BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **240,** 57–60 (1997)
ARTICLE NO. RC977606

Depletion of Endoplasmic Reticulum Calcium Stores Protects against Hypoxia- and Mitochondrial Inhibitor-Induced Cellular Injury and Death

Shayla L. Waters, Jeremy K. Wong, and Rick G. Schnellmann
Department of Pharmacology and Toxicology, University of Arkansas for Medical Sciences, 4301 W. Markham St., Slot 638, Little Rock, Arkansas 72205-7199

Received October 3, 1997

We have shown previously that intracellular Ca^{+2} chelation and calpain inhibitors block the influx of extracellular Ca^{+2} and Cl^- during the late phase of cell injury in renal proximal tubules (RPT) exposed to the mitochondrial inhibitor antimycin A. Since the endoplasmic reticulum (ER) is the major intracellular Ca^{+2} storage site, ER Ca^{+2} release/depletion may mediate the Ca^{+2} influx and cell death. Treatment of RPT suspensions with thapsigargin, an ER Ca^{+2}-ATPase inhibitor, increased cytosolic free Ca^{+2} (Ca_f^{+2}) levels from 122 ± 7 to 322 ± 55 nM within 10 sec of addition followed by a return to control levels within 3 min. A 5-min pretreatment of RPT suspensions with thapsigargin blocked antimycin A- and hypoxia-induced influx of extracellular Ca^{+2} and Cl^- and the resulting cell death/lysis. These data suggest that ER Ca^{+2} release/depletion during cell injury may trigger a signaling cascade that causes extracellular Ca^{+2} influx followed by Cl^- influx, cell swelling, and ultimately cell death/lysis.

The cascade of cellular events that occurs during anoxia/hypoxia- and toxicant-induced cellular injury leading to necrotic cell death has gradually been elucidated in numerous models. In rabbit renal proximal tubules (RPT), cessation of respiration caused by either mitochondrial inhibition or hypoxia/anoxia results in intracellular ATP and K^+ depletion and a rise in intracellular Na^+ levels (1,2). While these initial events are generally agreed upon, those that occur during the late phase of cell injury are not entirely understood.

Abbreviations: renal proximal tubules (RPT), lactate dehydrogenase (LDH), 3-(4-iodophenyl)-2-mercapto-(Z)-2-propenoic acid (PD150606), N-acetyl-leu-leu-norleucinal (calpain inhibitor 1), ethyleneglycol-bis(β-aminoethylether) N, N, N′, N′-tetraacetic acid (EGTA), ethyleneglycol-bis(β-aminoethylether) N, N, N′, N′-tetraacetoxymethyl ester (EGTA-AM), endoplasmic reticulum (ER).

The precise role of Ca^{+2} during the cell death cascade is still controversial (3-9). We recently demonstrated that extracellular Ca^{+2} influx occurs in the late phase of RPT cell injury through a nifedipine-sensitive, verapamil-insensitive pathway and plays a critical role in cellular injury and death/lysis (10). Furthermore, we showed that chelation of extracellular Ca^{+2} with EGTA or intracellular Ca^{+2} with EGTA-AM prevents mitochondrial inhibitor-induced cell death and extracellular Ca^{+2} influx. These data suggest that increases in Ca_f^{+2} levels and extracellular Ca^{+2} influx are both important components of the cell death cascade in RPT.

One potential mechanism of Ca^{+2}-induced cellular injury involves calpain activation. Calpains are Ca^{+2}-dependent non-lysosomal, neutral, cysteine proteases found in most cells (11). Investigators have suggested that calpain activation contributes to anoxia- and/or toxicant-induced cell death, and studies have shown that inhibiting calpains is cytoprotective (10, 12-18). Interestingly, recent data indicated that calpain inhibition also blocks mitochondrial inhibitor-induced extracellular Ca^{+2} influx (10). Thus, calpain activation may play a role in the signaling cascade that mediates the extracellular Ca^{+2} influx that occurs during the late phase of cell injury.

