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Deficiency of calcium and magnesium induces apoptosis via scavenger receptor BI

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Abstract

Aims—Cell undergoes apoptosis in stressed status such as intracellular calcium overload or extracellular calcium/magnesium deficiency. The mechanisms of how deficiency of the divalent metal ions induces apoptosis remain to be defined. Scavenger receptor BI (SRBI) is a high density lipoprotein (HDL) receptor. Recent studies demonstrated that SR-BI is a stress response molecule which induces apoptosis upon serum deprivation. In this study, we assessed our hypothesis that the deficiency of calcium/magnesium induces apoptosis via SR-BI apoptotic pathway.

Main methods—We employed CHO cell lines expressing vector and SR-BI to test the effect of SR-BI on apoptosis induced by deficiency of calcium, magnesium and zinc in culture medium. The regain of different metal ions in deficient medium was also performed, respectively. Cell death was detected by morphological changes and quantified by LDH cytotoxicity assay. Apoptosis was also assessed by DNA ladder assay and DNA condensation assay. The SR-BIC323G mutant cells which lack the apoptotic activity of SR-BI were employed to verify the SR-BI-dependent effect on calcium/magnesium induced apoptosis.

Key findings—The deficiency of calcium/magnesium induced cell apoptosis CHO-SR-BI cells, but not in CHO-vector cells. Moreover, no apoptotic cell death was observed in SR-BIC323G mutant cells, indicating that the deficiency of divalent metal ions induces apoptosis in a SR-BI-dependent manner. Furthermore, the restoration of calcium or magnesium, but not zinc, protected CHO-SR-BI cells from apoptotic cell death, in a dose-dependent fashion.

Significance—These findings extend our understanding about how calcium and magnesium deficiency induces apoptosis.

Keywords

calcium; magnesium; apoptosis; scavenger receptor BI

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Introduction

Divalent metal ions possess a large variety of biological functions, including structural, catalytic and regulatory roles, making these metal ions crucial to health conditions. Calcium regulates numerous cellular events including gene transcription, muscle contraction, exocytosis and cell survival (Bading, et al. 1993, Kostyuk and Verkhatsky 1994, Kristian and Siesjo 1998). Magnesium is involved in more than 300 essential metabolic reactions, including energy production, DNA and RNA synthesis (Perez-Giraldo, et al. 1990). The deficiency of these metal ions is considered to be a potential health hazard. Indeed, calcium deficiency has been implicated in skeletal abnormalities and hypertension (Tang, et al. 2007, Wang, et al. 2008, Wien and Schwartz 1983). Magnesium deficiency leads to an increased incidence of cardiovascular diseases, including hypertension, stroke and atherosclerosis (Cerklewski 1983, Saris, et al. 2000, Seelig 1994, Song, et al. 2005), and gastrointestinal disorders, including loss of appetite, nausea and vomiting. Loss of zinc can also result in multi-organ system disorder symptoms (Lobo, et al. 2009, Sensi, et al. 2009, Weiss, et al. 2000). These observations indicate that divalent metal ions are essential for normal cellular activities. However, the underlying mechanisms of these signs of metal ion deficiency are still not fully revealed.

One of the mechanisms involved is metal ion related apoptosis. Apoptosis is required for eliminating unneeded and damaged cells in physiological conditions. So it is essential for normal tissue development and homeostasis. However, apoptotic cell death also occurs in response to cellular stresses which contributes to many forms of pathological cell loss and disorders. It has been well-established that a moderated rise in intracellular ionized calcium triggers apoptosis in many experimental models (Dunlap, et al. 1995, McConkey, et al. 1995, Orrenius and Nicotera 1994, Rizzuto, et al. 2003). Interestingly, emerging evidence also indicates that not only excessive but also deficiency in extracellular calcium induces apoptosis in multiple cell lines (Chiesa, et al. 1998, Lin, et al. 1998, Turner, et al. 2007). Chiesa *et al.* (Chiesa, et al. 1998) demonstrated that the apoptotic machinery in astrocytes is activated similarly not only by increased calcium influx but also by the extracellular calcium deprivation. Kluck *et al.* (Kluck, et al. 1994) employed calcium chelators to induce apoptosis in cultured cells, providing evidence that a raised intracellular ionized calcium is not universally present during the induction of apoptosis, and the loss of calcium homeostasis is a more determining factor in apoptotic cell death. As for magnesium, severe magnesium deficiency provokes pro-oxidative and pro-inflammation changes (Rayssiguier and Mazur 2005, Weglicki, et al. 1996, Weglicki, et al. 1994), and also has been shown to be pro-apoptotic in liver, heart and thymus (Malpuech-Brugere, et al. 1999, Martin, et al. 2003, Tejero-Taldo, et al. 2007). Furthermore, zinc deficiency has also been reported to induce apoptosis in cell cultures (Hyun, et al. 2001, Maclean, et al. 2001, Sakabe, et al. 1998, Sunderman 1995, Wood and Osborne 2001). Despite these observations, the mechanisms of how divalent metal ion deficiency induces apoptosis are still unclear.

