

Research article

HIGH INTRACELLULAR Zn²⁺ IONS MODULATE THE VHR, ZAP-70 AND ERK ACTIVITIES OF LNCaP PROSTATE CANCER CELLS

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Abstract: Malignant prostate tissues have markedly reduced zinc (Zn²⁺) contents in comparison to non-malignant tissues. In this study, we restored a high intracellular Zn²⁺ level to LNCaP prostate cancer cells by culturing the cells in a growth medium supplemented with a supraphysiological concentration of Zn²⁺ (10 µg/ml) over 5 weeks. The intracellular Zn²⁺ level increased in the Zn²⁺-treated cells, and there was a marked increase in the presence of zinosomes, a Zn²⁺-specific intracellular organelle. The proliferation rate of the Zn²⁺-treated cells was markedly reduced. There was also a significant increase (36.6% ± 6.4%) in the total tyrosine phosphorylated proteins. Vaccinia H1-related (VHR) phosphatase, zeta chain-associated protein-70 (ZAP-70) kinase and phosphorylated extracellular signal-regulated protein kinase 1 and 2 (p-ERK 1 and 2) were also present in higher abundance. Treatment with TPEN, which chelates Zn²⁺, reduced the abundance of VHR phosphatase and ZAP-70 kinase, but increased the abundance of p-ERK 1. However, the TPEN treatment restored the Zn²⁺-treated LNCaP cell proliferation to a rate comparable to that of the non Zn²⁺-treated cells. These results highlight the importance of a high intracellular Zn²⁺ content and the VHR/ZAP-70-associated pathways in the modulation of LNCaP prostate cancer cell growth.

Key words: Cancer, ERK, Prostate, VHR, ZAP-70, Zn²⁺

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Abbreviations used: ERK – extracellular signal-regulated protein kinase; KAP – kinase-associated phosphatase; PTK – protein tyrosine kinase; PTP – protein tyrosine phosphatase; PTP1B – protein tyrosine phosphatase 1B; PTP1C/SHP-1 – protein tyrosine phosphatase 1C; TPEN – *N,N,N',N'*-tetrakis(2-pyridylmethyl)ethylenediamine; VHR – vaccinia H1-related; ZAP-70 – zeta chain-associated protein-70

INTRODUCTION

Prostate cancer is one of the most common malignancies affecting males in Western countries [1]. The etiology of the disease is complex and it has a multitude of potential contributing factors including genetic and environmental factors [2]. One of the consistent markers of prostate malignancy is the significantly low Zn^{2+} concentration in malignant prostate tissues [3]. It is presently unknown how prostate cells could lose the ability to accumulate high levels of intracellular Zn^{2+} , and whether this occurs as a consequence of acquiring cancerous properties. In a number of earlier studies, Zn^{2+} was reported to inhibit prostate cancer cell proliferation and invasion through several different pathways, including the induction of mitochondrial apoptogenesis [4, 5], the suppression of the NF- κ B signaling pathways [6], and the suppression of the invasive potentials of human prostate aminopeptidase N and urokinase-type plasminogen activator [7]. In other studies, Zn^{2+} is shown to enhance telomerase activity [8] and antagonize the inhibitory effects of bisphosphonates on tumor cell invasion potentials [9]. The diverse effects of Zn^{2+} on prostate cells reflect the diversity of the interaction of this ion with the various cellular kinases, phosphatases and transcription factors in the regulation of cellular processes [10]. These include the stimulation of protein tyrosine phosphorylation through the inhibition of phosphatases (PTPs) such as protein tyrosine phosphatase 1B (PTP1B), PTP1C (SHP-1) and vaccinia H1-related phosphatase (VHR) [11-14], and also the activation of the mitogen-activated protein kinase (MAPK) cascade [15-19]. These earlier findings strongly emphasize the potential importance of Zn^{2+} regulation in the pathogenesis of prostate cancers. However, most of these earlier findings are derived from the short-term exposure of prostate cancer cells to Zn^{2+} . These cellular responses may be transient responses and do not reflect the long-term effects of a high intracellular Zn^{2+} content. In this study, we simulated the effects of progressive long-term intracellular Zn^{2+} accumulation in prostate cells by prolonged culture of LNCaP prostate cancer cells in a growth medium supplemented with a supraphysiological concentration of Zn^{2+} as a model for the identification of the cell signalling pathways affected by the accumulation of high intracellular Zn^{2+} .

