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Transient receptor potential melastatin type 7 channels are involved in zinc-induced apoptosis in gastric cancer

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Transient receptor potential melastatin 7 (TRPM7) channels are novel Ca^{2+}-permeable non-selective cation channels that are ubiquitously expressed. Activation of TRPM7 channels has been shown to be involved in the survival of gastric cancer cells. Here we show evidence suggesting that TRPM7 channels play an important role in Zn^{2+}-mediated cellular injury. Using a combination of electrophysiology, pharmacological analysis, small interfering RNA (siRNA) methods and cell death assays, we showed that activation of TRPM7 channels augmented Zn^{2+}-induced apoptosis of AGS cells, the most common human gastric adenocarcinoma cell line. The Zn^{2+}-mediated cytotoxicity was inhibited by the non-specific TRPM7 blockers Gd^{3+} or 2 aminoethoxydiphenyl borate (2-APB) and TRPM7 specific siRNA. In addition, we showed that overexpression of TRPM7 channels in HEK293 cells increased Zn^{2+}-induced cell injury. Thus, TRPM7 channels may represent a novel target for physiological disorders where Zn^{2+} toxicity plays an important role.

Keywords: transient receptor potential melastatin 7; TRPM7; zinc; AGS cells; apoptosis

Introduction

Calcium has a fundamental role in countless cellular processes, including modulation of ion channels, receptors, G proteins, effector enzymes, cell proliferation, and survival (Volpe and Vezu 1993; Berridge et al. 2000; Romani and Scarpa 2000; Lee et al. 2010). Also, calcium toxicity is one of the key factors responsible for cell death (Choi 1998). Recent studies have demonstrated that zinc ions, like calcium, play an important role in cellular injuries associated with various physiological conditions (Koh et al. 1996; Calderone et al. 2004). The exact pathways mediating intracellular zinc accumulation and toxicity are, however, not clear. Zinc is one of the most crucial trace metals in cells. For example, zinc is required for the function of a broad range of enzymes involved in transcription, protein synthesis, and signal transductions (Beyersmann and Haase 2001). Although there is low level of free zinc in the cells, most zinc ions are bound to intracellular proteins (Cuajungco and Lees 1997). The mechanisms that affect the free zinc concentration are, therefore, pivotal for maintaining normal cellular function.

Transient receptor potential melastatin 7 (TRPM7) is a member of the large TRP channel superfamily expressed in almost every tissue and cell type (Nadler et al. 2001; Runnels et al. 2001; Clapham 2003). Increasing evidence suggests that activation of TRPM7 channels contributes to various physiological and pathophysiological processes (Schmitz et al. 2003; Jiang et al. 2007; Jin et al. 2008). Notably, we demonstrated that human gastric adenocarcinoma cells express TRPM7 channels whose presence is essential for cell survival (Kim et al. 2008). TRPM7 is, at present, the only known zinc-permeable channel among the TRP family of ion channels (Clapham 2003; Monteilh-Zoller et al. 2003; Inoue et al. 2010). It is reported that the zinc permeability for TRPM7 channels is 4-fold higher than for Ca^{2+} (Monteilh-Zoller et al. 2003). Despite these facts, it has not been established whether TRPM7 channels play a role in zinc-mediated cytotoxicity in AGS cells. In this study, we examined the potential role of TRPM7 channels in the survival of zinc-mediated AGS cells, the most common human gastric adenocarcinoma cell line. Our data suggest that TRPM7 channels have an important role in the survival of these zinc-mediated tumor cells.

Materials and methods

Cells

The most common human gastric adenocarcinoma cell line (AGS) was used. AGS cells were established

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at the Cancer Research Center, College of Medicine, Seoul National University, Korea. Cells were propagated in RPMI-1640 medium (Gibco-BRL) supplemented with 10% heat-inactivated fetal bovine serum and 20 μg/ml penicillin and streptomycin in an atmosphere of 5% CO_2 at 37°C.