SR-BI is a 75-kDa membrane protein expressed in the liver, endothelial cells, macrophages, and steroidogenic tissues (Krieger 1999). It is a well established high density lipoprotein (HDL) receptor and plays a key role in regulating plasma cholesterol levels and steroidogenesis (Acton, et al. 1996, Kozarsky, et al. 1997, Kraemer 2007). A number of recent studies suggest that SR-BI is a stress response molecule. Mice deficient in SR-BI failed to generate glucocorticosteroid in response to stresses such as endotoxemic or septic challenge and cold exposure (Cai, et al. 2008, Guo, et al. 2009, Hoekstra, et al. 2009, Li, et al. 2005). Using Chinese hamster ovary cell (CHO cell) system, we recently demonstrated that SR-BI induces apoptosis via the caspase-8 pathway in response to serum deprivation stress (Li, et al. 2005). We further demonstrated that SR-BI induces apoptosis in primary embryonic fibroblasts and aortic endothelial cells in the absence of serum, indicating that the

apoptotic activity of SR-BI is cell line independent (Li, et al. 2005). In this study, we proposed that the deficiency of divalent metal ions induces cell stress which triggers apoptotic cell death through SR-BI-mediated apoptotic pathway. To test this hypothesis, we employed CHO cell lines expressing SR-BI, and studied the role of divalent metal ions—calcium, magnesium, and zinc, by deprivation or addition of the divalent metal ions. We demonstrated that deficiency of calcium and magnesium but not zinc triggers apoptosis via SR-BI even in the presence of serum, and the restoration of calcium or magnesium effectively inhibits SR-BI induced apoptosis.

Materials and Methods

Materials

Normal F-12 nutrient mixture (Ham) and heat inactivated fetal bovine serum (FBS), and Prolong Gold antifade reagent with 4',6-diamidino-2-phenylindole (DAPI) were from Invitrogen. Slide-A-Lyzer dialysis cassettes were from Thermo Scientific. Calcium chloride dehydrate, magnesium chloride and zinc chloride were from Sigma-Aldrich. The cytotoxicity detection kit (lactate dehydrogenase (LDH) activity) and the apoptotic DNA ladder kit were from Roche Applied Science.

Deficient medium

F-12 nutrient mixture (Ham) without calcium chloride, magnesium chloride and zinc sulfate was custom made by Invitrogen. The medium was supplemented with 5% FBS, 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.3 mg/ml G418, and used as deficient medium for cell culture.

Cell Culture

The CHO cell lines stably expressing pLNCX2 (vector), wild type human SR-BI and SRBIC323G mutant were obtained as we described previously (Li, et al. 2002). As we indicated above, SR-BI-induced apoptosis is cell line independent so that we chose CHO cell system in this study. The CHO cells were cultured in complete medium, which is comprised of normal Ham's F-12 medium, 5% FBS, 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.3 mg/ml G418, to ~50% confluency at 37 °C. After that, cells were incubated in complete or the deficient medium or other reagents for 24 h and the apoptosis was determined by the assays described below.

Cytotoxicity assay

To quantitatively monitor cell damage, LDH activity in the cell culture supernatant was measured using the cytotoxicity detection kit following the manufacturer's instructions. Data are expressed as percentage of the total LDH activity.