MATERIALS AND METHODS

Cell cultures and treatments

Human prostate cancer cells with the LNCaP.FGC designation (American Type Culture Collection, Rockville, USA) were maintained in RPMI 1640 (Flowlab, Australia) supplemented with 10% Fetal Bovine Serum (FBS; Biowhittaker, Walkersville, USA). The cells were propagated in growth medium supplemented with Zn^{2+} (1 μ g/ml and 10 μ g/ml) using $ZnSO_4$ for 5 passages in 5 weeks. Cell cultures supplemented with only the Zn^{2+} diluent were propagated in parallel, and used as controls. Cells were also treated with 100 μ M *N,N,N',N'*-tetrakis

(2-pyridylmethyl)ethylenediamine (TPEN; Sigma, St. Louis, USA) for 90 min at 37°C to chelate Zn²⁺. In separate experiments, the Zn²⁺-supplemented cells were also treated with H₂O₂ (200 μM), a known inhibitor of phosphatases, for 40 min at 37°C for immunoblot analyses. The cell proliferation rate was measured over a period of 6 days in week 6 post-initiation of the Zn²⁺ treatment using a Cell Titer 96[®] Non Radioactive Cell Proliferation Assay Kit (Promega, Madison, USA). Cells at week 5 post-initiation of the Zn²⁺ treatment were used for all the other experiments.

Intracellular Zn²⁺ staining

Approximately 5 × 10⁴ LNCaP prostate cancer cells were seeded into a 24-well tissue culture plate and cultured to 70% confluency prior to staining. Zn²⁺ was chelated from the cell cultures by treating the cells with TPEN (100 μM) for 90 min at 37°C. In those experiments where the Zn²⁺ uptake was enhanced, the cells were pretreated with Zn²⁺ pyrithione, 1-hydroxypyridine-2-thione (4 μM; Sigma, USA) for 30 min at 37°C before the treatment with Zn²⁺ (1 μg/ml). The treated cells were fixed with 4% paraformaldehyde and stained with 25 μM Zinquin ethyl ester (Calbiochem, San Diego, USA) for 30 min at 37°C. The cells were then counter-stained with propidium iodide solution (10 μg/ml).

Colorimetric Zn²⁺ assay

The concentration of Zn²⁺ in the LNCaP cells was determined using the water-soluble pyridylazo dye, 2-(5-bromo-2-pyridylazo)-5-(N-propyl-N-sulfopropyl amino) phenol disodium salt dihydrate (5-Br-PAPS, Fluka, Japan) as described previously [20]. Briefly, cells were scraped from the tissue culture flasks, collected, and washed three times with ice-cold PBS. The cells were then sedimented by centrifugation at 800 × g for 5 min, resuspended in 10% trichloroacetic acid (TCA), and incubated on ice for 15 min. After another centrifugation at 4°C for 10 min, the pellet was saved and the resulting supernatant was removed and incubated with 0.08 mM 5-Br-PAPS and 29 mM salicylaldehyde at room temperature for 10 min. The absorbance of the mixture was then measured at 570 nm. The TCA-precipitated proteins were solubilized with a buffer containing 8 M Urea, 4% CHAPS, 40 mM Tris and 2 mM Tributylphosphine (TBP). Following an overnight incubation, the protein suspension was centrifuged at 40,000 × g for 1 hour. The supernatant was collected and quantitated using the Micro BCA assay (Pierce, Illinois, USA). The total cellular Zn²⁺ concentration was normalized to the cell protein concentration.