Patch-clamp experiments

Experiments were performed at room temperature (22–25°C) by using the whole-cell configuration of the patch-clamp technique. AGS cells were transferred to a small chamber on the stage of an inverted microscope (IX70; Olympus, Japan), and were constantly perfused within a solution containing (in mM) KCl 2.8, NaCl 145, $CaCl_2$ 2, glucose 10, $MgCl_2$ 1.2, and HEPES 10, adjusted to pH 7.4 with NaOH. The pipette solution contained (in mM) Cs-glutamate 145, NaCl 8, Cs-2-bis(2-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid 10, and HEPES-CsOH 10, adjusted to pH 7.2 with CsOH. An Axopatch I-D (Axon Instruments, Foster City, CA, USA) was used to amplify membrane currents and potentials. For data acquisition and the application of command pulses, pCLAMP software v.9.2 and Digidata 1322A (Axon Instruments) were used. Results were analyzed by using pClamp and Origin software (Microcal Origin version 6.0).

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay

Cell viability was assessed by MTT assay. The AGS cells were seeded into each well of 12-well culture plates and then cultured in RPMI-1640 supplemented with other reagents for 24 h. After incubation, 100 μl of MTT solution (5 mg/ml in PBS) was added to each well, and the plates were then incubated for 4 h at 37°C. After removing the supernatant and shaking with 200 μl of dimethyl sulfoxide (Jersey Lab Supply, Livingston, NJ, USA) for 30 min, absorbance was measured at 570 nm. All experiments were repeated at least three times.

RNA interference

All the synthetic siRNAs were designed at Qiagen using the BIOPREDsi algorithm licensed from Novartis. All siRNA target sequences for silencing of the *TRPM7* gene (GenBank accession number NM_017672) were as follows: TRPM7-siRNA-5′-CCCTGACGGTAGATACATTAA-3′. SiRNA transfections were performed in 12-well plates. Previously, we showed the TRPM7 knock-down effects of this TRPM7-specific siRNA (Kim et al. 2008).

Flow cytometric analysis

In order to investigate whether the cell cycle of AGS cells was redistributed, flow cytometric analysis was used with propidium iodine (PI) stain (Nicoletti et al. 1991; Wang et al. 2005). Cells (1×10^6) were placed in an e-tube. Ice-cold fixation buffer (ethyl alcohol) (700 μl) was slowly added with vortexing. Tubes were sealed with parafilm and incubated at 4°C overnight. Samples were spun for 3 min at 106 *g* at 4°C, and the supernatant was aspirated and discarded. The cell pellet was resuspended by 200 μl of PI staining solution (PI [5 mg/ml] 2 μl and RNase 2 μl in PBS 196 μl) at 20,817 *g* for 5 s. After 30 min in the dark at room temperature, samples were analyzed in a fluorescence-activated cell sorter (FACScan; Becton-Dickinson, Moutain View, CA, USA) at $\lambda = .488$ nm using Cell-Quest software (Becton-Dickinson). The DNA content distribution of normal growing cells is characterized by two peaks, the G1/G0 and G2/M phases. The G1/G0 phase comprises the normal functioning and resting state of the cell cycle with the most diploid DNA content, while the DNA content in the G2/M phase is more than diploid. Cells in the sub-G1 phase have the least DNA content in cell cycle distribution; this is termed hypodiploid. The hypodiploid DNA contents represent the DNA fragmentation (Wang et al. 2005).

Caspase-3 assay

Caspase-3 assay kits (Cellular Activity Assay Kit Plus) were purchased from BioMol (Plymouth, PA, USA). After experimental treatment, cells were centrifuged (1000 *g*, 4°C, 10 min) and washed with PBS. Cells were resuspended in ice-cold cell lysis buffer and incubated on ice for 10 min. Sample were centrifuged at 10,000 *g* (4°C, 10 min), and the supernatant was removed. Supernatant samples (10 μl) were incubated with 50 μl of substrate (400 μM Ac-DEVD-pNA) in 40 μl of assay buffer at 37°C. Absorbance at 405 nm was read at several time-points. pNA concentration in samples was extrapolated from a standard created with absorbances of sequential pNA concentrations.