Leaf (2) originally demonstrated that Cl^- influx occurs following mitochondrial inhibition and contributes to cell swelling and lysis. Subsequent studies in our laboratory have shown that Cl^- influx occurs after a lag period following mitochondrial inhibition and during the final phase of cell injury (19-21). Recent experiments demonstrated that inhibition of extracellular Ca^{+2} influx with the Ca^{+2} channel blocker nifedipine, as well as calpain inhibition with calpain inhibitor 1 and PD150606 inhibits this extracellular Cl^- influx (10). Furthermore, calpain inhibitor 1 and PD150606 blocked calcium ionophore (ionomycin)-induced Cl^- influx and cell death. Collectively, these studies suggest that calpain activation and extracellular Ca^{+2} influx precede and may mediate extracellular Cl^- influx.

0006-291X/97 $25.00

At present, it is unknown how mitochondrial inhibition elicits the initial calpain activation and extracellular Ca^{+2} influx. We hypothesized that the release/depletion of intracellular Ca^{+2} stores results in calpain activation and subsequent extracellular Ca^{+2} influx. Since the primary intracellular store of Ca^{+2} is the endoplasmic reticulum (ER), the goal of the current study was to determine whether 1) the ER Ca^{+2}-ATPase inhibitor thapsigargin (22,23) releases/depletes ER Ca^{+2} stores in rabbit RPT and 2) prior depletion of ER Ca^{+2} stores prevents antimycin A- and hypoxia-induced extracellular Ca^{+2} and Cl^- influx, and cell death/lysis.

MATERIALS AND METHODS

Reagents. $^{36}Cl^-(Na^+)$, $^{45}Ca^{+2}(2Cl^-)$ and [^{14}C]dextran were obtained from Dupont NEN (Boston, MA). Antimycin A and dimethylsulfoxide were purchased from Sigma Chemical Co. (St. Louis, MO). Thapsigargin was obtained from Calbiochem (San Diego, CA). Fura-PE3 was purchased from TEFLABS (Austin, TX). The sources of the remaining chemicals have been reported previously (24,25). All glassware was silanized and autoclaved prior to use. All media and buffers were sterilized by filtering prior to use.

Preparation and incubation of RPT. Rabbit RPT were isolated and purified as described previously (24,25) 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 (1 mg cellular protein/ml) were incubated at 37° C in an orbital shaking water bath (180 rpm) under 95% air/5% CO_2 (40 ml/min flow rate). All experiments contained a 15 min preincubation period with no experimental manipulations. Thapsigargin (5 μM) was added 5 min prior to antimycin A (1 μM) or initiation of hypoxia with a continuous stream of 95%N_2/5%CO_2, and the incubation continued for an additional 30 min. Aliquots were removed and lactate dehydrogenase (LDH) release determined as described previously (26).

Cl^- and Ca^{+2} influxes. Cl^- and Ca^{+2} influxes were determined by adding a tracer amount of $^{36}Cl^-$ (Na^+) or $^{45}Ca^{+2}$ ($2Cl^-$) to RPT suspensions 15 min after antimycin A addition or hypoxia initiation (19-21). Fifteen min later, aliquots were removed and RPT separated from the surrounding buffer by rapid centrifugation through a layer of dibutylphthalate:dioctylphthalate (2:1). RPT $^{36}Cl^-$ and $^{45}Ca^{+2}$ contents were determined by resuspending the pellets in Triton X-100 solubilization buffer (100 mM Tris, 150 mM NaCl and 0.05% Triton X-100 at pH 7.5) and aliquots taken for liquid scintillation spectrometry and protein determination. Extracellular $^{36}Cl^-$ and $^{45}Ca^{+2}$ were corrected for using the extracellular water marker [^{14}C]-dextran. Tubular protein concentration was determined using the biuret method (31).

