Award Number: DAMD17-01-1-0072

TITLE: Genetic Alteration of Metabolism and Tumorigenicity of Prostate Cancer Cells

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REPORT DATE: June 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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Genetic Alteration of Metabolism and Tumorigenicity of Prostate Cancer Cells

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In the prostate, overwhelming evidence now exists that zinc and citrate metabolism are important factors in the pathogenesis and progression of prostate cancer (PCA). This proposal is aiming to establish that the prostatic tumor cells obtained from PC-3 and LNCaP-derived tumors are citrate oxidizing cells; and to demonstrate that the genetic alteration of zinc accumulation and expression of m-aconitase will alter citrate oxidation and will correspondingly alter the tumorigenic capabilities of LNCaP and PC-3 cells. The second year study was focused on: 1) to study the zinc effect on the prostate tumorigenicity in vivo; 2) to establish and characterize ZIP1 over-expressed PC-3 cells; 3) To determine the tumorigenic capacity of ZIP1 over-expressed PC-3 cells; 4) To establish ZIP1 over-expressed LNCaP cells using stable transfection technique. At the present time the progress of this study is very promising, and we anticipate continuation of significant outcomes from this project. With this grant support, two abstracts were published and presented in international and local meetings, one paper was published and two manuscripts have been submitted.
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Introduction

In the prostate, overwhelming evidence now exists that zinc and citrate metabolism are important factors in the pathogenesis and progression of prostate cancer (Pca). This proposal is aiming to establish that the prostatic tumor cells obtained from PC-3 and LNCaP-derived tumors are citrate oxidizing cells; and to demonstrate that the genetic alteration of zinc accumulation and expression of m-aconitase will alter citrate oxidation and will correspondingly alter the tumorigenic capabilities of LNCaP and PC-3 cells. The second year study was focused on: 1) to study the zinc effect on the prostate tumorigenicity in vivo; 2) to establish and characterize ZIP1 over-expressed PC-3 cells; 3) To determine the tumorigenic capacity of ZIP1 over-expressed PC-3 cells; 4) To establish ZIP1 over-expressed LNCaP cells using stable transfection technique. At the present time the progress of this study is very promising, and we anticipate continuation of significant outcomes from this project. With this grant support, two abstracts were published and presented in international and local meetings, one paper was published and two manuscripts have been submitted.

Body

The research progress and accomplishments associated with Task 2: To determine the tumorigenic capability of m-aconitase deficient PC-3 cells and ZIP1 over-expressed PC-3 cells, and Task 3: To determine the tumorigenic capacity of m-aconitase over-expressed LNCaP cells and ZIP1 deficient LNCaP cells are reported as following:

Experiments and Results:

1. To study the zinc effect on prostatic tumorigenicity in nude mice

We continued the study of the zinc effect on prostatic tumorigenicity in nude mice. A summary of this study follows; please see the details in the Appendix in a related poster (Feng et al., Apoptosis 2003, Luxembourg) and a short paper (Feng et al., submitted 2003).

Normal prostate epithelial cells accumulate the highest zinc levels of any cells in the body. However, malignant prostate cells have lost this ability; and evidence indicates that zinc plays a critical role in the normal function and pathology of the prostate gland. Our recent studies have revealed that zinc induces mitochondrial apoptosis in cultured PC-3 (human prostatic malignant cell line) and BPH (benign prostatic hyperplasia) cells; and the exposure of isolated mitochondria from PC-3 and BPH cells to zinc resulted in the release of cytochrome c. To further understand the apoptotic effect of zinc in vivo, the objectives of this study are to investigate the effect of zinc on: 1) the tumorigenesis in nude mice, 2) the zinc accumulation in tumor tissues, and 3) the levels of mitochondrial membrane permeability related proteins, Bax/Bcl-2. Methods: Nude mice bearing PC-3 cell-induced tumors (two tumors/mice) were used, and osmotic pumps for delivering zinc/or saline in vivo were employed. The animals were divided into three groups: controls with pumps containing saline only, zinc treated animals carrying pumps
containing zinc sulfate at the concentrations of 10mg/ml (low dose) and 15 mg/ml (high dose), respectively. The implantation of pumps was performed at the time of inoculating PC-3 cells, and the experiment lasted four weeks. **Results:** Zinc-induced inhibition of tumor growth was evident by significantly smaller tumor size in zinc treated groups compared with the controls. However, no significant effect of zinc on animal body weight was observed. The incidence of tumor occurrence was 12/12 (control), 11/12 (low dose) and 9/12 (high dose). A higher zinc accumulation of tumor tissues was observed in zinc treated animals (16.3-16.4ng/mg) than that of controls (12.5ng/mg protein). Significantly higher zinc levels were also observed in pooled prostate tissues from zinc treated animals (36.3-38.2ng/mg protein) than in controls (29.7ng/mg protein). Western blot analyses indicated that zinc treatment in vivo significantly increased Bax levels in tumor tissues. **Conclusions:** In vivo treatment of zinc increased zinc accumulation in the tumor tissues and inhibited prostatic tumor growth. This inhibitory effect of zinc may result from zinc-induced apoptosis through regulation of mitochondrial membrane permeability.

2. To establish and characterize the ZIP1 over-expressed PC-3 cells

**Methods:**

**Cell Culture Methods and Transfection-** PC-3 cells (from ATCC) were grown in complete DMEM medium supplemented with 10% fetal bovine serum (Gibco). Cells were plated in T 75 flasks, incubated in a humidified atmosphere of 5% CO₂ and air and transfected with 10 μg of pRc-CMV or pCMV-hZIP1 vectors using Effectene reagents (Qiagen). Stably transfected clones were selected by the dilution plating technique in 350 μg/ml of G418. The stable transfectant cell lines were maintained in 350 μg/ml G418.

**Zn Uptake Assay-** Cells were grown to 70-75% confluence, harvested by trypsin digestion from the flasks, washed once in cold Hanks Balanced Salt Solution (HBSS) and counted by hemacytometer. The cells were resuspended in cold uptake-buffer (HBSS, 50 mM HEPES, pH 7.4) and maintained on ice. Uptake assays were initiated by addition of 100 μl of cell suspension to 150 μl of pre-warmed uptake-buffer containing the specified concentration of ZnCl₂ and a constant specific activity of ⁶⁵Zn (Amersham Pharmacia Biotech, Inc.). The cell suspension was incubated at 37°C. Assays were terminated by addition of 4 volumes (1 ml) of stop-buffer (50 mM HEPES, 250 mM sucrose, 1 mM EDTA, pH 7.2). Cells were collected by filtration through glass fiber filters (Whatman GF/C) on a Brandel cell harvester. The filters were washed six times with wash-buffer (1X PBS with 1 mM EDTA) and counted by liquid scintillation.

**Overexpression of hZIP1 in PC-3 Cells.** PC-3 cells were transfected with pCMV-hZIP1 or pRc-CMV (control) as described in Experimental Procedures. hZIP1 expression was assayed by RT-PCR and by Western blots and the accumulation of ⁶⁵Zn in hZIP1 transfected cells and CMV vector-only control cells measured.

![Figure 1a](image1.png)  
*Figure 1a* shows RT-PCR analysis of hZIP1 and GAPDH mRNA extracted from PC-3 cells stably transfected with hZIP1 or the CMV control vectors. The expected 212 bp fragments from mRNA samples and the overexpression of hZIP1 were observed. *Figure 1b* shows hZIP1 protein...
levels in cells overexpressing hZIP1 compared with CMV controls. Upper panel shows Western blot analysis of hZIP1 and β-actin from cell extracts of hZIP1 transfected and CMV control PC-3 cells. Bottom panel shows quantification of Western blot. hZIP1 transfected cells had ~2 fold more hZIP1 than the CMV control.

Figure 2 shows that overexpression of hZIP1 resulted in an increase in the uptake of $^{65}$Zn. Zinc uptake kinetics of CMV control (circles) and hZIP1 transfected (squares) cells. Control and hZIP1 cells were cultured under identical conditions, collected and uptake of $^{65}$Zn assayed. The kinetics of zinc transport showed an increase in Vmax for cells overexpressing hZIP1 compared with the CMV control (105±7.8 and 46±1.9 pmoles/min/10⁶ cells respectively) with no significant change in the Km (7.6 ± 2.3 and 6.6 ± 2.3 μM). These kinetics are consistent with an increase in the abundance of hZIP1.

Figure 3. To determine if the overexpression of hZIP1 resulted in the increased accumulation of cellular zinc, we incubated over-expressing PC-3 cells and CMV control cells with $^{65}$Zn for various periods. Zinc accumulation by hZIP1 (squares) and CMV control transfectants (circles) measured at 37 °C (open symbols) and at 4 °C (closed symbols). Cells were cultured under identical conditions, collected and zinc accumulation measured from 0 to 40 min. at a medium zinc concentration of 15 μM ZnCl₂. Cells transfected with hZIP1 accumulated more zinc than the CMV transfected controls. Values are mean ± s.e.m. of a representative experiment (n=3).

Figure 4. hZIP1 over expression inhibits growth of PC-3 cells. CMV control and CMV-hZIP1 transfected cells were plated in multi-well plates. The cells were cultured under standard conditions, collected at various times over a 136 h interval and the cell number determined. Each point is the mean ± s.e.m. for combined results from two experiments (n=6). Previously, we reported that zinc inhibited the growth of PC-3 cells [1, 2]. Therefore, we wanted to determine if overexpression of hZIP1 and the resultant higher rate of zinc uptake would affect the
growth of PC-3 cells. Figure 4 shows the cell number with time of culture for the CMV controls and the hZIP1 overexpressing cells. Cells that overexpress hZIP1 had slower growth rates than the CMV control.

