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Endoplasmic reticulum Ca^{2+} signaling and calpains mediate renal cell death

JF Harriman[1,4], XL Liu[1], MD Aleo[2], K Machaca[3] and RG Schnellmann*[,1,5]

[1] Department of Pharmacology and Toxicology, University of Arkansas for Medical Sciences, 4301 West Markham Street, Slot 638, Little Rock, Arkansas, AR 72205-7199, USA
[2] Drug Safety Evaluation, Pfizer, Inc., Eastern Point Road, Groton, Connecticut, CT 06340-4947, USA
[3] Department of Physiology, University of Arkansas for Medical Sciences, 4301 West Markham Street, Slot 505, Little Rock, Arkansas, AR 72205-7199, USA
[4] Current address: Wil Research, Inc., 1407 George Road, Ashland, Ohio, OH 44805-9281, USA
[5] Current address: Department of Pharmaceutical Sciences, Medical University of South Carolina, 280 Calhoun St., POB 250140, Charleston, SC 29425, USA
* Corresponding author: Rick G Schnellmann, Department of Pharmaceutical Sciences, Medical University of South Carolina, 280 Calhoun St., POB 250140, Charleston, SC 29425, USA. Tel: (843) 792-3754; Fax: (843) 792-2620; E-mail: schnell@musc.edu

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Abstract

The goal of the current study was to determine the roles of ATP content, endoplasmic reticulum (ER) Ca^{2+} stores, cytosolic free Ca^{2+} (Ca^{2+}_f) and calpain activity in the signaling of rabbit renal proximal tubular (RPT) cell death (oncosis). Increasing concentrations (0.3–10 μM) of the mitochondrial inhibitor antimycin A produced rapid ATP depletion that correlated to a rapid and sustained increase in Ca^{2+}_f, but not phospholipase C activation. The ER Ca^{2+}-ATPase inhibitors thapsigargin (5 μM) or cyclopiazonic acid (100 μM) alone produced similar but transient increases in Ca^{2+}_f. Pretreatment with thapsigargin prevented antimycin A-induced increases in Ca^{2+}_f and antimycin A pretreatment prevented thapsigargin-induced increases in Ca^{2+}_f. Calpain activity increased in conjunction with ER Ca^{2+} release. Pretreatment, but not post-treatment, with thapsigargin or cyclopiazonic acid prevented antimycin A-induced cell death. These data demonstrate that extensive ATP depletion signals oncosis through ER Ca^{2+} release, a sustained increase in Ca^{2+}_f and calpain activation. Depletion of ER Ca^{2+} stores prior to toxicant exposure prevents increases in Ca^{2+}_f and oncosis.

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Keywords: cell death; oncosis; renal proximal tubule; endoplasmic reticulum; calpain; calcium

Abbreviations: ER, endoplasmic reticulum; Ca^{2+}_f, cytosolic free Ca^{2+}; RPT, renal proximal tubule; SERCA, smooth endoplasmic reticulum Ca^{2+}-ATPase; LDH, lactate dehydrogenase; IP_3, inositiol trisphosphate; PLC, phospholipase C

Introduction

An initial effect of ischemia, hypoxia, or mitochondrial inhibition is the prevention of ATP production. When ATP production falls, protein kinases are unable to phosphorylate phosphoproteins and ATP-dependent ion transporters are inhibited. Further, the loss of oxidative phosphorylation and ATP depletion results in either oncotic (necrotic, rapid) or apoptotic (delayed) cell death, depending on the level of glycolytic ATP production. For example, in cultured mouse renal proximal tubules (RPT), Lieberthal *et al*.[1] determined that an 85% reduction in ATP levels was required to switch the mechanism of cell death from apoptosis to oncosis. RPT have low glycolytic activity and are very susceptible to ischemia- and toxicant-induced oncosis.

Many studies suggest that increases in cytosolic free Ca^{2+} (Ca^{2+}_f) mediate oncosis following anoxia/hypoxia and chemical exposure. For example, studies using concurrent measurement of Ca^{2+}_f and cell viability revealed that increases in Ca^{2+}_f occur prior to plasma membrane damage.[2,3] In addition, decreasing the extracellular Ca^{2+} concentration reduced the release of lactate dehydrogenase (LDH), a marker of cell death and membrane damage, from rabbit RPT subjected to anoxia or mitochondrial inhibition and rat RPT subjected to hypoxia.[4–6] Further, chelation of intracellular Ca^{2+} prevented extracellular Ca^{2+} uptake and cell death in mitochondrial inhibitor-exposed RPT.[7] Finally, evidence supporting a role for Ca^{2+} in cell injury comes from the observed cytoprotection of Ca^{2+} channel blockers in renal cell models subjected to anoxia or hypoxia.[6–8] These studies support an important role for Ca^{2+} in cell death and suggest that a rise in Ca^{2+}_f occurs before extracellular Ca^{2+} influx.

The endoplasmic reticulum (ER) contains the largest intracellular store of Ca^{2+} and is replenished by a high-affinity, low-capacity Ca^{2+}-ATPase uptake system (SERCA).[9,10] Physiological release of ER Ca^{2+} results from phospholipase C-mediated inositol trisphosphate (IP3) formation and its subsequent binding to IP3 receptors on the ER.[10] In many cells, depletion of ER Ca^{2+} stores triggers extracellular Ca^{2+} entry, resulting in a rise in Ca^{2+}_F and the refilling of the ER Ca^{2+} store. This influx pathway is termed capacitative Ca^{2+} entry or store-operated Ca^{2+} entry.[11] Waters *et al*.[12] suggested that ER Ca^{2+} release played a role in RPT cell death by demonstrating that depletion of ER Ca^{2+} stores prior to hypoxia or mitochondrial inhibitor exposure prevented cell death.