Measurement of Ca_f^{+2}. After a 15 min preincubation period at 37°C, RPT suspensions (2 mg/ml) were incubated with Fura-PE3 (5 μM) for 30 min at 25°C. RPT were then washed once (500 × g for 2 min) in incubation buffer warmed to 37°C. After resuspension in 37°C incubation buffer, a 1.5 ml aliquot was placed in a 1 cm^2 quartz cuvette and positioned in a Hitachi F2000 spectrofluorometer (Hitachi Instruments, Danbury, CT) equipped with a magnetic stirrer and a thermostatic cell holder. RPT were continuously stirred using a magnetic stir bar and maintained at 37°C. Following a 30 sec equilibration period, baseline readings were obtained for 30 additional sec before addition of the test compound. Thapsigargin (5 μM) or the diluent dimethylsulfoxide (< 0.1%) were added to the cuvette and readings collected for 5 min. Fluorescence measurements were made by alternating excitation wavelengths between 340 nm and 380 nm and continuously monitoring emission at 510 nm. In matched flasks, fluorescence due to extracellular Fura-PE3 was corrected for with EGTA (1 mM). Readings were taken every 0.5 sec and stored by a computer for later analysis. Maximum and minimum fluorescence were determined using Triton X-100 (0.2%) followed by the Ca^{+2} chelator EGTA (10 mM), respectively. Ca_f^{+2} levels were determined using the following formula: $[Ca^{+2}]_f = K_d(R - R_{min})/(R_{max} - R)$ (27), where K_d = 290 nM and R = the fluorescence emission ratio at 340 nm:380 nm excitation.

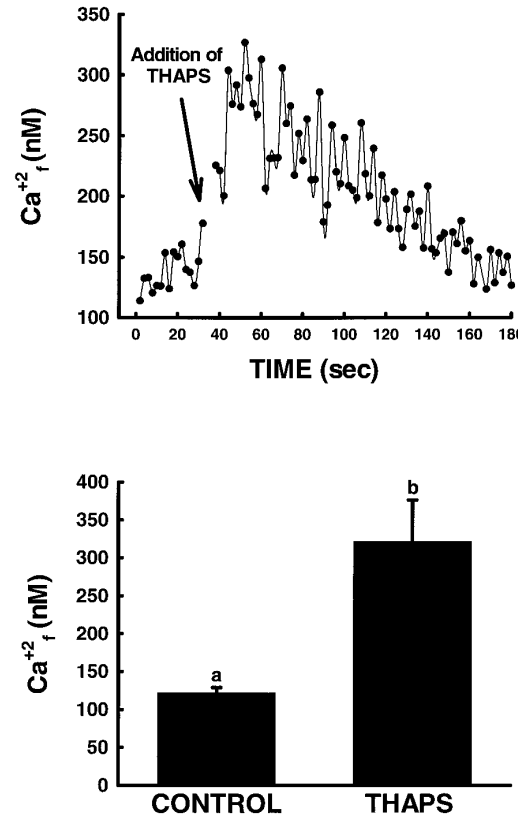

FIG. 1. The effect of thapsigargin addition on cytosolic Ca_f^{+2} levels in rabbit RPT suspensions. The upper panel is a representative tracing of 4 individual experiments. Baseline readings were obtained for 30 sec. Thapsigargin (5 μM) was added at 30 sec and readings were obtained for an additional 4.5 min. The lower panel illustrates cytosolic Ca_f^{+2} levels 10 sec post thapsigargin (THAPS, 5 μM) addition. Bars represent the means ± SE (N=4). Bars with different letters are significantly different from one another ($P < 0.05$).

Statistics. The data are presented as means ± SE. RPT suspensions isolated from one rabbit represented a separate experiment (N of 1). Data were analyzed by ANOVA and multiple means compared using Fisher's protected LSD test with $p < 0.05$.

RESULTS

To determine whether thapsigargin releases/depletes ER Ca^{+2} stores, Ca_f^{+2} levels were measured following a single or double exposure to thapsigargin. Figure 1 (upper panel) shows a representative tracing obtained from RPT exposed to thapsigargin. Quantitative results pooled from 3 separate experiments demon-