Statistical analysis

Data are expressed as mean ± SEM. Differences between the data were evaluated by Student's *t*-test. A *P*-value of 0.05 was taken to indicate a statistically

significant difference. The *n*-values reported in the text refer to the number of cells used in the patch-clamp experiments.

Results

Cell death by zinc in AGS cells

Functional TRPM7 channels are expressed in AGS cells where they play important roles in cell death (Kim et al. 2008). Also, zinc ions have been reported to permeate through TRPM7 channels in heterologous expression systems (Monteilh-Zoller et al. 2003). However, whether TRPM7 channels play a role in zinc-mediated cytotoxicity in AGS cells has never been explored. Therefore, we investigated whether the zinc influences the survival of AGS cells. First, we tested the effect of zinc on the survival of AGS cells. Addition of 100 and 200 μM zinc in the culture medium inhibited the survival of AGS cells by $21.2 \pm 1.2\%$ and $45.3 \pm 2.1\%$ with MTT assay ($n = 6$; Figure 1). To confirm the involvement of TRPM7 channels, we investigated the various pharmacological effects of these zinc-induced cell deaths in AGS cells. 2-APB and Gd^{3+} have been reported to block TRPM7 currents (Nadler et al. 2001; Monteilh-Zoller et al. 2003). In the presence of 100 μM 2-APB, the cell death was attenuated by $7.3 \pm 1.3\%$ in zinc 100 μM and $9.5 \pm 1.1\%$ in zinc 200 μM with the MTT assay ($n = 5$; Figure 1). In the presence of 10 μM Gd^{3+}, the cell death was attenuated by $6.7 \pm 1.2\%$ in zinc 100 μM and $9.0 \pm 1.3\%$ in zinc 200 μM with the MTT assay ($n = 5$; Figure 1A).

For determination that these effects were really mediated by activation of TRPM7 channels, we used RNA interference (RNAi). Previously, we created the 21-nucleotide siRNA specifically targeting human TRPM7 (Kim et al. 2008). Western blotting and electrophysiological measurements demonstrated that expression of TRPM7 in AGS cells was reduced by approximately 70–80% compared to control cells (Kim et al. 2008). Depletion of TRPM7 in AGS cells rendered the cells more resistant to zinc. In the presence of 500 μM Zn^{2+}, the cell death was attenuated by $25.5 \pm 2.5\%$ in siRNA with the MTT assay ($n = 3$; Figure 1B). In this experiment, a high concentration of zinc (500 μM) was used to induce more cell death, which allows for easier detection of the reduction of cell injury by the TRPM7 siRNA treatment.

To investigate the effect of zinc on the electrophysiological characteristics in AGS cells, we recorded the TRPM7-like current in AGS cells (Kim et al. 2008). In the presence of zinc in the bath solution, the outward current was partially inhibited by $27.3 \pm 1.1\%$ and the inward current was increased by $571.1 \pm 20.5\%$ ($n = 6$; Figure 2). Taken together, these data indicate that TRPM7 channels are involved in zinc-mediated cell death in AGS cells.

Overexpression of TRPM7 channels enhances the zinc toxicity in HEK293 cells

To provide further evidence supporting the contribution of TRPM7 channels to zinc toxicity, we investigated whether changing the expression level of TRPM7 channels influences the zinc-mediated cell injury. We used HEK293 cells with inducible expression of TRPM7 channels (Nadler et al. 2001; Jiang et al. 2007). In the absence of induced expression of TRPM7 channels (TRPM7(-) cells, tetracycline(tet)(-)),