Supraphysiological and/or prolonged increases in Ca^{2+}_f are thought to activate degradative enzymes such as calpains, Ca^{2+}-activated cysteine proteases. Ischemic/hypoxic injury in brain, kidney, liver and myocardium is thought to be mediated by calpains.[13–19] Using the RPT model, we demonstrated that dissimilar calpain inhibitors decrease RPT cell death produced by a variety of

toxicants.[7,20,21] However, there is limited data demonstrating increased calpain activity during cell injury and death. Edelstein *et al*.[16] reported an increase in calpain activity in rat RPT subjected to anoxia and Bronk and Gores[13] reported an increase in calpain-like protease activity in rat hepatocytes subjected to anoxia. However, increases in calpain activity have not been examined in relation to increases in $Ca^{2+}{}_f$ during cell injury and death. The pathway in Figure 1 illustrates our current hypothesis for the role of ATP content, ER Ca^{2+} stores, cytosolic free Ca^{2+} and calpains in cell death. However, several critical questions remain. Is ER Ca^{2+} released during cell injury and death? What is the signal that results in the release of ER Ca^{2+}? Is calpain activity increased as a result of the ER Ca^{2+} release? The goal of the studies in this manuscript is to answer these questions.

Results

Characterization of ER Ca^{2+} release with ER Ca^{2+}-ATPase inhibitors

To determine the kinetics of ER Ca^{2+} release with smooth endoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitors, thapsigargin (5 μM) or cyclopiazonic acid (100 μM) was added to RPT suspensions and $Ca^{2+}{}_f$ monitored. Thapsigargin is an irreversible inhibitor and cyclopiazonic acid is a reversible inhibitor of SERCAs.[22,23] Inhibition of SERCAs quickly results in increases in $Ca^{2+}{}_f$ through the ER Ca^{2+} leak. Increases in $Ca^{2+}{}_f$ occurred within 10 s after exposure to thapsigargin or cyclopiazonic acid, resulting in similar peak $Ca^{2+}{}_f$ levels (184 ± 7 nM baseline to 275 ± 20 and 292 ± 13 nM, respectively) before returning to the baseline level (30 s after addition) (Figure 2A,B). Both agents produced similar rises and decays in $Ca^{2+}{}_f$ (Figure 2A,B). To test whether the ER Ca^{2+} store was completely depleted by 5 μM thapsigargin or 100 μM cyclopiazonic acid, two experiments were performed. In the first, the agents were added again 1 min following the first exposure to thapsigargin. The second addition of thapsigargin or cyclopiazonic acid did not result in a second peak, indicating ER Ca^{2+} depletion (data not shown). The addition of ionomycin in the absence of extracellular Ca^{2+} non-specifically releases internal Ca^{2+} stores. In the second experiment, pretreatment with thapsigargin deceased $Ca^{2+}{}_f$ increases due to ionomycin (10 μM) by 89%. To determine whether extracellular Ca^{2+} influx contributed to the rise or decay of $Ca^{2+}{}_f$, extracellular Ca^{2+} was chelated with EGTA. Decreasing extracellular Ca^{2+} levels to 100 μM immediately prior to thapsigargin or cyclopiazonic acid did not significantly alter the peak or decay of $Ca^{2+}{}_f$, suggesting little or no extracellular Ca^{2+} influx during or immediately after ER Ca^{2+} depletion with these agents (Figure 2).

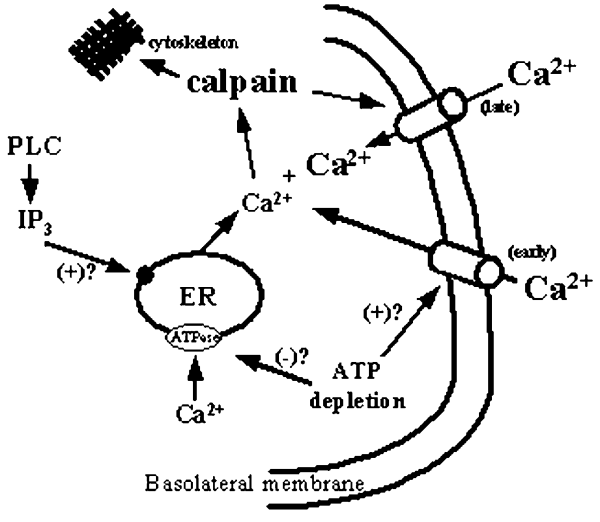

Figure 1 Hypothesis of $Ca^{2+}{}_f$ increases and calpain activation in RPT during antimycin A treatment. ER Ca^{2+} release may occur by either phospholipase C (PLC)-generated inositol trisphosphate (IP_3) binding to the IP_3 receptor on the ER or by inhibition of the ER Ca^{2+}-ATPase by ATP depletion. The early events upon antimycin A exposure include increases in $Ca^{2+}{}_f$ levels due to ER Ca^{2+} release and extracellular Ca^{2+} influx and resulting increase in calpain activity. Calpain activation leads to substrate hydrolysis, late stage Ca^{2+} influx and cell death