Figure 5 shows that hZIP1 overexpression increases the growth inhibitory effect of zinc. CMV and CMV-hZIP1 transfected cells were plated into multi-well plates and cultured overnight under standard conditions. The medium was changed to serum free medium containing 15 μM zinc. The cells were incubated for 24, 48 and 72 h, collected and the cell number determined. Bars show the % decrease in cell number of zinc treated cultures compared with no zinc treatment controls.

* stands for statistically significant difference; CMV vs. hZIP1, p<.05.

We determined the effect of zinc added to the culture medium on cell numbers for CMV controls and cells over-expressing hZIP1. Figure 5 shows that treatment with 15 μM ZnCl2 resulted in a decrease in cell numbers of approximately 50% for the CMV control in 24 h and the loss of cells increased to approximately 70% by 72 h. The effect of zinc on cell numbers in cells over-expressing hZIP1 was significantly greater at each time point compared with the CMV control with a maximum decrease of approximately 90% by 72 h.

**Fig. 6**

**Fig. 7**

**Zinc Atomic Absorption Assay**

**Zinc Uptake of 300 ng/ml for 1hr**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Zinc Conc. (mg/ml)</th>
</tr>
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<tbody>
<tr>
<td>PC-3</td>
<td>359.80</td>
</tr>
<tr>
<td>CMV</td>
<td>702.00</td>
</tr>
<tr>
<td>PCG</td>
<td>704.80</td>
</tr>
<tr>
<td>PCH</td>
<td>815.00</td>
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<tr>
<td>PCl</td>
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<td>PCG</td>
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<td>PCH</td>
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<td>PCl</td>
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<tr>
<td>CMV</td>
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<tr>
<td>PCG</td>
<td>355.20</td>
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<tr>
<td>PCH</td>
<td>391.10</td>
</tr>
<tr>
<td>PCl</td>
<td>391.10</td>
</tr>
</tbody>
</table>

| Mean      | 495.17            |
| SE        | 114.98            |

| Mean      | 593.13            |
| SE        | 94.62             |

**Cell Line**

**Mean**

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<th>567.3</th>
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<th>722.8</th>
<th>720.3</th>
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<td>45.11</td>
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<td>41.30</td>
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<td>12.57</td>
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</tr>
</tbody>
</table>
The overexpression of hZIP1 resulting in the increased accumulation of cellular zinc was further characterized in stable transfected cell lines (G, H and 11) (Fig.6 and 7). Fig.6 shows that no significant differences were detected in endogenous zinc levels between PC-3 cells and stable transfected PC-3 cells with ZIP1 and CMV vector only. The zinc concentrations were measured by Atomic Absorption assay as described previously. The results were obtained from three independent experiments. We further compared the abilities of zinc uptake in these cells (shown in Fig. 7). Cells were cultured in the growth medium until 70% confluence; and 24h before the zinc treatment cell culture medium was changed to serum free medium. Cells were treated with or without zinc (300ng/ml) for 1 h; and then the cells were harvested and prepared for zinc measurement using Atomic Absorption. The data showed that stable transfected cell line 11 appeared to have the highest ability for zinc uptake; and recently the results were repeated correspondingly by using Zn65.

Fig. 8

To further characterize if the ZIP1 transfection resulted in any functional alterations, the citrate acid production of the cell lines was determined (Fig.8). The cells were cultured in the 6 well/plate at density of 1x 10^5 /well in growth medium with 2.5% FBS for 3 days. Then the conditioned medium were collected and prepared for citrate acid assay as previously described. The data showed that ZIP1 transfection increases citrate acid production significantly. The results were from three independent assays. These results suggest that the aconitase activity of the cells overexpressed ZIP1 may be inhibited by elevated zinc uptake, even if the elevation of zinc concentration is very small.

3. To determine the tumorigenic capacity of ZIP1 over-expressed PC-3 cells

The experiments were carried out in Nude mice; and a preliminary experiment showed that ZIP1 transfected cells have similar tumorigenic capacity as PC-3 cells. The tumors derived from both cell lines were responding to zinc treatment. However, the in vivo experiments are still on going; in the in vivo experiment, cell line 11 are employed to compare with the cells with CMV vector only, as well as to the wild type PC-3 cells. The results of all tested parameters will be summarized and reported in a manuscript.

4. To establish ZIP1 over-expressed LNCaP cells
As the in vivo studies are on going, we are working on the project to establish ZIP1 over expressed LNCaP cells using the same method as we described above for the PC-3 cells. Once we establish the cell lines, we will immediately apply the cells in an in vivo study in SKID mice. Details of this experiment are not described herein, since the principle and techniques are the same as described in PC-3 cells.

Key Research Accomplishments:

- Studied the zinc effect on tumorigenicity in PC-3 cell induced tumors using ALZET pumps.
- Characterization of ZIP1 over expressed PC-3 cells
- Study the zinc effect on tumorigenicity in ZIP1 over expressed PC-3 cells
- To establish ZIP1 over expressed LNCaP cells

Reportable Outcomes:

- A paper published; Feng et al., "Direct effect of zinc on mitochondrial apoptosis in prostate cells" in The Prostate 52:311-318, 2002 (Appendix)
- Abstract and presentation; Feng et al., "Effect of zinc on prostatic tumorigenicity in nude mice" in the meeting of APOPTOSIS 2003, From signaling pathways to therapeutic tools by European Conference Center in Luxembourg, Jan. 2003 (Appendix)
- Abstract and presentation; Guan et al., "Kinetic evidence for the existence of a mitochondrial zinc uptake transporter in prostate cell" at the 25th Annual Research Meeting for Graduate Students in the University of Maryland, Baltimore, April 2003 (Appendices)
- Abstract and presentation of "Mechanism of zinc accumulation in prostate and its effect on prostate cell growth" at the meeting of Molecular Mechanisms as Targets in Prostatic Diseases, International Conference, Liverpool, UK 2002.
- Abstract and presentations of 1)" The Role of Zinc in the Pathogenesis of Prostate Cancer: A Cause and A Cure??" 2) "Zinc induces mitochondrial apoptosis in prostate cells" Costello, LC was an invited speaker. Zinc Signals 2003 International Conference, Grand Cayman,BWI May 2003.
- Abstract and presentations on Mechanism of zinc transporter in prostate cancer cells. Franklin, RB was an invited Speaker. Zinc Signals 2003 International Conference, Grand Cayman, BWI May 2003.
- Manuscript: Guan et al., "Kinetic identification of a mitochondrial zinc uptake transporter in prostate cells" J Inorg Biochem. 2003, accepted with revision (Appendices)
Conclusions:
With the support of this grant, the research project generated new information which was presented in our publications, meeting presentations and the communications with other scientists who are working in the field of prostate cancer research. The progress obtained from the second year study of this project is very promising as indicated in the research data presented in this report. It is very important to do the in vivo study on the mechanisms of the zinc effect on metabolism, which have a direct link to tumorigenicity in prostate. Although the in vivo study is generally more difficult than the in vitro study, the results will provide new insight to the pathogenesis of prostate cancer, eventually leading to a new clinical therapeutic method for prostate cancer.

References:


Personnel Receiving Pay From the Research Effort:

Pei Feng, M.D., Ph.D.
TieLuo Li, M.D.
ZhiXin Guan, Ph.D. Candidate
APPENDIX

P.I.  Pei Feng, M.D., Ph.D

DAMD 17-01-1-0072
Direct Effect of Zinc on Mitochondrial Apoptogenesis in Prostate Cells

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1Greenebaum Cancer Center, University of Maryland, Baltimore, Maryland
2Molecular and Cell Biology Section, Department of OCBS, Dental School, University of Maryland, Baltimore, Maryland

BACKGROUND. Prostate epithelial cells uniquely accumulate significantly higher levels of zinc than other mammalian cells. We previously showed that the accumulation of high intracellular zinc levels in specific prostate cells results in the induction of apoptosis and the inhibition of cell growth. The apoptotic effect is due to zinc induction of mitochondrial apoptogenesis. We now report additional studies that corroborate this effect of zinc and provide insight into the mechanism of this unique effect.

METHODS. The effect of exposure to physiological levels of zinc on apoptosis was determined for three human prostate cell lines (PC-3, BPH, and HPR-1). Zinc-induced apoptosis was identified by DNA fragmentation. The direct effect of zinc on isolated mitochondrial preparations from each cell line was determined. The mitochondrial release of cytochrome c was determined by Western blot.

RESULTS. Exposure to zinc induced apoptosis in PC-3 and BPH cells but not in HPR-1 cells. The zinc accumulation in PC-3 (4.3 ± 0.3) and BPH (2.8 ± 0.4) was higher than that in HPR-1 cells (1.8 ± 0.1). The apoptotic effect of zinc on PC-3 cells could be observed as early as 4–6 hr of zinc treatment, and this effect was not reversible. The exposure of isolated mitochondria from PC-3 and BPH cells to zinc resulted in the release of cytochrome c; but zinc had no effect on mitochondria from HPR-1 cells.

CONCLUSIONS. Exposure to zinc induces apoptosis in PC-3 and BPH cells, which accumulate high intracellular levels of zinc, but not in HPR-1 cells, which do not accumulate high levels of zinc. Once initiated, the induction of apoptosis is not reversed by the removal of zinc, i.e., it is an irreversible process. The apoptogenic effect is due to a direct effect of zinc on mitochondria that results in the release of cytochrome c. The cell specificity of zinc induction of apoptogenesis is dependent on the ability of the cells to accumulate high levels of intracellular zinc and on the ability of the mitochondria to respond to the direct effect of zinc.