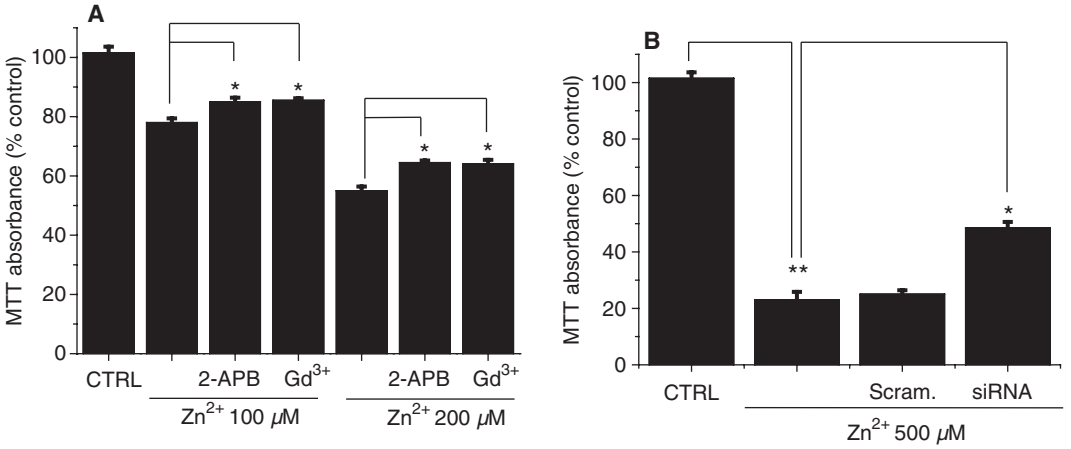

Figure 1. Effect of Zn^{2+} on cell viability with a MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide)-based viability assay in AGS cells. (A) Cell viability was significantly decreased by Zn^{2+}. Inhibition of TRPM7 channels by 2-APB and Gd^{3+} reduced zinc toxicity. (B) Inhibition of TRPM7 channels by siRNA reduced zinc toxicity. Values are mean ± SEM. CTRL, AGS cells; Scram., scrambled siRNA; siRNA, TRPM7-specific siRNA. $*P < 0.05$, $**P < 0.01$.

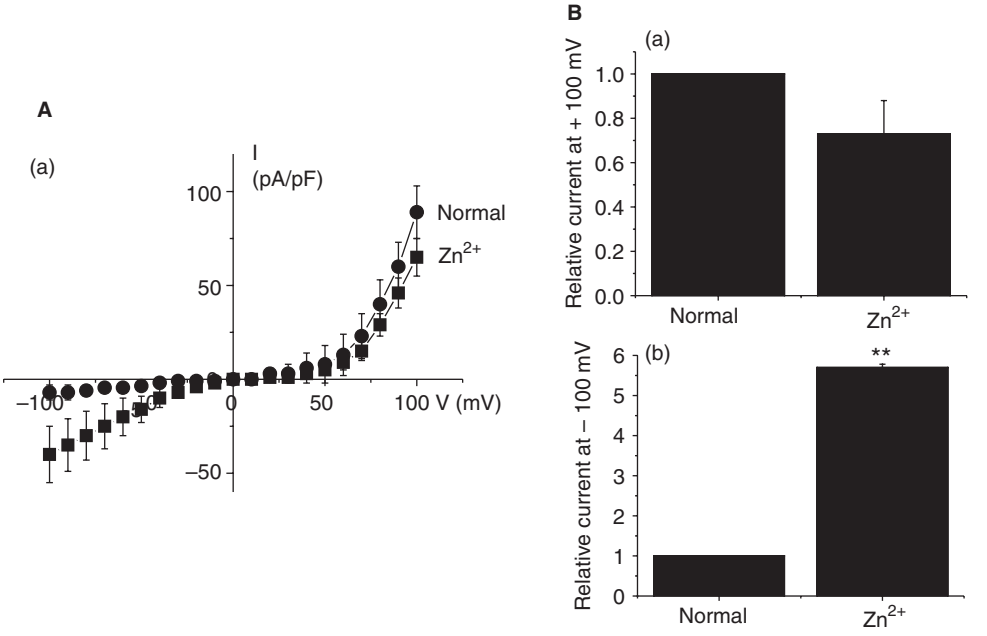

Figure 2. Effect of Zn^{2+} on transient receptor potential melastatin 7 (TRPM7)-like current in AGS cells. I–V curves (A) and summary bar graph (B) in the absence (●) and presence (■) of Zn^{2+}.