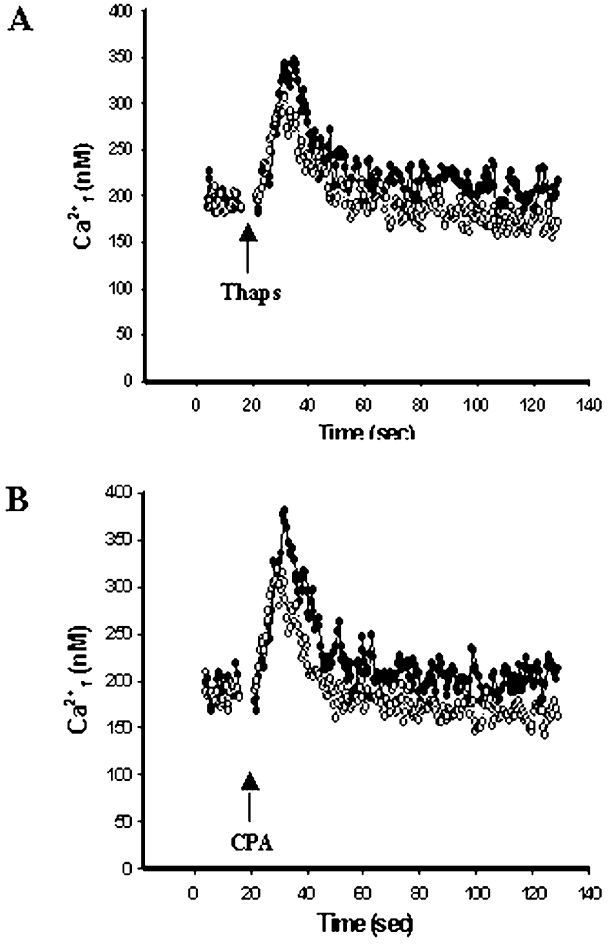

Figure 2 The effect of thapsigargin (Thaps) (**A**) and cyclopiazonic acid (CPA) (**B**) on cytosolic $Ca^{2+}{}_f$ levels in RPT. Thaps (5 μM) or CPA (100 μM) were added with or without addition of EGTA (900 μM) and readings obtained for an additional 2 min. Shown are representative traces (n=4). Closed circles are without EGTA, open circles are with EGTA

Effect of antimycin A exposure on Ca^{2+}_f levels

Antimycin A blocks the electron transport chain and has been used as a model of anoxia (i.e. chemical anoxia).[24] Antimycin A caused a concentration-dependent (0.3–10 μM) increase in Ca^{2+}_f levels, resulting in a sustained peak by 90 s (Figure 3A,B). Ca^{2+}_f measurements taken 15 and 30 min after addition of 10 μM antimycin A revealed that the initial increases in Ca^{2+}_f were sustained (data not shown).

Effect of antimycin A exposure on ATP levels

Experiments were conducted to determine the concentration- and time-dependent effects of antimycin A on ATP levels in RPT. Within 60 s of addition, antimycin A decreased ATP levels by 90% or greater at all concentrations used (0.3–10 μM) (Figure 4A). Time course experiments revealed that 10 μM antimycin A decreased ATP levels 89% within 30 s and 97% within 2 min (Figure 4B). The antimycin A-induced ATP depletion and increase in Ca^{2+}_f levels both occurred within 60 s following antimycin A addition, suggesting that depletion of ATP, the resulting inhibition of the ER Ca^{2+}-ATPase and ER Ca^{2+} release may account for the increases in Ca^{2+}_f.

Effect of ER Ca^{2+} depletion on antimycin A-induced Ca^{2+}_f increases

To determine whether antimycin A released the thapsigargin-sensitive ER Ca^{2+} pool and whether this release could account for the Ca^{2+}_f increase observed after antimycin A addition in the presence of extracellular Ca^{2+}, thapsigargin was added 100 s after antimycin A and Ca^{2+}_f levels were monitored. Thapsigargin addition did not result in additional Ca^{2+}_f increases after addition of antimycin A (Figure 5A). Likewise, when antimycin A was added 100 s after the depletion of ER Ca^{2+} with thapsigargin, antimycin A did not result in an increase in Ca^{2+}_f (Figure 5B). To determine whether the early release of ER Ca^{2+} prevented antimycin A-induced Ca^{2+}_f increases over an extended period of time, Ca^{2+}_f levels were measured 30 min after antimycin A with and without a 5-min pretreatment with thapsigargin or cyclopiazonic acid. Thapsigargin and cyclopiazonic acid also prevented the antimycin A-induced increases in Ca^{2+}_f observed at 30 min (data not shown).