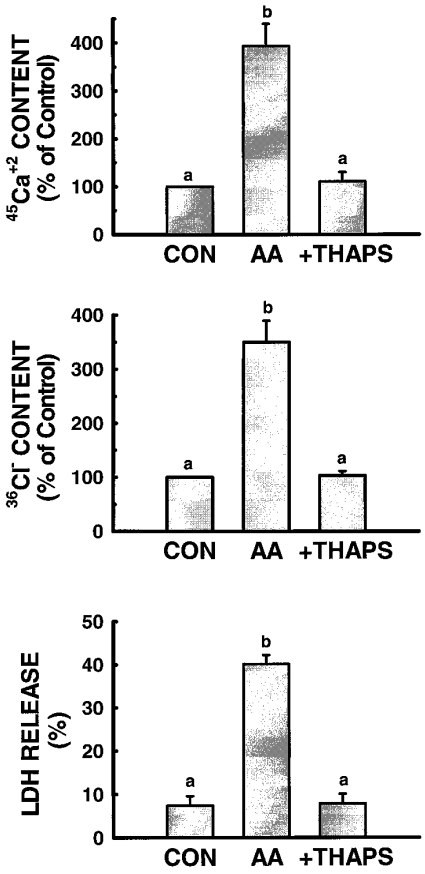

FIG. 2. The effect of thapsigargin (THAPS) on antimycin A (AA)-induced RPT $^{45}Ca^{+2}$ influx (upper panel), $^{36}Cl^{-}$ influx (middle panel), and LDH release (lower panel). THAPS (5 μM) was added 5 min prior to AA. $^{45}Ca^{+2}$ or $^{36}Cl^{-}$ was added 15 min after AA and RPT $^{45}Ca^{+2}$ or $^{36}Cl^{-}$ content determined 15 min later. LDH release was determined 30 min after AA addition. Control (CON) RPT $^{45}Ca^{+2}$ and $^{36}Cl^{-}$ content were 653 ± 57 and 812 ± 74 dpm/mg protein, respectively. Bars represent the means ± SE (N=4–5). Bars with different letters are significantly different from one another ($P < 0.05$).

strate that within 10 sec of thapsigargin addition, Ca_f^{+2} levels increase from 122 ± 7 nM to 322 ± 55 nM (Figure 1, lower panel). The increase in Ca_f^{+2} returned to control levels within 3 min, and a second addition of thapsigargin did not increase Ca_f^{+2} (data not shown). These data demonstrate that addition of the ER Ca^{+2}-ATPase inhibitor thapsigargin increases Ca_f^{+2} levels through the release/depletion of ER Ca^{+2} stores.

To determine if ER Ca^{+2} release/depletion plays a role in mitochondrial inhibitor- or hypoxia-induced RPT cell death, the effect of preincubation of thapsigargin on antimycin A (Figure 2, bottom panel)- and hypoxia (Figure 3, bottom panel)-induced LDH release was determined. RPT were preincubated with thapsigargin for 5 min and LDH release determined 30 min subsequent to the initiation of hypoxia or antimycin A addition. Antimycin A and hypoxia increased LDH release to 40 ± 2% and 39 ± 2%, respectively. LDH release in control RPT were 7 ± 2% and 10 ± 1% in antimycin A and hypoxia experiments, respectively. Thapsigargin pretreatment blocked LDH release under both conditions.

Our laboratory has demonstrated previously that antimycin A exposure results in the influx of extracellular Ca^{+2} and Cl^{-} in RPT (10, 19-21). Thus, experiments were conducted to examine the effect of thapsigargin pretreatment on antimycin A- and hypoxia-induced influxes of extracellular Ca^{+2} and Cl^{-}. Antimycin A and hypoxia resulted in approximately a 3.8-fold (Figure 2, upper panel) and 3.3-fold (Figure 3, upper panel) increase in RPT $^{45}Ca^{+2}$ content and a 3.4-fold (Figure 2, middle panel) and 2.9-fold (Figure 3, middle panel) increase in RPT $^{36}Cl^{-}$ content, respectively. Thapsigargin pretreatment of RPT blocked both responses. These