DNA ladder assay

DNA fragmentation is a biochemical hallmark of apoptosis (Hengartner 2000). Cells were cultured in 10 cm plates and treated with corresponding reagents for 24 h. The ladder DNA was isolated using the apoptotic DNA ladder kit. Both adherent and detached cells were harvested. The isolated DNA was treated with RNase for 15 min at room temperature and applied to a 1.0% agarose gel electrophoresis.

Nuclear morphology assay

Cells were cultured on cover glasses in 6-well plates and treated with corresponding reagents for 24 h. Then cells were fixed with 3% paraformaldehyde (pH 7.4) for 15 min and then washed with phosphate buffered saline. After stained with DAPI, cells were observed

under a fluorescence microscope (Nikon eclipse Ti, Japan). Cells with condensed chromatin were considered as apoptotic cells, and the percentage of apoptotic cells was obtained by counting 5 independent fields of each slide.

Statistical analysis

Each experiment was conducted 3–5 times with triplicate samples. Results were expressed as means with their standard deviations. The statistical significance of differences between means was assessed by Student's *t* test. Differences in the means were considered statistically significant at $p < 0.05$.

Results

The deficiency of calcium, magnesium and zinc triggered apoptosis in a SR-BI dependent manner

Using CHO cells stably expressing vector cDNA, SR-BI or SR-BIC323G mutant, we previously demonstrated that SR-BI induces apoptosis in response to the stress of serum deprivation and a mutant at C323 abolishes the apoptotic activity of SR-BI (Li, et al. 2005). In this study, we employed this convenient cell system to elucidate whether the deficiency of calcium, magnesium and zinc triggers apoptosis via SR-BI. In order to deprive metal ions in the cell culture, we custom made Ham's F-12 medium specifically deficient in calcium, magnesium and zinc.

When the cells were kept in normal complete medium, all of the cells grew well and displayed a healthy spindle-like morphology (Fig. 1a, Complete medium). However, when the cells were cultured in deficient medium for 24 h, CHO-SR-BI cells underwent a major morphological change consistent with cells undergoing cell death (Fig. 1a, Deficient medium). In contrast, the CHO-vector cells maintained a healthy morphological appearance similar to the cells kept in the presence of metal ions. Cell death was further quantified by LDH cytotoxicity assay. When the cells were cultured in deficient medium, CHO-SR-BI cells released 15% of total cellular LDH into the culture medium, while CHO-vector cells released only 5% (Fig. 1b). These data indicate that deficiency of calcium, magnesium and zinc causes cell damage in a SR-BI dependent manner. To clarify whether the cell damage is caused by apoptosis, DNA fragmentation was assessed after 24 h incubation in complete medium or deficient medium. Clear DNA ladders were obtained in CHO-SR-BI cells in deficient medium, while only a trace amount of DNA ladder was detected in the CHO-vector cells (Fig. 1c). Finally, we used the nuclear morphology assay to further confirm that the cell death is due to apoptosis. As shown in Fig. 1, d and e, upon 24h incubation in deficient medium, significantly more cells with condensed chromatin were observed in CHO-SR-BI cells than in CHO-vector cells.

To further determine whether deficiency of calcium, magnesium and zinc induces apoptosis in a SR-BI dependent manner, we employed CHO-C323G cells. Our early study demonstrated that C323G mutant SR-BI lacks apoptotic activity (Li, et al. 2005). After 24 h incubation with the deficient medium, the CHO-SR-BIC323G cells kept normal cell growth and morphology, and showed no significant sign of cell death, in contrast to CHO-SR-BI cells (Fig. 1a). The LDH leakage of CHO-SR-BIC323G cells was 3%, which is 5-fold less than CHO-SR-BI cells and even less than CHO-vector cells (Fig. 1b). Moreover, DNA fragmentation and nuclear staining results were in agreement with morphological changes that no significant apoptosis was detected in CHO-SR-BIC323G cells (Fig. 1c to e). Thus, we conclude that the absence of calcium, magnesium and zinc induces apoptosis in a SR-BI dependent manner.