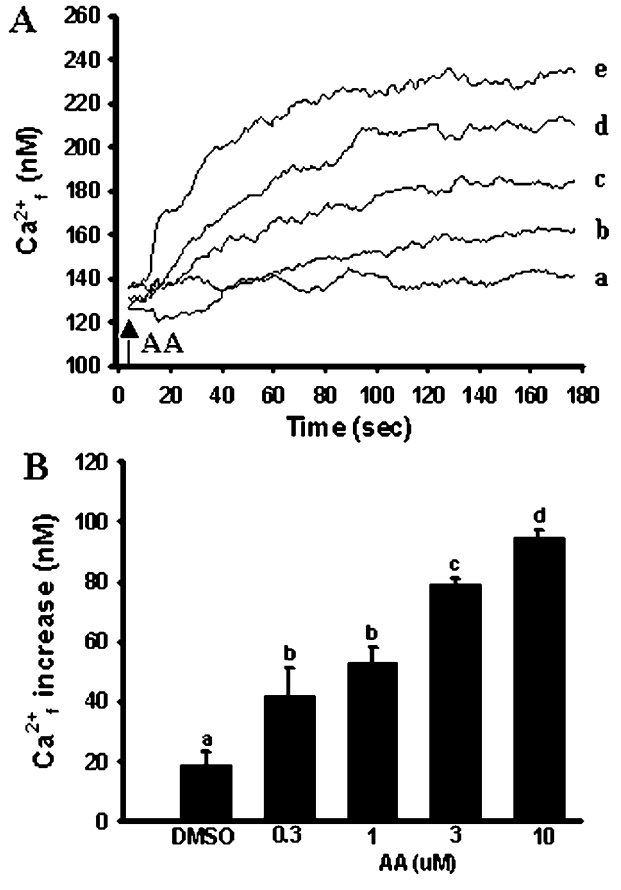

Figure 3 The effect of antimycin A (AA) on cytosolic Ca^{2+}_f levels in RPT. (**A**) Antimycin A was added at 15 s and readings obtained for an additional 3 min (a=dimethylsulfoxide control; b=0.3 μM; c=1.0 μM; d=3 μM; e=10 μM AA). Shown are representative traces (n=4). (**B**) Bars represent the means ± s.e. increase above baseline between 60 and 90 s after addition of AA (n=4). Bars with different letters are significantly different from one another ($P<0.05$)

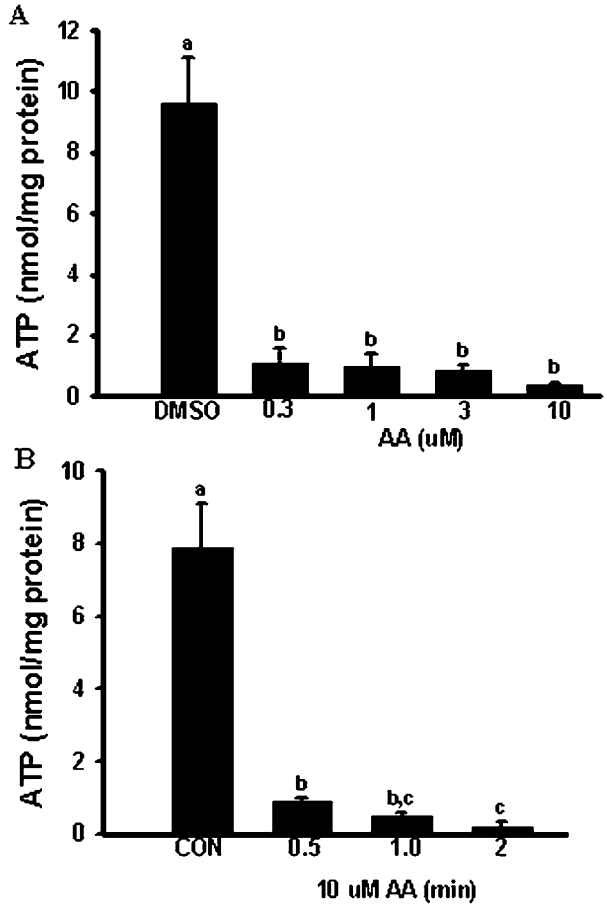

Figure 4 The effect of antimycin A (AA) on intracellular ATP content. (**A**) ATP content was determined 1 min after addition of the indicated concentration of AA. (**B**) ATP content was determined at the indicated time point after exposure to 10 μM AA. Bars represent means ± s.e. (n=3). Bars with different letters are significantly different from one another ($P<0.05$)

Effect of U73122 on antimycin A-induced $Ca^{2+}{}_f$ increases

The phospholipase C (PLC)-inositol phosphate pathway is a major Ca^{2+} signaling mechanism in cells.[25] Activation of PLC triggers the production of IP_3, the binding of IP_3 to IP_3 receptors on the ER and the release of ER Ca^{2+}. U73122 has been used in a number of cell types to inhibit PLC.[26–28] Inhibition of PLC results in the blockade of IP_3 production and thus serves as a useful pharmacological tool for determining whether IP_3-sensitive ER Ca^{2+} release plays a role in a cell response. To determine whether the antimycin A-induced Ca^{2+} response is mediated by PLC/IP_3, we pretreated RPT with 2 μM U73122 10 min prior to 10 μM antimycin A addition. Pretreatment with U73122 did not alter the antimycin A-induced increase in $Ca^{2+}{}_f$ (data not shown). As a positive control, ATP (50 μM) was added to RPT to activate membrane purinoceptors and cause the release of ER Ca^{2+} and a transient increase in $Ca^{2+}{}_f$ levels. A 10 min pretreatment with 2 μM U73122 inhibited the ATP-induced ER Ca^{2+} release by 76% but had no effect on thapsigargin-induced ER Ca^{2+} release (data not shown). These results suggest that antimycin A-induced ER Ca^{2+} release is through a PLC-independent mechanism.