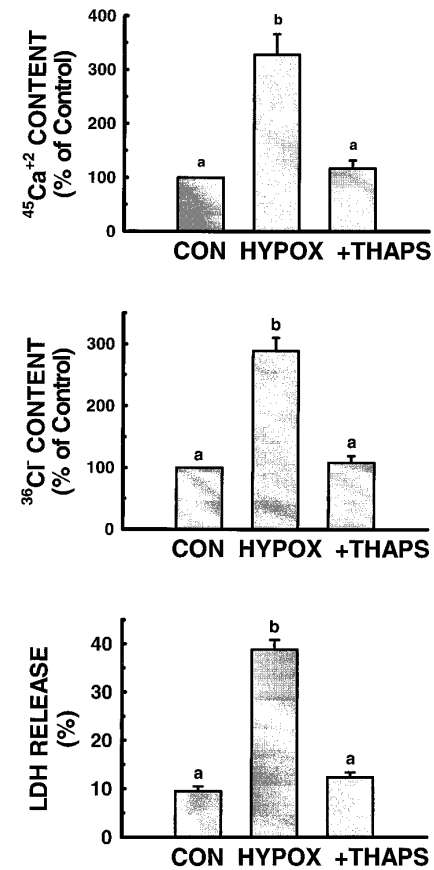

FIG. 3. The effect of thapsigargin (THAPS) on hypoxia (HYPOX)-induced RPT $^{45}Ca^{+2}$ influx (upper panel), $^{36}Cl^{-}$ influx (middle panel), and LDH release (lower panel). THAPS (5 μM) was added 5 min prior to the initiation of HYPOX. $^{45}Ca^{+2}$ or $^{36}Cl^{-}$ was added 15 min after HYPOX initiation and RPT $^{45}Ca^{+2}$ or $^{36}Cl^{-}$ content determined 30 min later. LDH release was determined 45 min after HYPOX initiation. Control (CON) RPT $^{45}Ca^{+2}$ and $^{36}Cl^{-}$ contents were 772 ± 81 and 993 ± 84 dpm/mg protein, respectively. Bars represent the means ± SE (N=4–5). Bars with different letters are significantly different from one another ($P < 0.05$).

data suggest that ER Ca^{+2} release/depletion plays a role in antimycin A- and hypoxia-induced influxes of extracellular Ca^{+2} and Cl^-, and cell death/lysis.

DISCUSSION

The current data demonstrate that thapsigargin elicits a 2.6-fold increase in RPT Ca_f^{+2} concentrations within 10 sec of addition with a return to control levels in approximately 3 min. A second addition of thapsigargin did not increase Ca_f^{+2} concentrations, suggesting that thapsigargin depleted ER Ca^{+2} stores. Thus, the release/depletion of ER Ca^{+2} stores results in an increase in Ca_f^{+2} concentrations followed by a return to control levels, most likely due to plasma membrane Ca^{+2}-ATPase mediated Ca^{+2} extrusion, under physiological conditions.

In addition, the current studies show that ER Ca^{+2} release/depletion with thapsigargin pretreatment blocks mitochondrial inhibitor- and hypoxia-induced extracellular Ca^{+2} and Cl^- influx and cell death/lysis. The mechanism(s) through which mitochondrial inhibition or hypoxia release ER Ca^{+2} under these pathological conditions is not clear. However, since previous studies demonstrated that an intracellular Ca^{+2} chelator and calpain inhibitors block antimycin A-induced extracellular Ca^{+2} influx (10), Ca^{+2} release/depletion may increase Ca_f^{+2}, activate calpain, and elicit calpain-mediated extracellular Ca^{+2} influx. The lack of ATP under these conditions would prevent removal of the accumulated Ca^{+2} and result in a pathological state.

Temporally, the loss of intracellular ATP, K^+ efflux, Na^+ influx and a more positive membrane potential are early events in cell injury (1,2). The current data suggest that in the late phase of rabbit RPT cell injury, ER Ca^{+2} release/depletion may trigger calpain activation and extracellular Ca^{+2} influx. We have shown previously that the influx of extracellular Ca^{+2} is followed by Cl^- influx through a 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB)- and diphenylamine-2-carboxylate (DPC)-sensitive pathway ultimately resulting in terminal cell swelling and lysis (21).

In summary, we have shown that the ER Ca^{+2}-ATPase inhibitor thapsigargin releases/depletes ER Ca^{+2} stores under physiological conditions in rabbit RPT, and that prior depletion of ER Ca^{+2} stores prevents antimycin A- and hypoxia-induced extracellular Ca^{+2} and Cl^- influx, and cell death/lysis. These novel data provide evidence suggesting that ER Ca^{+2} release plays a role in the necrotic cell death cascade in rabbit RPT.