incubation of HEK293 cells with zinc induced a decrease of the MTT assay ($n=6$; Figure 3A). However, when overexpression of TRPM7 channels was induced by adding tetracycline (TRPM7(+) cells, tet(+)), incubation of HEK293 cells with zinc induced a greater decrease of the MTT assay, suggesting that increased expression of TRPM7 channels exacerbates zinc-induced cell damage. Similar to the AGS cells, in the presence of 100 μM 2-APB, the cell death was attenuated by $12.5 \pm 0.6\%$ in zinc 100 μM and $24.5 \pm 1.2\%$ in zinc 200 μM with the MTT assay ($n=6$; Figure 3A). In the presence of 10 μM Gd^{3+}, the cell death was attenuated by $14.5 \pm 1.1\%$ in zinc 100 μM and $29.5 \pm 1.4\%$ in zinc 200 μM with the MTT assay ($n=6$; Figure 3A). For determination that these effects were really mediated by activation of TRPM7 channels, we used RNAi. Similar to the AGS cells in Figure 1B, depletion of TRPM7 in HEK293 cells with overexpressed TRPM7 channels rendered the cells more resistant to zinc. In the presence of 200 μM

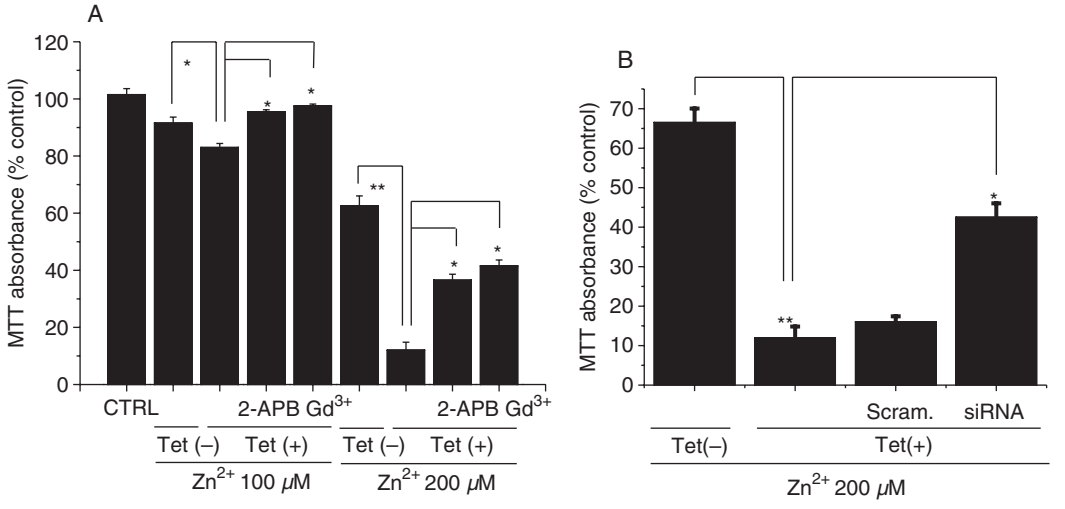

Figure 3. Effect of Zn^{2+} on cell viability with a MTT-based viability assay in HEK293 cells with overexpressed TRPM7 channels. (A) Cell viability was significantly decreased by Zn^{2+}. Inhibition of TRPM7 channels by 2-APB and Gd^{3+} reduced zinc toxicity. (B) Inhibition of TRPM7 channels by siRNA reduced zinc toxicity. Values are mean ± SEM. CTRL, AGS cells; Tet, tetracycline; Scram., scrambled siRNA; siRNA, TRPM7-specific siRNA. $*P<0.05$, $**P<0.01$.