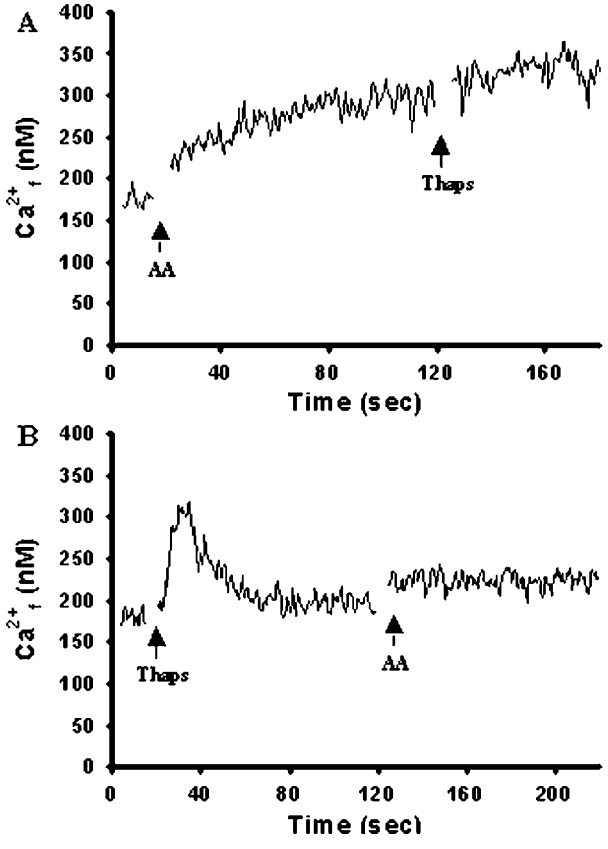

Figure 5 The effect of thapsigargin (Thaps) on antimycin A (AA)-induced increases in $Ca^{2+}{}_f$. (**A**) AA (10 μM) was added 100 s prior to Thaps (5 μM). (**B**) Thaps was added 100 s prior to AA. Shown are representative traces (n=4)

Examination of store-operated Ca^{2+} entry in rabbit RPT suspensions

To determine whether the increase in $Ca^{2+}{}_f$ following antimycin A addition was partly due to extracellular Ca^{2+} influx, 900 μM EGTA was added immediately prior to antimycin A and $Ca^{2+}{}_f$ levels determined between 60 and 90 s later. Chelation of extracellular Ca^{2+} significantly reduced the antimycin A-induced $Ca^{2+}{}_f$ increase from 182 ± 5 to 109 ± 10 nM. Extracellular Ca^{2+} influx was verified using a Mn^{2+} quench experiment, in which extracellular Mn^{2+} enters a cell similarly to Ca^{2+} but quenches the fluorescence of Fura.[29] After exposure to 10 μM antimycin A in the presence of 50 μM Mn^{2+}, Fura fluorescence was partially quenched, verifying that some extracellular Ca^{2+} influx occurred immediately after exposure to antimycin A (data not shown).

These experiments suggested that store operated Ca^{2+} entry may occur during RPT injury. To explore this possibility, control experiments were performed in which RPT in 1 mM extracellular Ca^{2+} were exposed to diluent (dimethylsulfoxide), thapsigargin, or antimycin A for 2 min before the addition of 10 mM Ca^{2+}. Addition of 10 mM Ca^{2+} after DMSO, thapsigargin and antimycin A resulted in $Ca^{2+}{}_f$ increases of 11, 56 and 10 nM, respectively. Although not typical of the large increases observed in other models,[11,30] a reproducible small increase in $Ca^{2+}{}_f$ levels was observed following addition of 10 mM Ca^{2+} in thapsigargin-treated *versus* diluent-treated RPT. However, the addition of 10 mM Ca^{2+} following 10 μM antimycin A exposure did not result in significant $Ca^{2+}{}_f$ increases, suggesting that store operated Ca^{2+} entry does not occur following mitochondrial inhibition and ATP depletion.

Effect of thapsigargin and antimycin A on calpain activity

To determine whether ER Ca^{2+} release can increase calpain activity, thapsigargin was added to RPT and calpain substrate hydrolysis measured. Thapsigargin 5 μM induced a significant increase in substrate hydrolysis within 2 min of addition, indicating that ER Ca^{2+} release increases calpain activity (Figure 6A). Antimycin A increased calpain activity numerically in a concentration-dependent manner with 10 μM antimycin A increasing calpain activity 25% in the first 2 min after its addition, in the same time frame as the ER Ca^{2+} release. (Figure 6B). Inhibition of calpain activity with calpain inhibitor 1 (300 μM) prevented the antimycin A-induced increase in calpain activity (data not shown).

Effect of ER Ca^{2+} depletion on antimycin A- and TFEC-induced cell death

A preliminary study demonstrated that thapsigargin-induced ER Ca^{2+} release prior to antimycin A exposure blocked RPTC cell death.[12] Additional experiments were conducted to further characterize the ER Ca^{2+} depletion and cell death induced by antimycin A and TFEC. Treatment of RPT with 10 μM antimycin A for 30 min resulted in an increase in LDH release (Figure 7A). A 5-min pretreatment with 5 μM thapsigargin or

100 μM cyclopiazonic acid prevented antimycin A-induced LDH release. Thapsigargin and cyclopiazonic acid pretreatment also decreased LDH release induced by a 3 h exposure to TFEC (Figure 7B). To document that the cytoprotective effect required ER Ca^{2+} depletion prior to toxicant exposure, thapsigargin was added after antimycin A or TFEC and LDH release measured. The addition of thapsigargin 15 min after antimycin A or 1 h after TFEC had no effect on antimycin A- or TFEC-induced LDH release (Figure 7).