KEY WORDS: zinc; prostate; mitochondria; apoptosis; PC-3; BPH; HPR-1

INTRODUCTION

Normal human prostate secretory epithelial cells accumulate the highest zinc levels of any cells in the body. In prostate cancer (PCa), the malignant prostate cells have lost this ability; compelling evidence now exists for the implication of zinc in the pathogenesis and progression of prostate malignancy [1–3]. Consequently, zinc plays a critical role in the normal function and pathology of the prostate gland. For detailed reviews of these relationships, see Costello and Franklin [3,4].

An important question is, "What are the consequences and role of the accumulation of high zinc levels in the prostate secretory epithelial cells that

Grant sponsor: Department of Defense; Grant number: PC001174; Grant sponsor: NIH; Grant numbers: CA 71207, CA 79903.
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Received 11 December 2001; Accepted 26 April 2002
Published online 19 July 2002 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/pros.10128

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possess the unique capability of transporting and accumulating high levels of zinc (i.e., zinc-accumulating prostate cells)? We recently demonstrated that zinc accumulation in prostate cells results in increased apoptosis [5,6] and that this effect was manifested by the zinc induction of mitochondrial apoptosis [6]. Exposure of these cells to zinc rapidly induces the translocation of cytochrome c from mitochondria into cytosol, which triggers caspase activation that leads to cascading cytosolic and nuclear apoptotic events. In contrast, exposure of cells to zinc that are incapable of high zinc accumulation does not result in apoptosis. This present report confirms and extends our earlier reports and addresses two important issues relating to the mechanism of zinc-induced mitochondrial apoptosis: (1) whether or not the effect of zinc is due to a direct effect of zinc on the mitochondria, and (2) if the cell-specific effect of zinc is due to the level of cellular zinc and/or in the capability of mitochondria to respond to accumulated zinc.

MATERIALS AND METHODS

Cell Lines and Cultures

The present studies were conducted with PC-3 cells, a human malignant prostate cell line obtained from ATCC as we previously described [5]; BPH cells (kindly provided by Dr. S. Haywood, University of California, San Francisco), an epithelial cell line that was derived from human benign prostatic hyperplasia (BPH) tissue; and HPR-1 cells (kindly provided by Dr. C. K. Choo, University of Hong Kong, Hong Kong, China), an epithelial cell line that was derived from normal human prostate tissue. PC-3 cells and BPH cells were cultured in RPMI-1640 medium with 10% (PC-3) and 5% (BPH) fetal bovine serum and 1% penicillin/streptomycin. HPR-1 cells were cultured in serum-free keratinocyte medium with EGF (2.5 μg/500 ml) and bovine pituitary extracts (25 mg/500 ml) (Gibco BRL, Life Technologies, Bethesda, MD) and 1% penicillin/streptomycin.

Mitochondrial Preparation and Detection of Cytochrome c Release

The cells were collected by centrifugation at 250 g for 5 min at 4°C. The cells were washed with ice-cold PBS twice and resuspended in 5 volumes of mitochondrial isolation buffer (MIB) composed of 220 mM mannitol, 68 mM sucrose, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 10 mM HEPES, 0.1% bovine serum albumin (BSA), with added fresh 1 mM DTT and protease inhibitors (pepsstatin A, 5 μg/ml; leupeptin, 10μg/ml; aprotonin, 2μg/ml), pH 7.4. The cells were homogenized gently on the ice with a glass homo-

genizer and followed by a centrifugation at 800 × g for 10 min. The resulting supernatant fluid was centrifuged at 10,000g for 5 min at 4°C. The pellet (mitochondria) was resuspended in MRB buffer composed of 200 mM mannitol, 50 mM sucrose, 10 mM succinate, 5 mM potassium phosphate, 10 mM HEPES, 0.1% BSA, pH 7.4, and kept on ice. Aliquots of the mitochondrial suspension (200 μg of protein/40-μl reaction) were exposed to zinc for various time periods at 30°C under conditions described in the Results section. At the conclusion of the incubation period, the mitochondria were separated from the reaction by rapid centrifugation at 10,000g for 5 min. The supernatant fluid was assayed for cytochrome c by Western blot. The protein concentration of the mitochondrial preparations was determined by the method of Bradford [7]. Western blot assays were performed with specific anti-cytochrome c and β-actin antibodies (BD Transduction Laboratories, San Diego, CA) under the conditions recommended by the manufacturer.

Detection of Cell Apoptosis

The extraction of DNA and detection of DNA fragmentation were performed as previously described [5]. The morphology of the cells treated with or without zinc in six-well culture plate for designated time periods and the characteristics of apoptotic cells were observed under an inverted microscope (Nikon, Eclipse TE200) and photographed.

Determination of Cellular Zinc

Prostatic cells were grown in 75 cm² flasks until 90% confluence of the culture. The cells were treated with or without zinc (1,000 ng/ml) in fresh serum-free medium for 3 hr. Before harvest, the cells were washed once with 1 × PBS and then washed twice after the collection to remove extracellular zinc. The cells were resuspended in sucrose buffer (250 mM sucrose, 20 mM HEPES, pH 7.4) and homogenized on ice. The nuclei and cell membranes were separated by centrifugation at 800g for 10 min. The supernatants were then centrifuged at 10,000g for 5 min, and these supernatants were used as cytosol samples. The protein concentrations of the samples were measured by Bradford method [7]. Thirty microliters of each sample (200 μg of protein) were placed in a 96-well plate and mixed well with 60 μl of TSQ buffer, which was composed of 1.9 g of sodium acetate, 2.9 g of sodium barbital, 1.5 mg of TSQ (Molecular Probes, Eugene, OR) dissolved in 100 μl of warmed ethanol, then double distilled H₂O added to 100 ml, pH 10. The fluorescence of zinc labeled by TSQ was detected by using a Fluoroskan Ascent, Labsystems, Microplate
Reader (Life Sciences International Company, USA) with excitation of 355 and emission of 485 [8,9].

In this study, all experiments were repeated three or more times to establish the consistency and reproducibility of the results. The data presented are representative of the results of the repeated experiments.

RESULTS

Zinc Induces Apoptosis in Human Prostatic Malignant (PC-3) and Benign Hyperplasia (BPH) Cells, but Not in HPR-1 Cells Derived From Normal Prostatic Epithelial Cells

We previously demonstrated that exposure of LNCaP and PC-3 cells to physiological levels of zinc results in increased apoptosis and decreased cell growth while having no effect on HPV-1 cells, which are derived from human normal prostatic epithelial cells [5,6]. Correspondingly, LNCaP and PC-3 cells will accumulate high zinc levels; whereas HPV-1 cells do not accumulate high zinc levels (unpublished information). To extend this important relationship, the effect of zinc on the induction of apoptosis was studied in BPH cells, in HPR-1 cells that, like HPV-1 cells, purport to be representative of normal human prostate secretory epithelial cells, and in PC-3 cells for comparison of the effects. Figure 1 reveals that zinc exposure (1 µg/ml for 24 hr) of PC-3 and BPH cells resulted in a marked decrease in the cell density while having no effect on HPR-1 cells. Correspondingly, cell apoptotic morphologic features were observed in both PC-3 and BPH cells treated with zinc but no similar effect was observed in HPR-1 cells. The apoptotic changes observed in PC-3 and BPH cells included the appearance of cell membrane “blebbing,” nuclear chromosome condensation, and eventually the formation of apoptotic bodies. Apoptosis was further confirmed by genomic DNA fragmentation (Fig. 2). Multiple 180-bp DNA fragments, resulting from internucleosomal cleavage, were clearly visible in PC-3 and BPH cells treated with 0.25, 0.50, and 1.0 µg/ml zinc for 24 and 48 hr. To determine that these effects were possibly associated with a cellular accumulation of zinc, the level of increased zinc accumulation in the zinc-treated cells vs. the control cells was determined to be 4.3-fold for PC-3 cells, 2.7-fold for BPH cells, and only 1.8-fold for HPR-1 cells. The values for PC-3 and HPR-1 cells are consistent with the cellular increases as measured by atomic absorption (unpublished information). Thus, zinc induction of apoptosis is a major factor in the inhibitory effect of zinc on prostate cell growth as we reported earlier [5].

A time-course study of zinc-induced apoptosis was further conducted in PC-3 cells (Fig. 3A). The results show that zinc induction of apoptosis in PC-3 cells is time dependent. Apoptotic cells appeared as early as 4 hr after zinc treatment, and apoptosis was clearly evident after 6 hr of zinc treatment. Between 6 and 24 hr after the start of zinc treatment, the increase in apoptotic cells was associated with significant decreased cell density.

In another study (Fig. 3B), we wanted to determine whether zinc-induced apoptogenesis could be reversed by removal of the zinc. PC-3 cells were recovered for 24 hr in normal growth medium after 2.
the prolonged period of 60 min of exposure during which the endogenous release of cytochrome c is increased, zinc treatment resulted in the additional (50%) release of cytochrome c. The effects of zinc on cytochrome c release from BPH mitochondria were essentially the same as the effects obtained for PC-3 mitochondria (Fig. 4B). Thus, it is clearly evident that zinc-induction of the release of cytochrome c is due to a direct and rapid effect of zinc on mitochondria. These results extend our earlier report, which showed that zinc induces mitochondrial apoptosis in intact PC-3 cells by means of a stimulatory effect on the release of cytochrome c [6].