Cell cycle distribution analysis

Cells were trypsinized from tissue culture flasks and stained with propidium iodide using the CycleTEST[™] PLUS DNA Reagent Kit strictly according to the manufacturer's protocol (BD Biosciences Pharmingen, San Diego, USA). Freshly stained cells were then subjected to flow cytometry analysis using the

BD FACS Calibur (BD Biosciences, San Diego, USA). Prior to data acquisition, the linearity and resolution of the flow cytometer were verified and adjusted using DNA Quality Control Particles (BD Biosciences, San Diego, USA). Data acquisition and analysis were performed using BD CellQuest™ Pro Software (BD Biosciences, San Diego, USA) and ModFit LT 3.1 (Verity Software House, Topsham, USA).

Protein tyrosine kinase (PTK) and phosphatase (PTP) assays

PTK and PTP assays were performed in separate experiments as described previously [12]. Cells were detached from the tissue culture flasks and washed three times with buffer A, consisting of 20 mM HEPES/NaOH and 20 mM MgCl₂, pH 7.5. For the PTK assay, cells were resuspended in Buffer A, sonicated and incubated at room temperature for 30 min to allow dephosphorylation. Sodium orthovanadate (10 mM) was added to inhibit phosphatase activities. Protein phosphorylation was initiated by adding ATP (1 mM) to the lysates, and at selected time points, the cell lysates (100 µl) were aliquoted and separated by SDS-PAGE. For the PTP assay, cell lysates obtained following sonication in Buffer A at selected time points were immediately removed and separated by SDS-PAGE. The total tyrosine phosphorylated proteins were detected by immunoblotting using a antibody specific against phosphotyrosine.

Immunoblotting

Immunoblotting was performed using antibodies specific against VHR, PTP-1B, PTP-1C/SHP-1, kinase-associated phosphatase (KAP), ERK and ZAP-70 (all from BDH Pharmingen, San Diego, USA), phosphotyrosine (P-Tyr-100; Cell Signaling Technology, Danvers, USA) and β-actin (Sigma, St. Louis, USA). Specific antibodies that distinctively differentiate phosphorylated extracellular signal-regulated protein kinase 1 and 2 (p-ERK1 and 2; BDH Pharmingen, San Diego, USA) were used. The protein band intensities on the immunoblot were determined using Quantity One version 4.2.2 (Bio-Rad Laboratories, Hercules, USA). Pairwise comparisons of the protein bands were performed using BioNumerics version 2.50 (Applied Maths, Belgium).

Statistical analysis

Statistical analyses were performed using GraphPad Prism Version 4.03 (GraphPad Software Inc., San Diego, USA). The data was expressed as the mean ± standard error of the mean (SEM). Statistical differences were analyzed using Student's *T*-test or one-way ANOVA as indicated. A *P*-value < 0.05 was considered statistically significant.

RESULTS

Prolonged Zn^{2+} supplementation restores a high intracellular Zn^{2+} level to LNCaP prostate cancer cells

LNCaP prostate cancer cells were continuously cultured in a growth medium supplemented with 1 $\mu\text{g/ml}$ and 10 $\mu\text{g/ml}$ Zn^{2+} as a means of restoring a high intracellular Zn^{2+} level to these cells. At week 5 post-initiation of the Zn^{2+} treatment, the Zn^{2+} -supplemented LNCaP cells and cells treated with 1 $\mu\text{g/ml}$ Zn^{2+} in the presence of Zn^{2+} pyrithione exhibited numerous intense fluorescing vesicular zincosome structures (Fig. 1C, D, E) when stained with Zinquin, a Zn^{2+} -specific fluorescent probe which stains for zincosomes in the cell cytoplasm. By contrast, PNT2 and LNCaP cells cultured in a growth medium without the additional Zn^{2+} showed a much lower presence of zincosomes (Fig. 1A, B). A dull homogenous fluorescence that was not representative of Zn^{2+} -specific staining was observed in cells where Zn^{2+} was chelated using TPEN (Fig. 1F).