It was the absence of calcium and magnesium, but not zinc, that triggers SR-BI mediated apoptosis

To identify which metal ion(s) is the responsible one that regulates the SR-BI induced apoptosis, the regain of different metal ions in deficient medium was performed. The cells were first kept in normal complete medium to ~50% confluency, and then treated with deficient medium or deficient medium supplemented with calcium (0.3 mM), magnesium (0.6 mM) or zinc (0.03 mM), respectively. As shown in Fig. 2a, the supplementation of calcium or magnesium rescued CHO-SR-BI cells from cell death. To the contrary, the addition of zinc failed to prevent CHO-SR-BI cells from apoptosis. These results indicate that it is the absence of calcium and magnesium, but not zinc, which triggers SR-BI mediated apoptosis.

We further employed LDH assay to elucidate the effects of regain of calcium, magnesium or zinc on SR-BI-induced apoptosis, as shown in Fig. 2b. The restoration of calcium or magnesium provided significant protection against LDH release of CHO-SR-BI cells. But the addition of zinc was unable to prevent SR-BI induced cytotoxicity. Moreover, both calcium and magnesium prevented the DNA fragmentation while zinc had no such effect (Fig. 3a). Similarly, fluorescent staining of nuclear shows that both calcium and magnesium, but not zinc, abolished SR-BI induced chromatin condensation (Fig. 3, b and c), which further confirms that calcium or magnesium triggers apoptosis via SR-BI.

We then treated the cells with different concentrations of calcium or magnesium to identify the protective concentration range. As shown in Fig. 4, the cytotoxicity of CHO-SR-BI cells reduced in parallel with the increase of the calcium concentration, and magnesium had similar protective effect. Thus, calcium and magnesium provide protection against SR-BI induced cell death in a dose-dependent manner.

Discussion

Apoptotic cell death occurs when cells are exposed to cellular stress such as intracellular calcium overload (Lynch, et al. 2000, Tombal, et al. 1999, Zirpel, et al. 1998). Given the critical roles of divalent metal ions in maintaining cellular functions, it is not surprising that deficiency of the divalent metal ions puts the cells in a stressed status and induces apoptotic cell death. However, the molecular mechanisms underlying deficiency of divalent ions triggering apoptosis is unclear. In this study, we demonstrate that extracellular calcium/magnesium deficiency triggers apoptosis via SR-BI. When the SR-BI expressing CHO cells were treated with deficient medium without calcium, magnesium and zinc, apoptosis was detected as morphological change, LDH leakage, DNA fragmentation, and nuclear decomposition. Moreover, the apoptosis induced by the deficient medium was inhibited by exogenous supplementation of calcium or magnesium. These results indicate that calcium and magnesium are important modulators of apoptosis and their deficiency induces apoptosis via SR-BI apoptotic pathway.

Our previous studies demonstrated that mutation at C323 abolishes the apoptotic activity of SR-BI completely (Li, et al. 2005). Therefore, the CHO-SR-BIC323G cell is an ideal cell model to determine whether apoptosis induced by calcium/magnesium deficiency is SR-BI-dependent. As expected, CHO-SR-BIC323G cells displayed no significant apoptotic cell death in the absence of calcium/magnesium, which confirms that the deficiency of calcium and magnesium induced apoptosis via SR-BI.

Some early studies used metal ion chelators, such as ethylene diaminetetraacetic acid (EDTA), N,N,N',N'-tetraacetic acid (EGTA) and N,N,N',N'-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN), to deplete metal ions in cell culture in order to determine the role

of metal ions in apoptosis (Hyun, et al. 2001, Sakabe, et al. 1998). A potential problem with this approach is that the metal ion chelator chelates most of the transition metals such as calcium, magnesium, zinc, iron, copper and manganese, and the chelator binds with different metal ions with distinct affinity. As a result, it is difficult to identify which metal ion is the responsible one. Moreover, it is commonly believed that the addition of chelator should be carefully considered because of its partial permeability to a dialysis membrane and the intracellular entry of chelator will promote cell death directly (Fraker and Telford 1997, Jiang, et al. 1995, Treves, et al. 1994). As an alternative approach, we custom made cell culture medium specifically deficient in calcium and magnesium, which allows us to exclusively determine how deficiency of calcium/magnesium contributes to apoptotic cell death.