ACKNOWLEDGMENTS

The authors thank Dr. Philip R. Mayeux for the use of his spectrofluorometer and Mr. Satinder S. Sarang for his technical assistance. S. L. Waters was supported by an American Heart Association Arkansas Affiliate Predoctoral Fellowship.

REFERENCES

1. Gullans, S. R., Brazy, P. C., Soltoff, S. P., Dennis, V. W., and Mandel, L. J. (1982) *Am. J. Physiol.* **243,** F133–F140.
2. Leaf, A. (1959) *Ann. New York Acad. Sci.* **72,** 396–404.
3. Herman, B., Gores, G. J., Nieminen, A. L., Kawanishi, T., Harman, A., and Lemasters, J. J. (1990) *Crit. Rev. Toxicol.* **21,** 127–148.
4. Farber, J. L. (1990) *Chem. Res. Toxicol.* **3,** 503–508.
5. Weinberg, J. M. (1991) *Kidney Int.* **39,** 476–500.
6. Orrenius, S., and Nicotera, P. (1994) *J. Neural Transmission. Suppl.* **43,** 1–11.
7. Harman, A. W., and Maxwell, M. J. (1995) *Ann. Rev. Pharmacol. Toxicol.* **35,** 129–144.
8. Choi, D. W. (1995) *Trends Neurosci.* **18,** 58–60.
9. Trump, B. F., and Berezesky, I. K. (1995) *FASEB J.* **9,** 219–228.
10. Waters, S. L., Sarang, S. S., Wang, K. K. W., and Schnellmann, R. G. (1997) *J. Pharmacol. Exper. Thera.* **283.**
11. Mehdi, S. (1991) *Trends in Biol. Sciences.* **16,** 45–453.
12. Bronk, S. F., and Gores, G. J. (1993) *Am. J. Physiol.* **264,** G744–G751.
13. Edelstein, C. L., Wieder, E. D., Yaqoob, M. M., Gengaro, P. E., Burke, T. J., Nemenoff, R. A., and Schrier, R. W. (1995) *Proc. Natl. Acad. Sci.* **92,** 7662–7666.
14. Nicotera, P., Hartzell, P., Baldi, C., Svensson, S. A., Bellomo, G., and Orrenius, S. (1986) *J. Biol. Chem.* **261,** 14628–14635.
15. Saido, T. C., Sorimachi, H., and Suzuki, K. (1994) *FASEB J.* **8,** 814–822.
16. Wang, K. K. W., and Yuen, P.-W. (1994) *Trends Pharm. Sci.* **15,** 412–419.
17. Schnellmann, R. G. (1997) *in* Diseases of the Kidney (Schrier, R. W., and Gottschalk, C. W., Eds.), pp. 1049–1068, Little, Brown, and Co., Boston, MA.
18. Schnellmann, R. G., Yang, X., and Cross, T. J. (1994) *Canad. J. Physiol. Pharmacol.* **72,** 602.
19. Miller, G. W., and Schnellmann, R. G. (1993) *Life Sci.* **53,** 1211–1215.
20. Miller, G. W., and Schnellmann, R. G. (1995) *Toxicol. Lett.* **76,** 179–184.
21. Waters, S. L., and Schnellmann, R. G. (1996) *J. Pharmacol. Exper. Thera.* **278,** 1012–1017.
22. Thastrup, O., Cullen, P. J., Drobak, B., Hanley, M. R., and Dawson, A. P. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 2466–2470.
23. Sagara, Y., and Inesi, G. (1991) *J. Biol. Chem.* **266,** 13503–13506.
24. Rodeheaver, D. P., Aleo, M. D., and Schnellmann, R. G., (1990) *In Vitro Cell Dev. Biol.* **26,** 898–904.
25. Groves, C. E., and Schnellmann, R. G. (1996) *in* Methods in Renal Toxicology (Zalups, R. K., and Lash, L. H., Eds.), pp. 147–162, CRC Press, Boca Raton, FL.
26. Moran, J. H., and Schnellmann, R. G. (1996) *J. Pharmacol. Toxicol. Meth.* **36,** 41–44.
27. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) *J. Biol. Chem.* **260,** 3440–3450.