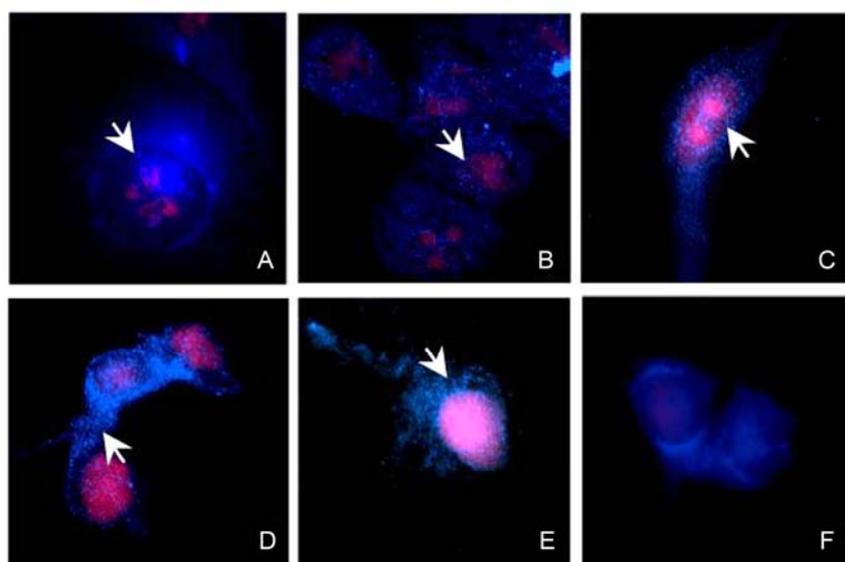


Fig. 1. The detection of intracellular Zn^{2+} in LNCaP and PNT2 cells using a Zn^{2+} -specific Zinquin fluorescent probe. Normal PNT2 prostate cells cultured in the growth medium without additional Zn^{2+} (A), and LNCaP cells cultured in the growth medium without additional Zn^{2+} (B), or with 1 $\mu\text{g/ml}$ Zn^{2+} (C) or 10 $\mu\text{g/ml}$ Zn^{2+} (D) are shown. Also shown are the positive control LNCaP cells supplemented with 1 $\mu\text{g/ml}$ Zn^{2+} in the presence of Zn^{2+} pyrithione to enhance Zn^{2+} uptake (E) and negative control LNCaP cells cultured in the growth medium supplemented with 1 $\mu\text{g/ml}$ Zn^{2+} in the presence of Zn^{2+} pyrithione and then treated with TPEN to chelate the Zn^{2+} (F). Arrows indicate the zincosomes. The cell nuclei were stained with propidium iodide and viewed under 100 \times magnification.

At the beginning of the study, the concentrations of Zn^{2+} when measured using pyridylazo dye in the PNT2 normal prostate cells and in all the LNCaP cell cultures were respectively 7.8 nmol/mg and ~6.5 nmol/mg protein (Tab. 1). At week 5 post-initiation of the Zn^{2+} -treatment, the concentration of Zn^{2+} in the Zn^{2+} -supplemented LNCaP cells increased to an average of ~10.0 nmol/mg protein, consistent with the increase in the number of zincosomes (Fig. 1B, C). By contrast, the Zn^{2+} concentration in the control non-supplemented LNCaP cells remained at ~6.2 nmol/mg protein, and the cells showed a much lower number of zincosomes (Fig. 1A). These results suggested that high intracellular Zn^{2+} was restored to the LNCaP cells propagated continuously in the growth medium supplemented with Zn^{2+} .

Tab. 1. Concentrations of intracellular Zn^{2+} in LNCaP prostate cancer and normal PNT2 prostate cells.

Passage No.	Total intracellular Zn^{2+} (nmol/mg protein) ^a			
	LNCaP			PNT2
	0 ^b	1 ^c	10 ^d	
0	6.45 ± 0.07	6.51 ± 0.01	6.54 ± 0.04	7.87 ± 0.06
5	6.21 ± 0.03	10.1 ± 0.08*	10.6 ± 0.05*	nd

^aValues are means ± SD, n = 2. *significantly different, $P < 0.05$, ^bCells cultured in the growth medium without additional Zn^{2+} , ^cCells cultured in the growth medium supplemented with 1 µg/ml Zn^{2+} , ^dCells cultured in the growth medium supplemented with 10 µg/ml Zn^{2+} , nd, not determined

Prolonged Zn^{2+} supplementation increases protein tyrosine phosphorylation activities in LNCaP cells