It is extremely important to note that (Fig. 4C), in sharp contrast to PC-3 and BPH, neither 38 μM nor 76 μM zinc had an effect on the release of cytochrome c from isolated mitochondria from the HPR-1 cells. This finding is consistent with the absence of zinc induction of apoptosis in these cells under conditions that induce apoptosis in PC-3 and BPH cells (Fig. 1). The results reveal that after zinc treatment the lower cytosol accumulation of zinc by HPR-1 cells as shown in Figure 1 was not the only reason for the absence of an apoptotic effect of zinc. The endogenous level of cytosol zinc is higher in HPR-1 cells than in BPH and PC-3 cells; yet these HPR-1 cells do not exhibit apoptosis (Fig. 1). It is now evident that the mitochondria in these cells, unlike PC-3 and BPH mitochondria, are not responsive to the direct effects of zinc on the release of cytochrome c.

The results presented in Figure 4 indicated that PC-3 and BPH mitochondrial exposure to zinc for 20 min was optimal for the release of cytochrome c. It was then important to conduct a dose-response study to determine the minimal concentration and the range of zinc that could result in the release of cytochrome c (Fig. 5). Studies with BPH mitochondria reveal that a dose-response effect of 20-min exposure to zinc on the release of cytochrome c is evident over the range of 0.002–0.040 μmol zinc/mg protein (equivalent to 7.6–152 μM zinc). Over this range, the cytochrome c released increased from 44% at 0.002 μmol/mg protein) to approximately 300% (at 0.04 μmol/mg protein) when compared with control mitochondria.

**DISCUSSION**

These studies extend our previous observations that zinc accumulation in prostate cells induces mitochondrial apoptosis. Most importantly, it is now evident that the induction of mitochondrial apoptosis is due to a direct effect of zinc on the mitochondria. Heretofore, it was thought that the cellular accumulation of zinc could have inducible effects that were prior to and required for the manifestation of.
mitochondrial response. That zinc induces the release of cytochrome c from isolated prostate mitochondria eliminates any required interaction of zinc on non-mitochondrial conditions or components before its mitochondrial effect. The next critical issue to investigate is the mechanism by which zinc alters mitochondrial function that leads to the release of cytochrome c and the onset of apoptosis.

It is extremely important to note the difference between the PC-3 and BPH cells vs. the HPR-1 cells. PC-3 cells, BPH cells, and, as previously reported [10], LNCaP cells accumulate high zinc levels when exposed to physiological levels of extracellular zinc. Correspondingly, the accumulation of zinc induces mitochondrial apoptosis in these cells. In addition, freshly prepared rat ventral prostate epithelial cells also undergo mitochondrial apoptosis when exposed to zinc [6]. Clearly, in zinc-accumulating prostate cells, exposure to zinc results in apoptosis. In contrast, the exposure of PZ-HPV cells (purported to be derived from normal peripheral zone) to zinc does not result in the cellular accumulation of high zinc levels and, therefore, does not undergo zinc-induced apoptosis [6]. Similarly, HPR-1 cells do not accumulate high zinc levels and do not exhibit zinc-induced apoptosis. Evidently the
ability of cells to accumulate high zinc levels is a requirement for the induction of apoptosis by exposure to extracellular zinc (i.e., plasma levels).

However, the present studies reveal another significant relationship. The issue raised is whether the lack of a pro-apoptotic effect of zinc on HPR-1 cells is due to an insufficient level of intracellular zinc and/or due to a lack of responsiveness of the mitochondria to zinc. Under the same experimental conditions that induced the release of cytochrome c from isolated PC-3 and BPH mitochondria, the isolated mitochondria from HPR-1 cells were not responsive to the direct exposure to zinc. It is now evident that the cell-specific effects of zinc induction of mitochondrial apoptosis as demonstrated in this and earlier reports [3,6] are dependent upon two factors: (1) the ability of cells to accumulate sufficiently high levels of mobile

Fig. 4. Zinc induction of mitochondrial cytochrome c release in prostatic cell-free system. Mitochondria were prepared from PC-3 cells (A), BPH cells (B), HPR-1 cells (C) as described in the Materials and Methods section and were incubated with or without zinc (0.01 – 0.02 μM/mg protein) for the time periods indicated. The levels of released cytochrome c determined by Western blot are presented by the relative O.D. from densitometry scan of the cytochrome c (Cyto c) bands corrected by the values of β actin as internal controls.

Fig. 5. Zinc induction of mitochondrial cytochrome c release in BPH cell-free system is concentration dependent. Mitochondria prepared from BPH cells were incubated with zinc at the concentrations indicated for 20 min at 30°C. The cytochrome c (Cyto c) release was determined as described in Figure 4.
reactive zinc necessary to induce mitochondrial release of cytochrome c; and (2) the ability of mitochondria from different cells to respond to the direct effect of zinc. An important issue that has to be pursued is the reason for the different zinc-responsivenesses of mitochondria from different cells. In this regard, we have shown that zinc-altered mitochondrial aconitate equilibrium is unique to prostate mitochondria due to a specific protein not associated with other mitochondria [11].

A direct effect of zinc on the mitochondrial release of cytochrome c was evident at zinc concentrations as low as 7.6 μM (0.004 μmol zinc/mg mitochondrial protein). Although the free zinc concentrations of mammalian cells is unknown, the cytosolic pool of free zinc has been estimated to be $10^{-5}$–$10^{-12}$ M and even as low as $10^{-15}$ M [12,13]. The total cellular zinc has been calculated to be in the range of approximately $10^{-3}$ M. These values are likely higher for prostate cells, because prostate accumulates approximately three to five times more zinc than other tissues. Moreover, cellular reactions of zinc are dependent upon mobile reactive zinc, not solely free zinc, through intermolecular exchange of zinc. This pool of zinc, although its cytosolic concentration is unknown, is magnitudes greater than the free zinc pool. Consequently, the effectiveness of zinc levels in the range of $10^{-6}$ M zinc as used in this study likely is representative of in situ cytosolic reactive zinc levels and is, therefore, a physiological effect of zinc in the prostate cells.

The present studies combined with our previous reports demonstrate a consistent and persistent effect of zinc in the induction of mitochondrial apoptosis in specific prostate epithelial cells. This effect is observed when the cells are exposed to conditions that are representative of the plasma level of zinc (approximately 2–15 μM). In contrast, most reports with other mammalian cells describe a role of zinc in the inhibition of apoptosis [14,15]. In the overwhelming majority of those reports, the levels of zinc to which the cells were exposed were in the millimolar range, i.e., unphysiological concentrations to which cells in situ would never be exposed. This, in part, could account for the difference in the zinc effects on apoptosis. This concern is also applicable to the report that zinc induces necrosis in prostate cells. In that study, 200–500 μM zinc (conditions that would never exist in situ) was required to induce necrosis; and the necrotic effect was not evident at lower, but still unphysiologic, levels of zinc [16]. However, similar to our observations with prostate cells, a few reports exist that also demonstrate zinc induction of apoptosis in select mammalian cells [17–19]. In support of our observations, Untergasser et al. recently reported that zinc induces mitochondrial alterations in prostate cells that result in apoptosis [20]. The divergent reported effects of zinc as an inhibitor of apoptosis in some cells or conversely a stimulator of apoptosis in other cells might be due, in part, to the ability or lack thereof of the mitochondria in different cells to respond to the direct apoptogenic effect of zinc as demonstrated in this present study. Consequently, the generalization that zinc is a physiological inhibitor of apoptosis in mammalian cells is not tenable. Recognition of the cell-specific role of zinc in apoptosis and cell growth is essential. Moreover, our earlier report combined with this present report with prostate cells provides the first demonstration in any mammalian cells (to our knowledge) that the zinc-induced apoptotic effect is targeted directly at the induction of mitochondrial apoptosis.

REFERENCES

Effect of zinc on prostatic tumorigenecity in nude mice

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Normal prostate epithelial cells accumulate the highest zinc levels of any cells in the body. However, malignant prostate cells have lost this ability; and evidence indicates that zinc plays a critical role in the normal function and pathology of the prostate gland. Our recent studies have revealed that zinc induces mitochondrial apoptosis in cultured PC-3 (human prostatic malignant cell line) and BPH (benign prostatic hyperplasia) cells; and the exposure of isolated mitochondria from PC-3 and BPH cells to zinc resulted in the release of cytochrome c. To further understand the apoptotic effect of zinc in vivo, the objectives of this study are to investigate the effect of zinc on: 1) the tumorigenesis in nude mice, 2) the zinc accumulation in tumor tissues, and 3) the levels of mitochondrial membrane permeability related proteins, Bax/Bcl-2. Methods: Nude mice bearing PC-3 cell-induced tumors (two tumors/mice) were used, and osmotic pumps for delivering zinc/or saline in vivo were employed. The animals were divided into three groups: controls with pumps containing saline only, zinc treated animals carrying pumps containing zinc sulfate at the concentrations of 10mg/ml (low dose) and 15 mg/ml (high dose), respectively. The implantation of pumps was performed at the time of inoculating PC-3 cells, and the experiment lasted four weeks. Results: Zinc-induced inhibition of tumor growth was evident by significantly smaller tumor size in zinc treated groups compared with the controls. However, no significant effect of zinc on animal body weight was observed. The incidence of tumor occurrence was 12/12 (control), 11/12 (low dose) and 9/12 (high dose). A higher zinc accumulation of tumor tissues was observed in zinc treated animals (16.3-16.4ng/mg) than that of controls (12.5ng/mg protein). Significantly higher zinc levels were also observed in pooled prostate tissues from zinc treated animals (36.3-38.2ng/mg protein) than in controls (29.7ng/mg protein). Western blot analyses indicated that zinc treatment in vivo significantly increased Bax levels in tumor tissues. Conclusions: In vivo treatment of zinc increased zinc accumulation in the tumor tissues and inhibited prostatic tumor growth. This inhibitory effect of zinc may result from zinc-induced apoptosis through regulation of mitochondrial membrane permeability.
Effect of zinc on prostatic tumorigenicity in nude mice

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Prostate epithelial cells accumulate the highest zinc levels of any cells in the body. However, malignant prostate cells have lost this ability; and evidence indicates that zinc plays critical roles in the normal function and pathology of the prostate gland. We have identified two important effects of zinc in the prostate epithelial cells: the inhibition of m-aconitase; and the induction of mitochondrial apoptosis (1,2,3). In normal prostate, the zinc inhibition of m-aconitase activity results in suppressing citrate oxidation, thus, increasing citrate production. Our recent studies have demonstrated that zinc induces mitochondrial apoptosis in cultured PC-3 (human prostatic malignant cell line) and BPH (benign prostatic hyperplasia) cells; and the exposure of isolated mitochondria isolated from these cells to zinc resulted in the release of cytochrome c and redistribution of Bax/Bcl-2 on mitochondrial membranes (Bax/Bcl-2 data unpublished). However, at the present time, the effects of zinc on prostatic cells in in vivo conditions have not yet been reported.