Discussion

Increases in Ca^{2+}_f have been demonstrated to play a critical role during oncosis in numerous models. For example, a study using concurrent measurement of Ca^{2+}_f and cell viability with propidium iodide revealed that increases in Ca^{2+}_f occur prior to plasma membrane damage in hypoxia-treated RPT.[3] In addition, in RPT exposed to antimycin A for 30 min, chelation of intracellular and extracellular Ca^{2+}, as well as the Ca^{2+} channel blocker nifedipine, inhibited Ca^{2+} uptake and were cytoprotective.[7] Depletion of ER Ca^{2+} stores prior to antimycin A exposure or hypoxia prevented RPT cell death, indicating the important role of ER Ca^{2+}.[12] This study suggested that ER Ca^{2+} release, increased Ca^{2+}_f levels and extracellular Ca^{2+} influx are important mediators of oncosis. Further, the observation that inhibitors of the Ca^{2+}-activated protease calpain are cytoprotective and block extracellular Ca^{2+} influx in RPT exposed to diverse toxicants suggest that calpains are a downstream target of elevated Ca^{2+}_f.[7,20,21] These studies resulted in the model illustrated in Figure 1.

In the first series of experiments, antimycin A produced a rapid and sustained increase in Ca^{2+}_f. The relative increase in Ca^{2+}_f was similar to that produced by known inhibitors of the ER Ca^{2+}-ATPase, thapsigargin and cyclopiazonic acid. Further, neither the addition of thapsigargin prior to antimycin A nor the addition of antimycin A prior to thapsigargin resulted in an increase in Ca^{2+}_f when the second agent was added. These results show that the increase in Ca^{2+}_f observed following antimycin A exposure is due to ER Ca^{2+} release. Thapsigargin and cyclopiazonic acid produced a transient increase in Ca^{2+}_f since ATP is present and the plasma membrane Ca^{2+}-ATPase decreases Ca^{2+}_f. In contrast, the

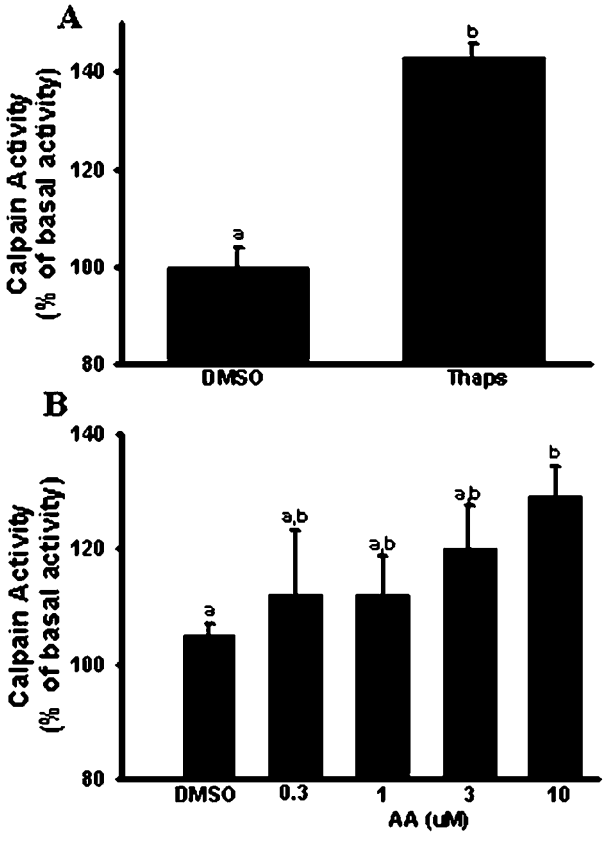

Figure 6 The effect of thapsigargin (Thaps) and antimycin A (AA) on calpain activity. After an initial baseline reading, Thaps (5 μM) or AA was added and readings were continued an additional 2 min. Bars represent the percentage of control calpain activity during the first 2 min after addition of Thaps or AA. Bars represent means $\pm$ s.e. (n=3–4). Bars with different letters are significantly different from one another ($P<0.05$)