The effects of the restoration of a high intracellular Zn^{2+} level to LNCaP prostate cancer cell signaling pathways were investigated by examining the effects on tyrosine phosphorylation activities. This was done by initially measuring the PTK and PTP activities in these cells. In the PTK assay, all the PTP activities in the cell lysates were first inhibited with sodium orthovanadate. From the assay, it was shown that protein tyrosine phosphorylation increased in both the Zn^{2+} -supplemented and non-supplemented cell lysates when stimulated with ATP. However, the total tyrosine phosphorylated proteins in the lysates of cells supplemented with 1 µg/ml and with 10 µg/ml Zn^{2+} were found to be significantly higher (22.9 ± 1.8% and 28.3 ± 2.3%, respectively; $P < 0.05$, Student's *T*-test) when compared to the non-supplemented cells at 5 min post-stimulation with ATP (Fig. 2A, lanes 2, 5 and 8; Fig. 2B, C). These findings suggest that in general, the tyrosine phosphorylation activities were higher in the Zn^{2+} -supplemented LNCaP prostate cancer cells.

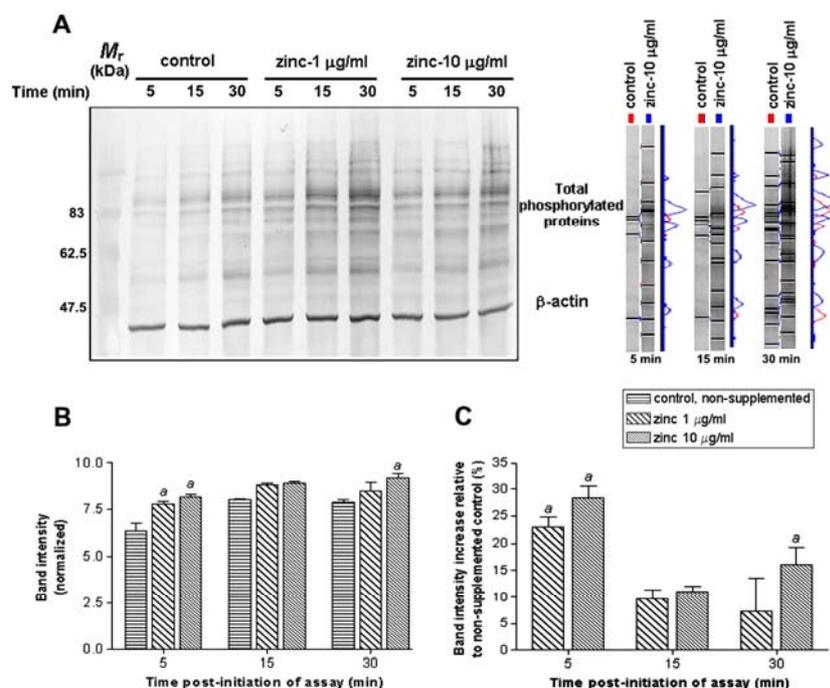


Fig. 2. The tyrosine kinase activities of Zn^{2+} -treated LNCaP prostate cancer cells. A time-dependent increase in the tyrosine kinase activity was measured using the PTK assay. A representative gel from three independently performed experiments is presented on the left in (A). On the right, there is a pairwise comparison of the protein band intensity for the control cells (red line) and the cells supplemented with $10 \mu\text{g/ml}$ Zn^{2+} (blue line). The tyrosine phosphorylated protein band intensity was normalized to the actin level and then extrapolated (B). The percentage increase in protein band intensity of the Zn^{2+} -supplemented cells in comparison to the non-supplemented cells is shown in (C). Each value is expressed as the mean \pm SEM of three independent experiments. ^a $P < 0.05$ (paired T -test), compared with control cells at the respective time-point. M_r , protein marker (NEB, Beverly, USA).