The objectives of this in vivo study were to investigate the effect of zinc on: 1) tumorigenicity in nude mice, 2) zinc accumulation in tumor tissues, and 3) the levels of mitochondrial membrane permeability related proteins, Bax/Bcl-2.

The experiments were carried out using male nude mice (4-6 weeks old) as a tumorigenicity animal model. The mice were housed in a pathogen-free environment under controlled light and humidity. Tumors were established by inoculation of PC-3 cells (5-10x10⁶/ml) prepared in 10% Matrigel and using 0.1 ml for each injection (s.c.) at both flanks of the animals. The size of tumors was measured weekly. Zinc treatment was given by ALZET osmotic pumps (Durect Corporation), with a releasing rate of 0.25 μl/hr for 28 days. The pumps were filled with PBS (control), zinc sulfate 5 mg/ml (low dose) and 7.5 mg/ml (high dose), respectively, and were implanted s.c. at the lower back of the animals. The operations of implanting pumps and inoculating PC-3 cells were carried out simultaneously. The animal body weights were examined weekly, and their general health conditions were monitored closely. After 28 days of treatment, the mice were sacrificed, and the samples were collected for further studies. Zinc concentrations were determined by Atomic Absorption Spectrophotometer method. Frozen sections of tumor tissues (8μm) were prepared for TUNEL assay (according to the manufactory protocol) and stained by
DAB. The results were examined and recorded by a light microscopy. The levels of Bax and Bcl-2 in the tumor tissues were determined by Western blot analyses with specific antibodies, and the amount of protein loaded for each sample was justified by α actin as an internal control. The citrate level in the tumor tissues was assayed using a previously established fluoroenzymatic method (3).

The results showed that zinc-induced inhibition of tumor growth was evident by relatively smaller size and lighter weight of tumors in zinc treated animals compared with the controls. However, there was no significant effect of zinc on animal body weight. The incidence of tumor occurrence was 12/12 (control), 11/12 (low dose) and 9/12 (high dose). A higher zinc accumulation of tumor tissues was observed in zinc treated animals (16.3-16.4 ng/mg protein) than that of controls (12.5ng/mg protein), leading to about 20-40% increase of citrate production in zinc treated tumor tissues. In order to investigate the inhibitory effect of zinc on PC-3 cell induced tumors, frozen sections of tumor tissues were examined by a TUNEL assay (Fig.1). The results showed that extensive cell apoptosis was observed in zinc treated tumor tissues compared to the controls, in which there were only few apoptotic cells detected. Most recently we have identified that zinc induces PC-3 cell apoptosis through regulating the mitochondrial outer membrane permeability related proteins, Bax/Bcl-2 (data not shown) in accordance with previously reported (4,5). Thus, to further ascertain the mechanism of zinc induced apoptosis in vivo, Bax and Bcl-2 levels in the tumor tissues were determined by Western Blot (Fig.2). The results showed that Bax levels significantly increased in zinc treated tumor tissues compared with those of the controls (about 3 fold), and Bcl-2 levels only increased slightly. The ratio of Bax/Bcl-2 in zinc treated groups was significantly elevated compared to that of the controls.

Our study demonstrated that in vivo treatment of zinc increased zinc accumulation and citrate production in PC-3 cell induced tumor tissues and inhibited tumor growth. The inhibitory effect of zinc appears to be resulted from zinc-induced apoptosis by regulation of mitochondrial membrane permeability related bax/bcl-2 proteins.

Acknowledgement: We thank Dr. JuRen He for her expert help with TUNEL assay and Ms. Maggie Pierce for her assistance with in vivo studies. This project is supported by a DOD grant, DAMD17-01-1-0072.

References


5. M.G.V. Heiden, N.S. Chandel, XX. Li, P.T. Schumacker, M. Colombini, and C.B. Thompson  Outer mitochondrial membrane permeability can regulate coupled respiration and cell survival. PNAS 97(9): 4666-4671, 2000
Zinc treatment induces apoptosis in tumor tissues

Figure 1. Frozen sections of the tumor tissues (8 lm) were stained for apoptotic DNA-fragmentation with an in situ cell death detection kit (TUNEL) (manufacturer instruction), followed by a DAB staining. The positive control was treated with DNase I, and the negative control was assayed without TdT. The results showed that the in vivo treatment of zinc significantly induced tumor cell apoptosis comparing to the tumors without zinc treatment.
The effect of zinc on the ratio of Bax/Bcl-2 levels

Figure 2. A. After 28 days of treatment, the levels of Bax in tumor tissues were detected by Western Blot. Significantly increased Bax levels were identified in both zinc treated groups compared to controls. B. In contrast, Bcl-2 levels increased slightly compared to controls. C. The Bax/Bcl-2 ratio significantly increased in zinc treated groups, increasing mitochondrial outer membrane permeability and leading to cytochrome c release. (Data shown as representatives of the analyzed samples)
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KINETIC EVIDENCE FOR THE EXISTENCE OF A MITOCHONDRIAL ZINC UPTAKE TRANSPORTER IN PROSTATE CELLS

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Prostate cells accumulate high cellular and mitochondrial concentrations of zinc, generally 3-10 fold higher than other mammalian cells. However the mechanism of mitochondrial import and accumulation of zinc from cytosolic sources of zinc has not been established for these cells or for any mammalian cells. Since the cytosolic concentration of free Zn++ ions is negligible (estimates vary from 10-9-10-15 M), we postulated that loosely bound zinc-ligand complexes (ZnLigands) serve as zinc donor sources for mitochondrial import. Zinc chelated with citrate (ZnCit) is a major form of zinc in prostate and represents an important potential cytosolic source of transportable zinc into mitochondria. The objectives of this study were to: 1. identify transportable ZnLigands; 2. characterize transport process in prostate mitochondria. The mitochondria and mitoplasts were isolated from rat ventral prostate and liver. Zinc transport was characterized by 65Zn uptake assay in isolated mitochondria and mitoplast. Total zinc accumulation was determined by atomic absorption. The results obtained from liver and ventral prostate mitochondria bshowed that the uptake of zinc from 1-100 uM ZnCl2 (free Zn++ ion) and from ZnCit was identical. No zinc uptake occurred from ZnEDTA. Zinc uptake exhibited Michaelis-Menten kinetics and characteristics of a functional energy-independent facilitative transporter associated with the mitochondrial inner membrane. The uptake and accumulation of zinc from various ZnLigand preparations with log Kf (formation constant) values less than 11 was the same as for ZnCl2; and was dependent upon the total zinc concentration and independent of the free Zn++ ion concentration. ZnLigands with log Kf values greater than 11 were not zinc donors. Therefore the putative zinc transporter exhibits an effective log Kf~11 and involves a direct exchange of zinc from ZnLigand to transporter. Conclusion: the results demonstrate the existence of a mitochondrial zinc uptake transporter that exists for the import of zinc from cytosolic ZnLigands. The uniquely high accumulation of mitochondrial zinc in prostate cells appears to be due to their high cytosolic level of zinc-transportable ligands, particularly ZnCit.

