye, FluoZin-3. Impaired zinc homeostatic mechanisms in transformed cells may facilitate the retention of excess intracellular Zn^{2+}, which may explain the increase in the frequency of FluoZin-3^+ cells. A majority of islet cells (60–85%) are insulin-producing β cells and bind FluoZin-3 with high affinity due to high levels of intracellular zinc [24]. However, the remaining non-β cells present in islets may not retain excess zinc as the transformed cells, which may explain the lack of increase in FluoZin-3^+ cells when islet cells were exposed to high doses of zinc. Further work is necessary to fully elucidate the homeostatic mechanisms involved in the regulation of intracellular levels of Zn^{2+} in normal islet cells and their alteration during transformation.

Our observation that the association of exaggerated expression of *ZNT1* and cell death in transformed pancreatic cells responding to zinc treatment is in apparent contradiction to the previous observation that overexpression of *ZNT1* in baby hamster kidney cells increased the zinc efflux, reduced the intracellular steady-state zinc concentration and conferred resistance to zinc-mediated toxicity [57]. Although ZnT-1 is located on the plasma membrane [10–12,54], it is also found in intracellular compartments of rat enterocytes [10] and in the insulinoma cell line, INS-1E [58]. It is possible that increased intracellular expression of ZnT-1 may facilitate the accumulation of toxic levels of zinc leading to mitochondrial damage and cytotoxicity in transformed cells. Imaging studies are required to determine whether exaggerated expression of ZnT-1 in intracellular organelles could explain the aberrant zinc homeostasis in transformed pancreatic cells.

Our study has also revealed the expression of *ZNT7* mRNA in human islets and pancreatic ductal adenocarcinomas. Interestingly, zinc treatment decreased the expression of *ZNT7* in human islets but increased it in S2013 carcinoma. The abundance of *znt2* was increased by zinc treatment in the rat insulinoma cell line, RINm5f. Since ZnT-2 and ZnT-7 are, respectively, located in vesicles and perinuclear vesicles such as Golgi apparatus [10,12], they could transport zinc into intracellular vesicles. Increased concentrations of zinc induce the dissipation of mitochondrial transmembrane potential and elevation of ROS in neuronal cells [31,32] and Hep-2 cells [33]. Exposure of pancreatic cancer cells to high levels of extracellular zinc may lead to overloading of zinc into mitochondria, and subsequent mitochondrial membrane depolarization, generation of ROS, intracellular acidification and eventual cell death. Since ZnT-1 is consistently up-regulated in rodent and human-transformed pancreatic cells and other ZnT genes in a cell type-specific manner, the relative contribution of these zinc transporters to pancreatic cancer cell death may be unraveled by siRNA-mediated ablation of *ZNT1*, *ZNT2* and *ZNT7* genes.

A comparison of the expression profiles of *ZNT1* to *ZNT9* in THP-1, Molt-4 and Raji cell lines indicated lower expression of these genes than in primary human T and B cells [54]. Similarly, a lower steady-state level of *ZNT1* mRNA expression was reported in chemically induced mammary tumors compared to normal rat mammary gland tissues [55]. However, we found that the expression profile of *znt1*, *znt2*, *znt4*, *znt5* and *znt8* in the rat insulinoma cell line RINm5F was similar to whole rat pancreas. While the expression levels of *ZNT1* and *ZNT5* were similar in purified human islets and pancreatic carcinomas, the steady-state level of *ZNT7* mRNA was comparably lower in human pancreatic carcinomas. Although it was reported that the steady-state level of *ZIP4* was higher in human pancreatic cancer tissues [22], our data do not indicate up-regulation of the *ZNT* genes analyzed in rat and human-transformed pancreatic cell lines compared to, respectively, whole pancreas and purified islets. A thorough analysis of the expression profiles of these *ZNT* genes in human pancreatic carcinoma biopsies may unravel additional useful biomarker(s) for the diagnosis as well as prognosis of pancreatic cancers.

Our data show that zinc treatment induced cytotoxic but not apoptotic death in pancreatic carcinomas and insulinomas as indicated by the lack of proteolytic processing of pro-caspase-9 and PARP cleavage, characteristic features of apoptosis [51]. In addition, the survival factors Bcl-X_L, XIAP, C-Abl and Cbl-b were not affected by zinc treatment. Although the MAPK family member ERK-1/2 has been implicated in survival of pancreatic cancer cells [50], zinc-induced cytotoxicity was not associated with either degradation of ERK-1/2 or its phosphorylation. In addition, only the antioxidant GSH but not the inhibitors of enzymes implicated in apoptosis could block zinc-induced carcinoma cell death even though caspases were activated during zinc treatment as indicated by the binding of FITC-VAD-FMK [25]. These data are consistent with the notion that transformed pancreatic ductal adenocarcinoma cells do not undergo caspase-dependent apoptosis, similar to the observation in Hep-2 cells [59]. However, zinc has been reported to induce caspase-dependent apoptosis in neuronal cells [31], HL-60 cells [38] and leukemia cells [39]. In addition, our data are also at variance with a previous report showing the participation of MAPK in zinc-induced cell death in HL-60 cells [39]. Taken together, these data suggest that zinc can engage caspase-dependent and -independent cell death pathways in a cell type-specific manner. Importantly, our data indicate that zinc treatment led to increased

ubiquitination of proteins, a process crucial for cell death [53,60]. It is possible that zinc influx may facilitate ubiquitination of survival factors not investigated herein and accelerate their degradation via the proteasomal pathway. Further work is necessary to determine the identity of target proteins for ubiquitination during zinc-induced cytotoxicity in pancreatic cancer cells.

Our data indicating that normal human β cells are resistant to treatment with extracellular zinc are at variance with a previous report that proposed that zinc is toxic to normal pancreatic cells [8]. Although the basis of this discrepancy is not clear, the striking difference is that in the previous study, a small number (n=3) of frozen islet preparations were studied [8] while we analyzed eight different freshly isolated islet preparations. Freezing and thawing of dispersed islet cells, as in the previous study [8], could render islet cells more susceptible to cell death upon subsequent culture and zinc treatment. We verified that the primary β cells, as stained by using FluoZin-3 [24], are not susceptible to physiological zinc concentrations, up to 0.5 mM. Therefore, our results do not support the previous proposal that zinc is a paracrine effector in normal β cell death and therefore may play a role in diabetes [8]. Rather, our data demonstrate that pancreatic carcinoma cells and not primary β cells are exquisitely sensitive to increases in extracellular zinc concentrations. This raises an interesting possibility of modulating pancreatic carcinomas by using the transition metal zinc and without causing undue toxicity to normal β cells.

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