While we clearly demonstrated that low levels of calcium (< 0.3 mM) or magnesium (< 0.03 mM) trigger apoptosis via SR-BI, a critical question is whether our observations are physiological or pathological relevant. Indeed, under physiological circumstances, the circulating calcium (1.1–1.3 mM) or magnesium (0.46–0.6 mM) levels are high enough to block SR-BI apoptotic pathway (Heaney 2000, Ising, et al. 1995). However, under some pathological circumstances, such as severe ischemia or transplanted organs during cold preservation where no blood flows and the storage solution could hardly penetrate into the individual cell in the organs, the extracellular calcium and magnesium levels could be low enough to induce apoptosis in SR-BI expressing cells. Further study is warranted to determine whether SR-BI mediates apoptosis in these pathological circumstances.

Conclusions

In summary, we demonstrate that the deficiency of calcium and magnesium but not zinc triggers apoptotic cell death via SR-BI. Our findings indicate that the disruption of calcium/magnesium homeostasis, not only a sustained rise in intracellular calcium but also a decrease in extracellular calcium/magnesium, is a determining factor in apoptosis.

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Figure 1. The absence of calcium, magnesium and zinc triggered apoptotic cell death through SR-BI

CHO cells expressing wild type SR-BI (SR-BI), point mutant SR-BI (SR-BI-C323G), or pLNCX2 (Vector) were grown in normal complete medium to ~50% confluency. Then the cells were maintained in normal complete medium (Complete medium) or in medium deficient in calcium, magnesium and zinc (Deficient medium) for 24 h. **a**, the morphology of the cells was visualized by phase-contrast microscopy (X10). Representative data are from four independent experiments. **b**, cytotoxicity was determined by measuring LDH activity in the cell culture medium. * $P < 0.05$ and ** $P < 0.01$ versus vector. $n = 5$. **c**, DNA ladder detection. Both adherent and detached cells were collected to extract DNA. Representative

data are from three independent experiments. **d** and **e**. DAPI fluorescent nuclear staining of cells. Condensed chromatin (white arrowheads) represents typical apoptotic characteristics and the percentage of apoptotic cells was obtained by counting 5 independent fields of each slide. $*P < 0.05$ and $**P < 0.01$ *versus* vector. Data are from three independent experiments.

Figure 2. Calcium and magnesium, but not zinc, prevented SR-BI induced cell death

CHO cells were grown in complete medium to ~50% confluency, after which the culture medium was changed to deficient medium or deficient medium supplemented with calcium 0.3 mM or magnesium 0.6 mM or zinc 0.003 mM for 24 h. **a**, the morphology of the cells was visualized by phase-contrast microscopy (X10). Representative data are from four independent experiments. **b**, cytotoxicity was determined by measuring LDH activity in the cell culture medium. * $P < 0.05$ and ** $P < 0.01$ versus vector in the same treatment group; # $P < 0.05$ and ## $P < 0.01$ versus SR-BI in the deficient medium group. Data are from three independent experiments.

Figure 3. Effects of calcium, magnesium and zinc on SR-BI induced DNA fragmentation and chromatin condensation

CHO cells were grown in complete medium to ~50% confluency, after which the culture medium was changed to deficient medium or deficient medium containing calcium 0.3 mM or magnesium 0.6 mM or zinc 0.003 mM for 24 h. **a**, DNA ladder detection. Both adherent and detached cells were collected to extract DNA. **b**, DAPI fluorescent nuclear staining of cells. Condensed chromatin (white arrowheads) represents typical apoptotic characteristics and the percentage of apoptotic cells was obtained by counting 5 independent fields for each slide. * $P < 0.05$ and ** $P < 0.01$ versus vector. Data are from three independent experiments.

Figure 4. Calcium and magnesium prevented SR-BI induced cell death in a dose-dependent manner

CHO cells were grown in normal medium to ~50% confluency, after which the culture medium was changed to deficient medium or deficient medium containing calcium or magnesium at various concentrations. After 24 h, cytotoxicity was determined by measuring LDH activity in the cell culture medium. * $P < 0.05$ and ** $P < 0.01$ versus vector in the same treatment group. # $P < 0.05$ and ## $P < 0.01$ versus SR-BI in the deficient medium group. $n = 4$.