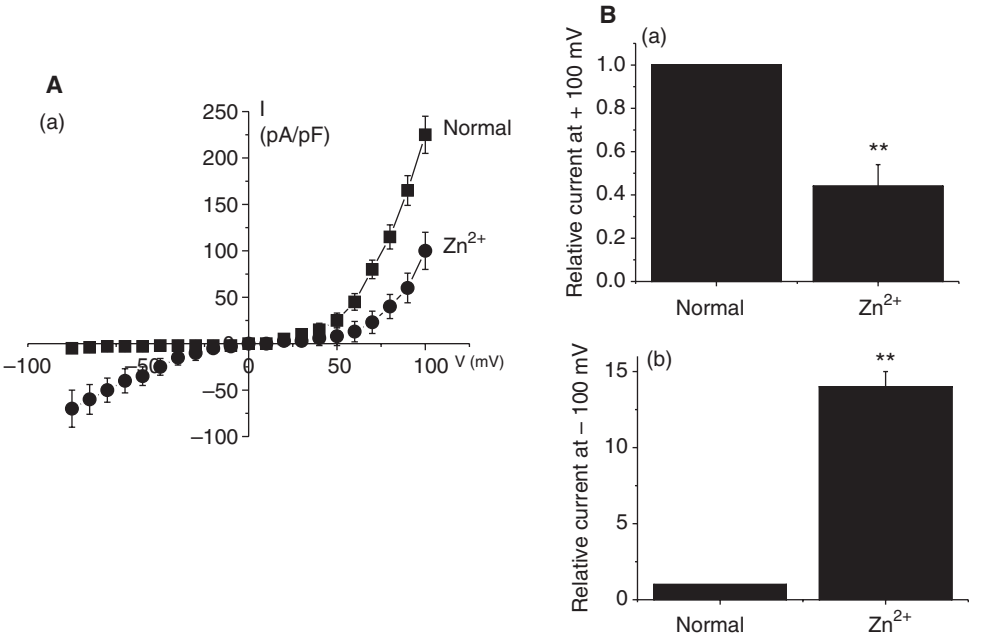

Figure 4. Effect of Zn^{2+} on transient receptor potential melastatin 7 (TRPM7)-like current in AGS cells. I–V curves (A) and summary bar graph (B) in the absence (■) and presence (●) of Zn^{2+}.

Zn^{2+}, the cell death was attenuated by $40.5 \pm 1.5\%$ in siRNA with the MTT assay ($n = 3$; Figure 3B). To investigate the effect of zinc on the electrophysiological characteristics in TRPM7 overexpressing cells, we recorded the TRPM7 currents. In the presence of zinc in the bath solution, the outward current was inhibited by $56.2 \pm 5.1\%$ and the inward current was increased by $140.1 \pm 10.5\%$ ($n = 5$; Figure 4). Taken together, these data are evidence that TRPM7 channels have a role in zinc-induced injury of HEK293 cells with overexpressed TRPM7.

Cell death by zinc leads to increased apoptosis

To determine whether AGS cell death occurred by apoptosis, we used sub-G1 analysis, and caspase-3 activity. As a method to analyze the mode of cell death in AGS cells by zinc, we used sub-G1 analysis (Hotz et al. 1994; Vermes et al. 2000). In this protocol, AGS cells with zinc are stained with a fluorescent DNA stain (such as PI). Due to the action of endogenous endonucleases in apoptotic cells, the DNA is cleaved into endonucleosomal fragments of typical sizes. These DNA fragments are extracted from the cells. This loss of DNA is detectable by FACS analysis, as the reduced nuclear staining of apoptotic cells results in a novel (sub-G1) fluorescence peak to the left of the regular fluorescence peak. The sub-G1 in AGS cells with zinc was markedly increased by $16.5 \pm 2.4\%$ (Figure 5A). However, in the presence of 2-APB and Gd^{3+}, the sub-G1 in AGS cells was decreased by $6.2 \pm 1.1\%$ and $9.1 \pm 2.1\%$, respectively (Figure 5A). Caspase-3 activation is one of the hallmarks of apoptotic cell death (Faleiro et al. 1997). We measured the enzyme activity in AGS cells with zinc. In AGS cells with zinc, caspase-3 activity was increased approximately 1.5-fold, but, in the presence of 2-APB and Gd^{3+}, caspase-3 activity decreased (Figure 5B).