ACKNOWLEDGEMENT These studies were supported by NIH research grants CA71207, CA79903 and DOD grant PC001174.
Kinetic Identification of a Mitochondrial Zinc Uptake Transporter in Prostate Cells

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Running Title: Zinc transporter in prostate mitochondria
ABSTRACT

Prostate cells accumulate high cellular and mitochondrial concentrations of zinc, generally 3-10 fold higher than other mammalian cells. However the mechanism of mitochondrial import and accumulation of zinc from cytosolic sources of zinc has not been established for these cells or for any mammalian cells. Since the cytosolic concentration of free Zn++ ions is negligible (estimates vary from $10^{-9}$-$10^{-15}$ M), we postulated that loosely bound zinc-ligand complexes (ZnLigands) serve as zinc donor sources for mitochondrial import. Zinc chelated with citrate (ZnCit) is a major form of zinc in prostate and represents an important potential cytosolic source of transportable zinc into mitochondria. The mitochondrial uptake transport of zinc was studied with isolated mitochondrial preparations obtained from rat ventral prostate. The uptake rates of zinc from ZnLigands (Citrate, Aspartate, Histidine, Cysteine) and from ZnCl$_2$ (free Zn++) were essentially the same. No zinc uptake occurred from either ZnEDTA, or ZnEGTA. Zinc uptake exhibited Michaelis-Menten kinetics and characteristics of a functional energy-independent facilitative transporter associated with the mitochondrial inner membrane. The uptake and accumulation of zinc from various ZnLigand preparations with log Kf (formation constant) values less than 11 was the same as for ZnCl$_2$, and was dependent upon the total zinc concentration independent of the free Zn++ ion concentration. ZnLigands with log Kf values greater than 11 were not zinc donors. Therefore the putative zinc transporter exhibits an effective log Kf~11 and involves a direct exchange of zinc from ZnLigand to transporter. The uptake of zinc by liver mitochondria exhibited transport kinetics similar to prostate mitochondria. The results demonstrate the existence of a mitochondrial zinc uptake transporter that exists for the import of zinc from cytosolic ZnLigands. This provides the mechanism for mitochondrial zinc accumulation from the cytosol which contains a negligible concentration of free Zn++. The uniquely high accumulation of mitochondrial zinc in prostate cells appears to be due to their high cytosolic level of zinc-transportable ligands, particularly ZnCit.
INTRODUCTION

Prostate secretory epithelial cells have the function and capability of accumulating extremely high zinc levels, generally 3-10 fold higher than other mammalian cells [see 1-3] for recent reviews of zinc relationships in prostate. In addition to the high total cellular zinc level, the mitochondria of the prostate cells also accumulate high zinc levels. The accumulation of high zinc levels results in altered mitochondrial function such as inhibition of m-aconitase and citrate oxidation and induction of mitochondrial apoptogenesis [4,5]. Therefore, a significant component of the total cellular zinc must exist in a mobile reactive form.

Estimates place the total cellular zinc content of mammalian cells at approximately 0.2 mM. In prostate this value approximates 1-5 mM depending upon the source of prostate tissue (eg rat or human prostate). For this discussion we would define three pools of zinc that comprise the total cellular zinc: a) Tightly bound zinc (mainly protein and nucleic acid bound) that is an immobile unreactive pool; b) loosely bound zinc (such as amino acid and citrate bound) which constitute a mobile reactive pool; c) free Zn++ ion which would be a reactive pool. The immobile unreactive pool comprises >95% of the total cellular zinc. The free Zn++ ion concentration is negligible; estimated to be in the nanomolar to picomolar range, and even as low of femtomolar concentration [6,7]. Therefore, zinc bound to mainly low molecular ligands (ZnLigands) comprises the major mobile reactive pool of zinc. Even if this pool represents as little as 0.1-1% of the total cellular zinc, its concentration would approximate 0.2-2 μM in mammalian cells; and about 1-10 μM in prostate cells.

These relationships raise the important question, “How is zinc trafficked through the cytosol for uptake and accumulation in mitochondria of prostate cells?” Prostate cells, unlike other mammalian cells, contain a high cellular concentration of citrate which is a major ligand for zinc that
represents as much as 30% of the total cellular zinc [8-10]. Since cytosolic free Zn++ ion concentration is negligible (10^{-9}-10^{-15} M), we postulated that the mitochondrial accumulation of zinc must involve a mitochondrial zinc uptake process that is not dependent upon the transport of zinc from a cytosolic free Zn++ ion pool. However, as of this time, the existence of a specific mitochondrial zinc uptake transporter or transport process has not been reported in any mammalian cells. We now present evidence that prostate mitochondria contain a specific zinc uptake transport process that involves a zinc exchange from donor ZnLigands to a putative zinc transporter located on the inner mitochondrial membrane; and that the transport does not require free Zn++ ion transport. Preliminary evidence is presented that this zinc transport process also exists in liver mitochondria.

**EXPERIMENTAL PROCEDURES**

**Isolation of Mitochondria:** Young adult male Wistar rats weighing between 300 and 350 g were employed as the source of tissues for these studies. The handling and treatment of animals were in accordance with the regulations and guidelines of the National Institutes of Health and the University of Maryland. The preparation of prostate and liver mitochondria has been described previously [4,5]. All procedures were carried out at 2-4°C on ice. Generally, rat ventral prostate (VP) and liver tissue were chopped into 1mm pieces in isolation buffer (250 mM sucrose, 10 mM HEPES and 1 mM EDTA, pH 7.35), homogenized in a motor glass homogenizer, and centrifuged at 500 x g for 5 minutes. The supernatant fluid was centrifuged for 7 minutes at 12,000 x g and the resulting pellet was washed twice in isolation buffer containing 0.25% fatty acid-free BSA, and washed once in reaction buffer (250 mM sucrose, 10 mM HEPES and 5 mM KH_{2}PO_{4}). The final mitochondrial pellets were suspended in reaction buffer and adjusted to provide a mitochondrial concentration around 20 mg protein/mL. Protein assay was performed by the method of Bradford [11]. The condition of the mitochondrial preparations was checked by determination of oxygen consumption and respiratory control with the aid of a fiber optic oxygen monitoring system. Preparations that did
not meet the criteria of no detectable endogenous respiration and a succinate-stimulated respiratory control ratio > 2.5 were generally excluded from the studies.

**Isolation of Mitoplasts:** Liver mitoplasts were prepared as described by Ye et al. [12] and Greenawalt [13]. The mitochondria were isolated in buffer medium containing 70 mM sucrose, 220 mM mannitol, 2 mM HEPES and 0.25% BSA, pH 7.35. The mitochondrial suspension was adjusted to 50 mg protein/mL and treated with 0.6 % digitonin by stirring on ice for 15 minutes. The suspension was centrifuged at 12,000 x g, and the resulting mitoplast pellets were washed once and re-suspended in reaction buffer.

**Zinc Uptake Assay:** Zinc transport was determined by $^{65}$Zn uptake in mitochondria. All $^{65}$Zn solution were dissolved in medium containing 250 mM sucrose and 10 mM HEPES, pH 7.35. Generally 75 µl mitochondria suspension containing 250 µg mitochondrial protein was added to 75 µl reaction buffer containing $^{65}$Zn in a 250 µl microfuge tube. After an appropriate incubation time at 37°C, the reaction tubes were loaded on a BRANDEL harvesting system (BRANDEL, Gaithersburg, MD), rapidly aspirated onto filter discs and immediately washed with 20 mL cold isolation buffer. The filters containing the mitochondria were placed into vials containing liquid scintillation cocktail, and the $^{65}$Zn was counted in a liquid scintillation counter. The same protocol was used for $^{14}$C assay. Generally, the ZnLigand solutions were prepared by the addition of the ligand to the stock ZnCl$_2$ solution containing $^{65}$Zn to provide a Zn/Ligand molar ratio of 1/3. For total zinc accumulation the mitochondria were digested and the zinc content was determined by atomic absorption as previously described [14].

**Statistics analysis:** Zinc accumulation and kinetic experiments were repeated two or three times to ensure the reproducibility of the results. The data and plots were analyzed by SigmaPlot 8.0 with the Enzyme Kinetics Module. The representative results are presented.

**RESULTS**

1. Studies with Prostate Mitochondria:
In these initial studies, zinc uptake rates were determined with the following zinc substrates: ZnCl$_2$ as a source of free Zn$^{++}$ ions; zinc citrate (ZnCit), zinc aspartate (ZnAsp), zinc histidine (ZnHis), and zinc cysteine (ZnCys) as potential zinc-transportable ZnLigands; and ZnEDTA and ZnEGTA as a low molecular weight sources of tightly bound zinc. ZnCit was selected because it is an important zinc ligand form in prostate cells. ZnAsp was selected because cellular aspartate concentration, like citrate, is high in prostate cells [15]. Fig.1 shows a time-course study of zinc uptake by prostate mitochondria. The uptake of $^{65}$Zn from 20 μM ZnCl$_2$ (ie 20 μM free Zn$^{++}$ ion) was compared with zinc uptake from ZnCit, ZnHis, ZnCys and ZnEDTA. The concentration of total zinc was 50 μM and the ligand was 60 μM. The estimated free Zn$^{++}$ ion concentration for ZnCit is ~4 μM, and is negligible for ZnEDTA. The results demonstrate that zinc uptake rates from ZnCl$_2$, ZnCit, ZnHis and ZnCys were identical and increased with time over the 15-minute uptake period. Evidently, these chelated forms of zinc were as effective as free Zn$^{++}$ ions as donors for zinc uptake. In contrast, no demonstrable uptake of zinc from ZnEDTA was evident. This indicates that undissociated ZnEDTA is not permeable across the mitochondrial inner membrane; and that zinc is not released from EDTA for availability for uptake. These observations are confirmed and extended by studies described below.

We then determined the relationship of prostate mitochondrial uptake of zinc versus zinc concentration with ZnCl$_2$, ZnCit, ZnAsp and ZnEDTA as substrates (Fig.2). Except for ZnEDTA, the rates of zinc uptake were dependent upon the concentration of zinc. No uptake of zinc was detectable from ZnEDTA over the range of zinc concentrations that was employed. Zinc uptake from the other substrates exhibited Michaelis-Menten kinetics that demonstrated the existence of a transport process. The $K_m$ (μM zinc) and $V_{max}$ (nmol zinc/mg mitochondrial protein/min) values with ZnCl$_2$ and ZnAsp as substrates were essentially identical ($K_m$ ~55-60; $V_{max}$ ~63-67; Table 1). The $K_m$ ~31 and $V_{max}$ ~0.42 with ZnCit were significantly different from ZnCl$_2$ and ZnAsp. However at zinc concentrations up to ~50 μM, the uptake rates were essentially the same for all three substrates (Fig.2A). Because
the ratio of ligand to zinc was maintained at 3/1, the free Zn++ ion concentrations for ZnCl₂, ZnCit, and ZnAsp preparations were 5-50 μM, 1-10 μM, and 0.05-0.5 μM, respectively (Fig.2C). Therefore, the uptake of zinc, as in Fig.1, was independent of the concentration of free Zn++ ion concentration; but was dependent upon the total zinc concentration.