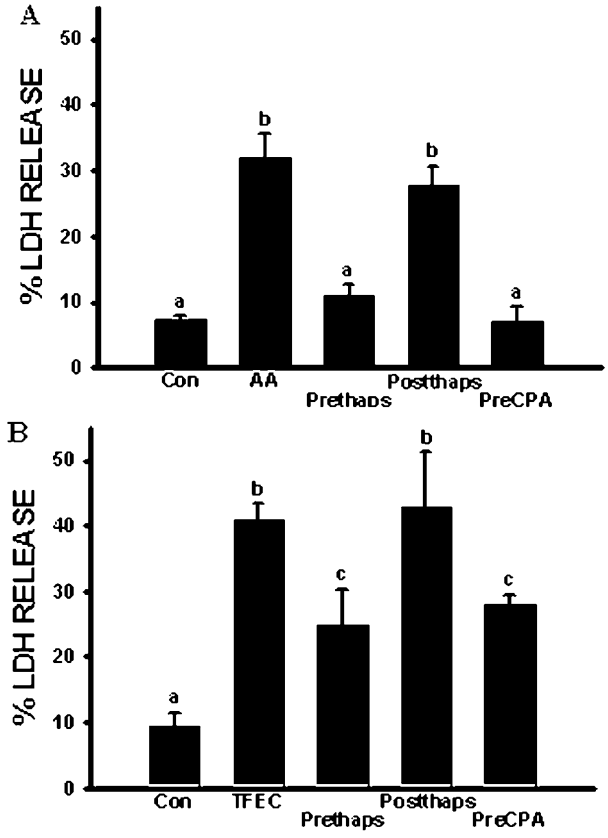

Figure 7 (**A**) The effects of thapsigargin (Pre-thaps or Post-thaps) and cyclopiazonic acid (CPA) on antimycin A (AA)-induced LDH release. Thapsigargin (5 μM) was added 5 min prior to AA (Pre-thaps) or 15 min after AA (Post-thaps). CPA (100 μM) was added 5 min prior to AA. LDH release was determined 30 min after AA addition. (**B**) The effect of thapsigargin (Pre-thaps or Post-thaps) and CPA on tetrafluoroethyl-L-cysteine (TFEC)-induced LDH release. Thapsigargin (5 μM) was added 5 min prior to TFEC (Pre-thaps) or 60 min after TFEC (Post-thaps). CPA (100 μM) was added 5 min prior to TFEC. LDH release was determined 180 min after TFEC addition. Bars represent means $\pm$ s.e. (n=3–4). Bars with different letters are significantly different from one another ($P<0.05$)

sustained increase in Ca^{2+}_f observed following antimycin A exposure is likely due to the absence of ATP and the inability of the plasma membrane Ca^{2+}-ATPase to pump Ca^{2+} out of the cell. In addition, antimycin A-induced Ca^{2+} influx is likely to contribute to the sustained Ca^{2+} rise.

Emptying of intracellular Ca^{2+} stores has been linked to store operated Ca^{2+} entry across the plasma membrane in numerous cell types.[31] For example, in a study by Demaurex *et al.*,[32] cyclopiazonic acid treatment of HL-60 cells in the presence of extracellular Ca^{2+} resulted in a sustained increase in Ca^{2+}_f, indicating a Ca^{2+} influx pathway secondary to ER Ca^{2+} release. A Ca^{2+} influx pathway also was observed in MDCK cells, accounting for half of the thapsigargin-evoked Ca^{2+} signal.[30] The data in the present manuscript suggest that store operated Ca^{2+} entry may occur to a limited degree in freshly-isolated rabbit RPT upon ER Ca^{2+} store depletion with thapsigargin, but does not occur during antimycin A-induced cell injury and death. These findings indicate the absence of store operated Ca^{2+} entry during injury and support prior evidence demonstrating an ATP requirement for store operated Ca^{2+} entry.[33]

In the second series of experiments we determined whether the antimycin A-induced ER Ca^{2+} release was the result of ATP depletion or PLC/IP_3 pathway. Pretreatment with the phospholipase C inhibitor U73122 had no effect on the antimycin A-induced Ca^{2+}_f increase, suggesting that the PLC/IP_3 pathway is not responsible for the observed ER Ca^{2+} release. However, antimycin A produced a rapid fall in ATP levels in the time frame of ER Ca^{2+} release. Within 1 min ATP depletion was 96% in RPT treated with 10 μM antimycin A. This degree of ATP depletion corresponds to an intracellular ATP concentration of approximately 150 μM (using the data in Figure 4 and an intracellular water volume of 2.4 μl/mg protein). Since the reported K_m for ATP on the ER Ca^{2+}-ATPase is 50–300 μM,[34] the ATP depletion produced by antimycin A would result in inhibition of ER Ca^{2+}-ATPase activity within the time frame of ER Ca^{2+} release. Further, this level of ATP depletion also would inhibit the plasma membrane Ca^{2+}-ATPase activity, resulting in the sustained Ca^{2+}_f increase.

ATP depletion and Ca^{2+}_f increases both occurred within 1 min following antimycin A exposure. The trend of ATP depletion suggests that Ca^{2+}_f would begin to increase when ATP depletion exceeds 80 to 85%. A number of studies over the past 15–20 years have noted that extensive ATP depletion is required for the initiation of oncosis. For example, Lieberthal *et al.*[1] found that oncosis occurred in cultured mouse RPT if ATP was depleted greater than 85%. Our results are consistent with their observations and provide an explanation relating ATP depletion to ER Ca^{2+}-ATPase inhibition, ER Ca^{2+} release and the onset of oncosis.

We have shown that diverse calpain inhibitors are cytoprotective and that calpain inhibition blocks antimycin A-induced extracellular Ca^{2+} influx.[7,21] These studies suggested that for calpains to be activated, Ca^{2+}_f must increase from an intracellular source. ER Ca^{2+} release produced by thapsigargin or antimycin A resulted in a rapid increase in calpain activity. Thus, antimycin A-induced ATP depletion inhibits the ER Ca^{2+}-ATPase, releasing the ER Ca^{2+} store and resulting in increases in Ca^{2+}_f and calpain activity. The increase in calpain activity following thapsigargin exposure does not cause oncosis because ATP levels are maintained. Calpains have a number of substrates, including cytoskeletal proteins, which may mediate the cell injury/death process. The identification of these substrates is the next step in understanding the mechanism of oncosis.