Discussion

Zinc is an essential trace element not only for humans, but for all organisms. Optimal nucleic acid and protein metabolism, as well as cell growth, division, and function, require sufficient availability of zinc (Vallee and Falchuk 1993). The human body contains 2–3 g zinc, and nearly 90% is found in muscle and bone (Wastney et al. 1986). On the cellular level, 30–40% of zinc is localized in the nucleus, 50% in the cytosol and the remaining part is associated with membranes (Vallee and Falchuk 1993). Cellular zinc underlies an efficient homeostatic control that avoids accumulation of zinc in excess. The cellular homeostasis of zinc is mediated by two protein families; the zinc importer (Zip; Zrt-, Irt-like proteins) family, containing 14 proteins that transport zinc into the cytosol, and the zinc transporter (ZnT) family, comprising 10 proteins transporting zinc out of the cytosol (Lichten and Cousins 2009). Zinc has many physiological functions. Among them, the exact role of zinc in the regulation of apoptosis is ambiguous. A variety of studies indicate that,

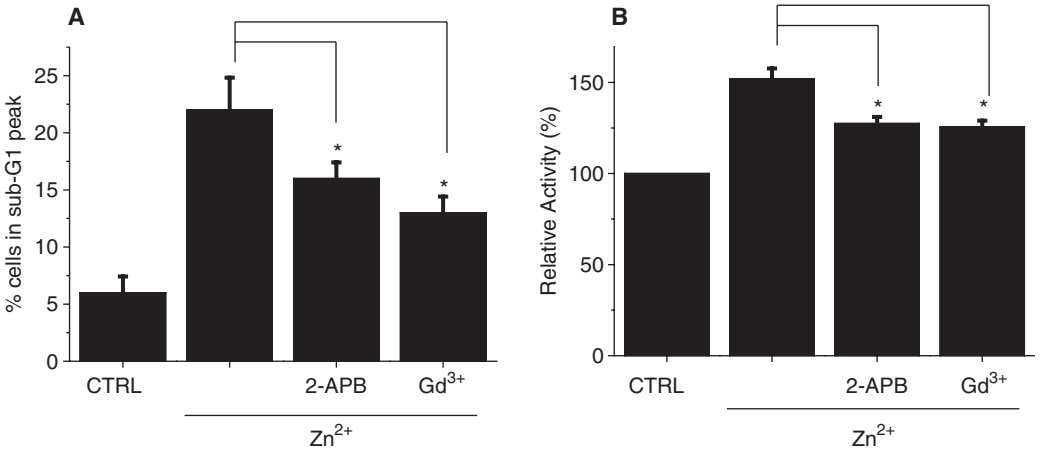

Figure 5. Cell death by Zn^{2+} lead to increased apoptosis. (A) Sub-G1 peak measured by FACScan. In the presence of 2-APB and Gd^{3+}, the sub-G1 in AGS cells was decreased. Quantitative data of three independent experiments. (B) Caspase-3 activities measured by enzyme assays. In the presence of 2-APB and Gd^{3+}, caspase-3 activity decreased. The specific activity was obtained from three samples per group. CTRL, AGS cells. $*P < 0.05$.