The effect of varying the citrate/zinc ratio on the uptake of zinc was then determined (Fig.3). The concentration of zinc (ZnCl₂) was maintained at 20 μM. Over the range of citrate/zinc ratios of 0.5/1-15/1 (ie the addition of 10-300 μM citrate), the uptake of zinc was the same as in the absence of citrate. Over this range the estimated free Zn++ ion concentration varied from ~11 μM at 0.5/1 ratio to ~1 μM at 10/1 ratio. Consequently the uptake of zinc from ZnCit was independent of the free Zn++ ion concentration. Since zinc uptake remained identical to the uptake from ZnCl₂ in the absence of any citrate, it is the total zinc (ZnCit plus free Zn++ ions) that constitutes the transportable zinc pool.

The possibility existed that the uptake of zinc from ZnLigand (ie ZnCit, ZnAsp) might result from uptake of the undissociated ZnLigand complex. Therefore we determined the simultaneous uptake of zinc and citrate. Citrate exists predominately as a trianion at the physiological pH range and is virtually impermeable across the mitochondrial membrane in well-maintained coupled mitochondrial preparations. The simultaneous uptake of ⁶⁵Zn and ¹⁴C citrate from ZnCit was determined (Fig.4). The results show that virtually no citrate uptake accompanied the zinc uptake by the mitochondria. In fact the results also demonstrate the integrity of the mitochondria since neither undissociated ZnCit (~16 μM) nor free citrate (~44 μM) entered the mitochondria by diffusion or by transport. These results, along with other results presented, indicate that the transport of zinc involves the direct transfer of zinc from ZnLigand to the putative zinc transporter; ie an inter-molecular zinc transfer that does not necessitate the involvement of a free Zn++ ion donor pool.

The composite studies described above demonstrated the ability of ZnCit, ZnHis, ZnCys and ZnAsp, but not ZnEDTA nor ZnEGTA (Fig.1 and 11, described below), to act as effective zinc donors for mitochondrial transport. This indicated that the formation constant (Kf) of ZnLigands is
an important determinant for the availability of zinc for mitochondrial transport. The log K_f value for each ligand is ZnCit~5, ZnAsp~6, ZnHis~6, ZnCys~10, ZnEGTA~12 and ZnEDTA ~16. Therefore all the ZnLigands with log K~10 or lower served as effective donors of zinc for mitochondrial transport. ZnLigands with log K_f~12 or higher did not donate zinc for transport. These results indicate that the putative zinc transporter has an effective log K_f~11.

The above observations lead to the conclusion that mitochondrial zinc uptake under the conditions employed was the result of the existence of a transport process; ie a putative zinc transporter. The following studies were then conducted to characterize further the transport properties. To obtain some indication of the metal specificity of the zinc transport, the effects of Ca++, Mg++, and Cd++ on zinc uptake was determined (Fig.5). Ca++ and Mg++ at concentrations up to 7-8 fold greater than zinc had no effect on the uptake of zinc from ZnCit. The absence of any effect by Ca++ also indicates that the uptake of zinc does not involve a Ca++ ion channel. However, Cd++ at equimolar concentration with zinc exhibited ~20% inhibition of zinc uptake; which increased to a maximum inhibition ~40% by increasing the Cd++ to 5-fold greater than zinc. Lineweaver-Burk plot (Fig.6) revealed a K_f~126 μM Cd++, and a noncompetitive inhibition. Because some zinc transporters evolved as zinc/ferrous iron transporter (eg ZIP transporter family), it was important to determine if Fe++ competed with the mitochondrial import of zinc. To insure that Fe++ was not oxidized, dithionite was also added; and Fe++ with or without dithionite had no effect on zinc uptake by mitochondria (results not shown).

The transport studies described above were conducted with non-respiring coupled mitochondrial preparations that contained no added utilizable energy sources. This suggested that the uptake of zinc might be energy-independent. To establish this relationship zinc uptake was determined under conditions that would alter the energy and respiratory state of the mitochondria (Fig.7A). The addition of succinate alone or with ADP had no effect on the uptake of zinc. Neither the addition of the uncoupling agent dinitrophenol nor the addition of cyanide to inhibit cytochrome
oxidase and respiration altered the uptake of zinc. We conducted parallel studies (not shown) of the respiration and terminal oxidation of the mitochondrial preparations to verify the treatment effects. Consequently, zinc transport is not energy-dependent. In addition, over the range of pH 6.0-8.0, there occurred no significant change in the zinc uptake rate (Fig. 7B). Therefore, the collective results of these studies strongly indicate that the mitochondrial uptake of zinc is the result of a facilitative zinc transporter and not an active transport process.

2. Studies with Liver Mitochondria

The studies described above establish the existence of a zinc transport process (putative zinc uptake transporter) involved in the mitochondrial uptake and accumulation of zinc in prostate cells. Whether or not this zinc transport process is specific and unique to prostate mitochondria needed to be addressed. Consequently, some zinc uptake studies were conducted with liver mitochondria. Fig. 8 reveals that liver mitochondria, like prostate mitochondria (Fig. 1), exhibited zinc uptake from ZnCit as well as ZnCl₂, but not from ZnEDTA or ZnEGTA. Lineweaver-Burk analysis (Fig. 10) of zinc uptake results in \( K_m \approx 80 \mu M \) and \( V_{max} \approx 0.90 \) nmol zinc uptake/min/mg mitochondrial protein for ZnCl₂; and \( K_m \approx 27 \) and \( V_{max} \approx 0.48 \) for ZnCit. It is interesting to note that the \( K_m \) and \( V_{max} \) values of liver and prostate mitochondria are quite similar for ZnCit (Table 1); but the values are highly dissimilar for ZnCl₂. However at zinc concentrations up to \( \sim 50 \mu M \), liver mitochondria zinc uptake from ZnCl₂ and ZnCit was identical (Fig. 9); as was also the case for prostate mitochondria (Fig. 2). These studies demonstrate that the uptake of zinc by liver mitochondria is not dependent upon a free Zn++ ion pool; and that ZnLigands provide an effective zinc donor source for zinc uptake. Therefore, it is evident that liver mitochondria, like prostate mitochondria, also contain a zinc uptake transport process (i.e. a zinc transporter).

It became essential to establish that the zinc transport process as determined by the \(^{65}\)Zn uptakes studies was associated with an increased net accumulation of zinc in the mitochondria.
Therefore we determined, under the conditions employed for the $^{65}\text{Zn}$ experiments, the level of total zinc in liver mitochondria after incubation with ZnCl$_2$, ZnCit, ZnCys, and ZnEDTA (Fig.10A). A rapid accumulation of high zinc levels was evident within two minutes and reached a peak by ten to fifteen minutes with ZnCl$_2$, ZnCIT and ZnCys as the sources of zinc. With ZnEDTA, no zinc accumulation resulted. These results parallel the $^{65}\text{Zn}$ uptake studies. Importantly, the net accumulation of zinc is not dependent upon a pool of free zinc since free zinc ions are negligible in the presence of cysteine. The absence of accumulation with ZnEDTA further indicates that the relative zinc-binding affinity for ZnLigand and putative transporter is a key factor in the mitochondrial transport process. The absence of accumulation with ZnEDTA is also evidence of the integrity of the mitochondrial preparations. A subsequent study (Fig.10B) demonstrates that prostate and liver mitochondria accumulate similar levels of total zinc. It is noteworthy that the maximal total zinc accumulation of about 5-6 nmol/mg protein at 10-15 minutes approximates the calculated zinc accumulation from the $^{65}\text{Zn}$ uptake studies. When adjusted for 50 µM zinc in medium, the estimated total zinc accumulation represented in fig.1, 8, and 11 is about 5-7 nmol/mg protein. This confirms that the uptake of $^{65}\text{Zn}$ was retained and accumulated in the mitochondria.

Since low molecular weight solutes such as the ZnLigands employed in these studies would be permeable across the mitochondrial outer membrane, the likely site for the putative zinc transporter would be the mitochondrial inner membrane. To investigate this likelihood mitoplasts were prepared from liver mitochondria to eliminate the outer membrane. Zinc uptake by the mitoplasts was compared with the intact mitochondria (Fig.11). Zinc uptake from ZnCl$_2$ and ZnCit by the mitoplast paralleled the uptake by the intact mitochondria. The absence of any zinc uptake from ZnEDTA and ZnEGTA demonstrates that the integrity of the inner membrane was retained in the mitoplast preparation. It is also significant that the mitoplast uptake of zinc from ZnCit was slightly less than from ZnCl$_2$; which is a characteristic that occurred in all of the intact mitochondrial
preparations from prostate and from liver. These results support the expectation that the putative zinc uptake transporter is associated with the mitochondrial inner membrane.

DISCUSSION

These studies reveal that zinc uptake and accumulation by prostate and liver mitochondria occurs via a zinc transport process. The uptake of zinc is saturable, energy-independent, and exhibits kinetic characteristics that are representative of the existence of a facilitative zinc transporter. The transporter appears to be associated with the mitochondrial inner membrane. The putative transporter exhibits a high specificity for zinc since neither Ca++, nor Mg++, nor low concentration of Cd++ altered the uptake of zinc. To our knowledge this is the first identification of a specific mitochondrial zinc uptake transport process in mammalian cells. It will be important to identify the putative transporter that is responsible for this zinc transport activity. To date, no genetic or proteomic identification of such a transporter has been established.