We extended our previous findings[12] and determined whether ER Ca^{2+} depletion protects RPT from TFEC toxicity. TFEC is a nephrotoxicant that is metabolized in RPT to a reactive electrophile that is capable of binding to nucleophiles and producing oncosis.[35] Pretreatments with thapsigargin or CPA were protective against antimycin A- and TFEC-induced cell death. However, thapsigargin did not alter LDH release if given after the initial exposure to the toxicants. Since oncosis produced by diverse mechanisms (reactive electrophile, mitochondrial inhibition, hypoxia) is blocked by ER Ca^{2+} depletion, ER Ca^{2+} signaling appears to be critical for oncosis.

From these data, we suggest that during ischemic/toxicant induced injury, rapid ATP depletion results in ER Ca^{2+} release, extracellular Ca^{2+} influx and a sustained increase in Ca^{2+}_f. The sustained increase in Ca^{2+}_f activates calpains to mediate the late stage cell death events, including a second phase of extracellular Ca^{2+} influx, cell swelling and the loss of plasma membrane integrity (Figure 1).

Materials and Methods

Reagents

Antimycin A, dimethylsulfoxide, thapsigargin and cyclopiazonic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). U73122 and Fura-PE3-2AM were obtained from Biomol (Plymouth Meeting, PA, USA) and TEFLABS (Austin, TX, USA), respectively. Tetrafluoroethyl-L-cysteine (TFEC) was synthesized according to the method of Moore and Green[36] and was a gift from Dr. Edward A Lock (Zeneca, Cheshire, UK). The sources of the remaining chemicals have been reported previously[37,38] or were obtained from Sigma Chemical Co. All glassware was silanized and autoclaved prior to use. All media were sterilized by filtering prior to use.

Preparation and incubation of RPT

Rabbit RPT were isolated and purified by the method of Rodeheaver *et al.*[37] and suspended in an incubation buffer containing 1 mM alanine, 4 mM dextrose, 2 mM heptanoate, 4 mM lactate, 5 mM malate, 115 mM NaCl, 15 mM $NaHCO_3$, 5 mM KCl, 2 mM NaH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$ and 10 mM HEPES (pH 7.4, 295 mOsm/kg). RPT suspensions (2 mg cellular protein/ml) were incubated at 37°C in an orbital shaking water bath (180 r.p.m.) under 95% air/5% CO_2 (40 ml/min flow rate). All experiments contained a 15-min preincubation period prior to any experimental manipulations.

Measurement of Ca^{2+}_f

RPT suspensions were incubated with 2 μM Fura-PE3-2AM and 0.1% pluronic acid for 60 min at 25°C. Fura-PE3-2AM resists the rapid

leakage and compartmentalization seen with Fura-2AM, yet has similar spectral properties.[39,40] RPT were washed and resuspended in 37°C incubation buffer. $Ca^{2+}{}_f$ was measured with a Hitachi F2000 spectrofluorometer (Hitachi Instruments, Danbury, CT, USA) equipped with a magnetic stirrer and a thermostatic cell holder (37°C). Following a 30 s equilibration period, baseline readings were obtained for 30 s. Antimycin A (0.3–10 μM), thapsigargin (5 μM), cyclopiazonic acid (100 μM), or the diluent (dimethylsulfoxide, $<0.5\%$ of total volume) was added and $Ca^{2+}{}_f$ was monitored for 2 min. For calibration, EGTA (1 mM) was added to correct for any extracellular Fura-PE3-2AM. After adding back $CaCl_2$ (2 mM), maximum and minimum fluorescence were determined using Triton X-100 (0.2%) followed by the Ca^{2+} chelator EGTA (20 mM). Readings were taken every 0.5 s and fluorescence measurements were made by alternating excitation wavelengths between 340 and 380 nm with continuous monitoring of emission at 510 nm. $Ca^{2+}{}_f$ levels were determined using the following formula: $[Ca^{2+}]_f=K_d(R\text{-}R_{min})/(R_{max}\text{-}R)\times(F_{min(380)}/F_{max(380)})$[41], where K_d=290 nM, R=the fluorescence emission ratio at 340 nm: 380 nm excitation and ($F_{min(380)}/F_{max(380)}$) is the ratio of minimum to maximum fluorescent intensity measured at 380 nm. For Mn^{2+} quench experiments, Mn^{2+} (50 μM) was added prior to antimycin A. Fluorescence was measured at the excitation wavelength of 360 nm and emission at 510 nm to achieve Ca^{2+}-independent fluorescence.[29]

Biochemical assays

Release of LDH into the medium was used as a marker of cell death as described previously.[42] ATP levels were analyzed by reverse-phase high performance liquid chromatography as described previously.[37] Cellular calpain activity was measured as described previously[21] with the following modifications. Baseline calpain activity was determined for 15 min after substrate (SLLVY-AMC) addition. Antimycin A or thapsigargin was then added and additional readings taken for the next two min. Calpain activity is reported as a percentage of the control linear change in fluorescence.

Statistics

Data are presented as means $\pm$ s.e.m. of at least three experiments. RPT suspensions isolated from one rabbit represent a separate experiment (N of 1). Data were analyzed by ANOVA and multiple means compared using Student-Newman-Keuls test with $P<0.05$ indicating significance.

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