depending on its concentration, zinc can either be pro- or anti-apoptotic, and both zinc deprivation and excess can induce apoptosis in the same cell line (Haase et al. 2001; Formigari et al. 2007; Cummings and Kovacic 2009). The induction of apoptosis by high levels of intracellular zinc has been shown in different tissues and cell types (Truong-Tran et al. 2001; Watjen et al. 2002). Reports indicate that accumulation of intracellular zinc, as a consequence of either exogenous administration or release from intracellular stores by reactive oxygen species or nitrosation, activates pro-apoptotic molecules like p38 and potassium channels, leading to cell death (Kim et al. 1999; McLaughlin et al. 2001; Truong-Tran et al. 2001). Increased intracellular zinc levels may also induce cell death by inhibition of the energy metabolism (Brown et al. 2000; Sheline et al. 2000). In the context of its apoptosis-inducing properties, zinc has been shown to increase the expression of Bax, leading to a decrease in the Bcl-2/Bax ratio (Feng et al. 2008). As a consequence, dissipation of the mitochondrial membrane potential leads to the release of cytochrome-*c* from mitochondria into the cytosol (Kim et al. 1999; Bitanihirwe and Cunningham 2009). Zinc has an important role in gastric function and pathology. Zinc is important for gastric acid secretion reduction and tissue healing (Kadakia et al. 1992; Mann et al. 1992; Watanabe et al. 1995; Troskot et al. 1997; Tagliati et al. 1999). A few clinical and animal studies have shown the effect of zinc components in treatment and prevention of peptic ulcer (Cho et al. 1985; Bandyopadhyay and Bandyopadhyay 1997; Santos et al. 2004). In one study, the zinc serum level of people with peptic ulcer was lower than normal population while zinc amount in gastric mucus was higher probably because of zinc aggregation in injured mucus (Bandyopadhyay et al. 1995). Also, zinc content in the plasma of gastric cancer patients was lower than in the healthy group and the difference was statistically significant (Lu et al. 1999; Sempertegui et al. 2007).

In this study, we provided evidence suggesting that TRPM7 is an important player in zinc-induced cytotoxicity. (1) The MTT assay revealed that addition of zinc induced the death of AGS cells (Figure 1A). In the presence of 2-APB or Gd^{3+}, TRPM7 blockers and TRPM7-specific siRNA attenuated the death of AGS cells (Figure 1). By the patch clamp method, the TRPM7-like inward current was increased, but the outward was decreased (Figure 2). (2) Overexpression of TRPM7 channels enhances the zinc toxicity in HEK293 cells (Figure 3A). In the presence of 2-APB or Gd^{3+}, TRPM7 blockers and TRPM7-specific siRNA attenuated the death of HEK cells with overexpressed TRPM7 channels (Figure 3). By the patch clamp method, the TRPM7 inward current was increased, but the outward was decreased (Figure 4). (3) By FACScan and caspase-3 activity, zinc-mediated cell damage was by apoptotic processes.

Studies by Monteilth-Zoller et al. first indicated zinc permeation through recombinant TRPM7 channels, using electrophysiological measurement (Monteilh-Zoller et al. 2003; Frederickson et al. 2005). The studies were performed using millimolar concentrations of zinc, which are several orders of magnitude higher than the physiological/pathophysiological concentrations of zinc (Monteilh-Zoller et al. 2003; Frederickson et al. 2005). Recently, Inoue et al. (2010) explored the role of native TRPM7 channels in intracellular zinc accumulation at physiologically/

pathologically relevant concentrations (Frederickson et al. 2005), and, more importantly, in zinc-induced neuronal injury. They showed that in cultured mouse cortical neurons, TRPM7 was involved in Zn^{2+}-mediated neurotoxicity. In HEK293 cells with over-expressd TRPM7 channels, zinc could permeate HEK293 cells through TRPM7 and induce neurotoxicity. These results were inhibited by Gd^{3+}. Therefore, they suggested that TRPM7 channels may represent a novel target for neurological disorders where Zn^{2+} toxicity plays an important role.

We demonstrated that human gastric adenocarcinoma cells express functional TRPM7 channels that are involved in cell growth and survival (Kim et al. 2008). Therefore, we investigated the involvement of TRPM7 channels in zinc-mediated cell death in AGS cells. In AGS cells, TRPM7 channels have an important role in zinc-induced cell death. However, the cellular mechanisms in zinc-mediated cell death require more investigation. In future, the Bcl-2/Bax ratio, the change in cytochrome-*c* from mitochondria, or the involvement of other channels will be good themes to explore.

In summary, we have demonstrated that functional TRPM7 channels are involved, at least partially, in zinc-mediated cell survival in human gastric adenocarcinoma cells.

Acknowledgements

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