This study reveals that the availability of free Zn++ ions is not a required source of donor zinc for transport and accumulation in mitochondria. Zinc derived from ZnLigands was directly transferred to the transporter, which provides the mechanism for zinc uptake by the mitochondria. This is further verified by the mitochondrial transport of zinc from ZnLigand without an accompanying uptake of the ligand. Also, the absence of zinc uptake from either ZnEDTA or ZnEGTA indicates that undissociated low molecular weight ZnLigands are not permeable or transportable across the mitochondrial inner membrane. An important determinant of the availability of zinc for transport is the formation constant (Kf) of the ZnLigand; ie the binding affinity of ligand for zinc. The effective log Kf of the transporter is estimated to be ~11 since ZnLigands with log Kf<11 were equally effective zinc donors available for transport, and ZnLigands with log Kf>11 were ineffective zinc donors.
The effectiveness of ZnLigands to donate zinc for mitochondrial import in prostate cells is consistent with the fact that the free Zn++ ion concentration of cytosol is in the nanomolar to femtomolar range; and, therefore, does not provide a sufficient zinc donor pool for mitochondrial uptake leading to zinc accumulation. Moreover the \( K_m \) for zinc uptake from \( \text{ZnCl}_2 \) is \( \sim 60 \ \mu \text{M} \) zinc, which is 1000-fold or greater than the cytosolic concentration of free Zn++ ions. In contrast the effective ZnLigands (eg amino acids, citrate) likely constitute a zinc pool in the micromolar range that would be functional at the \( K_m \) range exhibited by the prostate and liver mitochondrial transport.

These studies show that both prostate and liver mitochondria are similarly capable of importing and accumulating zinc from ZnLigands. Moreover, both exhibit similar values of maximal zinc accumulation, i.e. about 2-7 nmols Zn/mg protein in the presence of zinc in the range of 20-50 \( \mu \text{M} \). The in situ level of zinc in ventral prostate cells approximates 2 nmol/mg protein [14], which is virtually identical to the experimental value obtained in this study. Liver mitochondria in situ contains only about 0.6 nmol Zn/mg protein although the capacity for zinc accumulation is similar to prostate mitochondria [12,14]. This difference is consistent with our concept that the higher cytosolic concentration of zinc-transportable ZnLigands (particularly ZnCit) in prostate cells likely accounts for the characteristically higher zinc accumulation of prostate mitochondria compared to other cells. The similarity of the experimental level of zinc accumulation to the in situ mitochondrial zinc level provides additional support that the mitochondrial zinc uptake transport process identified in this study most likely is functionally operational in situ.

Despite the fact that zinc uptake and accumulation in mitochondria has long been known to be an important cellular functional relationship, few reported studies have addressed the issue of the mechanisms and processes of zinc entry into mitochondria. Most of the early studies of zinc uptake and its effects on mitochondria employed free Zn++ ions at concentrations that are now known to be highly unphysiological. Brierley and Knight [16] reported that heart mitochondria accumulated zinc by energy-dependent and by passive processes depending upon the conditions employed; but free
Zn++ in the range of 100-500 μM was employed in those studies. It has been suggested that free Zn++ ions enter the mitochondria via the calcium uniporter [17]. However those studies were also conducted with μM concentrations of free Zn++ ions, with no evidence that Zn++ at levels more representative of cytosol (10⁻⁹ M or lower) would successfully traverse the ion channel resulting in sufficient accumulation of intramitochondrial zinc. Also no direct measurements of mitochondrial uptake of zinc were provided. It is notable that Ye et al. [12] reported that metallothionein was an important chaperone for the delivery of cytosolic zinc to liver mitochondria. They showed that cytosolic metallothionein enters the intermembrane space where it relinquishes zinc that inhibits respiratory components at that location. Although they report that metallothionein does not enter into the mitochondrial matrix, no information was presented regarding the possible accumulation of zinc in the matrix. Our studies show that ZnCys (log Kf~10) is an effective zinc donor for the mitochondrial transport of zinc. Metallothionein also has a log Kf~10, and could possibly serves as a zinc donor for the zinc uptake transporter. We are making preparations to investigate the potentially important role of metallothionein in the exchange of zinc with the putative mitochondrial transporter for the uptake of zinc.

Our findings and this discussion are not intended to eliminate the involvement of other mitochondrial zinc import mechanisms that might exist. The process that we have now identified is operational under conditions that mimic the likely in situ availability of transportable zinc forms that exist in cytosol, especially in regard to the unique zinc relationships of prostate cells. Clearly much additional study of the mitochondrial zinc-uptake transport process and the putative transporter that we have described is essential. This initial report establishes the existence of such a mitochondrial transporter and provides the basis for further research.
ACKNOWLEDGEMENT

These studies were supported by NIH research grants CA71207, CA79903 and DOD grant PC001174.
Abbreviations

The abbreviation used are: EDTA, Ethylenediaminetetraacetic Acid; EGTA, ethylene-glycol-bis(β-aminoethyl)ether)N,N,N,N-tetraacetic acid; HEPES, N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid; ADP, Adenosine 5'-diphosphate; BSA, bovine serum albumin,
References


Table 1. Kinetic Parameters of Zinc Uptake in Rat Ventral Prostate and Liver Mitochondria.

<table>
<thead>
<tr>
<th>Mitochondria</th>
<th>Zn</th>
<th>Km</th>
<th>Vmax</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>SE</td>
</tr>
<tr>
<td>V. P.</td>
<td>ZnCl₂</td>
<td>59.93</td>
<td>9.98</td>
</tr>
<tr>
<td></td>
<td>ZnASP</td>
<td>54.74</td>
<td>8.53</td>
</tr>
<tr>
<td></td>
<td>ZnCIT</td>
<td>31.14</td>
<td>3.23</td>
</tr>
<tr>
<td>Liver</td>
<td>ZnCl₂</td>
<td>80.12</td>
<td>10.71</td>
</tr>
<tr>
<td></td>
<td>ZnCIT</td>
<td>26.52</td>
<td>2.22</td>
</tr>
</tbody>
</table>

Km = uM Zn; Vmax = nmol Zn/min/mg mitochondrial protein

1 ZnCIT vs ZnCl₂, p < 0.01;
2 ZnCIT vs ZnASP, p<0.01
Figure 1. Time course of $^{65}$Zn uptake from ZnCl$_2$, ZnCitrate, ZnHistidine, ZnCysteine and ZnEDTA by VP mitochondria. Substrates contained 20 uM ZnCl$_2$ and 60 uM ligand. Incubation at 37°C.

Figure 2. Zinc uptake from ZnCl$_2$, ZnAspartate and ZnCitrate in rat ventral prostate mitochondria. Uptake was performed at 37 °C, 15 minutes incubation. A. Hyperbolic plot B. Lineweaver-Burk plot inset; C. Free Zinc ion concentration.

Figure 3. Effect of added citrate on $^{65}$Zn uptake from 20 uM ZnCl$_2$ by rat ventral prostate mitochondria. Uptake was for 15 minutes at 37°C.

Figure 4. $^{65}$Zn and $^{14}$C-Citrate uptake from ZnCitrate at 37°C. ZnCitrate was prepared with 20 uM ZnCl$_2$ and 60 uM citrate.

Figure 5. Divalent cation effects on $^{65}$Zn uptake from ZnCitrate. VP mitochondrial uptake at 37°C, 15 minutes incubation. Indicated concentration of CaCl$_2$, MgCl$_2$ and CdCl$_2$ was added to reaction buffer containing 20 uM ZnCl$_2$ and 60 uM citrate.

Figure 6. Cd$^{2+}$ (60 uM) inhibition of zinc uptake from ZnCitrate. VP mitochondria at 37°C and 15 minutes incubation. Lineweaver-Burk plot and hyperbolic plot.

Figure 7. pH and energy effect effect on $^{65}$Zn uptake from ZnCitrate by VP mitochondria. 15 minutes incubation at 37°C. ZnCitrate preparation contained 20 uM ZnCl$_2$ and 60 uM citrate.

Figure 8. Time course of $^{65}$Zn uptake from ZnEDTA, ZnEGTA, ZnCitrate and ZnCl$_2$ by rat liver mitochondria. Substrates contained 50 uM ZnCl$_2$ and 150 uM ligand.

Figure 9. Zinc uptake from ZnCl$_2$ and ZnCitrate in rat liver mitochondria. 15 minute incubation at 37 °C.

Figure 10. Total zinc accumulation in mitochondria. Zinc concentration was 30 uM and ligands were 90 uM. After incubation, reaction tubes were rapidly centrifuged and mitochondrial pellets were digested and assayed for total zinc by atomic absorption. A) Zinc accumulation by liver mitochondria. B) Comparison of total zinc accumulation in liver and prostate mitochondria.

Figure 11. Time course of zinc uptake from ZnEDTA, ZnEGTA, ZnCitrate and ZnCl$_2$ in rat liver mitochondria vs mitoplasts at 37°C. Substrates contained 50 uM ZnCl$_2$ and 150 uM ligand.
Figure 1
Figure 2.
Figure 3
Figure 5.
$V_{\text{max}} = 0.4099$
$K_m = 29.5$
$K_I = 126.8$

Figure 6.
Figure 7.
Figure 8.
Figure 9.
Figure 10.
Figure 11.