Prolonged Zn^{2+} supplementation does not inhibit protein tyrosine dephosphorylation in LNCaP cells

When no ATP was added in the PTP assay, the intrinsic total tyrosine phosphorylated proteins level of cells supplemented with $10 \mu\text{g/ml}$ Zn^{2+} were found to be $36.6 \pm 6.4\%$ and $55.2 \pm 9.3\%$ higher than those for the non-supplemented cells at 5 and 15 min post-initiation of the assay, respectively (Fig. 3A, lanes 2-3 and 8-9; Fig. 3B, C; $P < 0.05$, Student's T -test). Protein dephosphorylation occurred gradually within 30 min in all the cell lysates. The total tyrosine phosphorylated protein level in the lysates of cells supplemented with $10 \mu\text{g/ml}$ Zn^{2+} remained significantly higher ($91.5 \pm 6.3\%$) at 30 min post-initiation of the assay when compared to the non-supplemented cells (Fig. 3A,

lanes 4 and 10; Fig. 3B, C; $P < 0.05$, Student's T -test). These observations suggest that prolonged culturing of LNCaP cells in $10 \mu\text{g/ml Zn}^{2+}$ resulted in an increase in the total tyrosine phosphorylated protein level, and that the increase was not due to a simple inhibition of dephosphorylation by Zn^{2+} . Immunoblot analyses of the total cell proteins separated by PAGE showed a marked increase ($\sim 47\%$) in the abundance of VHR phosphatase and an increase ($\sim 21\%$) for PTP1B and PTP1C phosphatases (Fig. 4A, lane 3 and Fig. 4B). No change in the KAP level in the cells supplemented with $10 \mu\text{g/ml Zn}^{2+}$ was observed when compared to the non-supplemented control. The abundance of VHR, PTP1B, PTP1C and KAP in these cells decreased (12, 49, 27 and 14%, respectively)

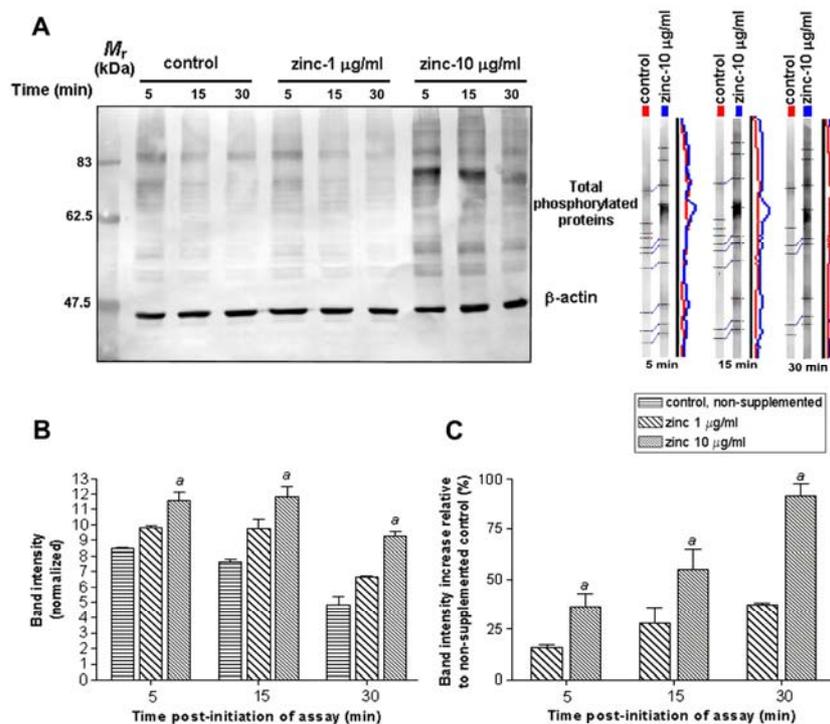


Fig. 3. The tyrosine phosphatase activities of Zn^{2+} -treated LNCaP cells. The PTP activity and time-dependent decrease in phosphorylation in the cell lysates were compared. A representative gel of three independently performed experiments is shown on the left in (A), and on the right, there is a pairwise comparison of the protein band intensity for the control cells (red line) and cells supplemented with $10 \mu\text{g/ml Zn}^{2+}$ (blue line). The protein bands representing the intensity of the phosphorylated proteins was normalized to the actin level and extrapolated, as shown in (B). The percentage increase in protein band intensity of the Zn^{2+} -supplemented cells in comparison to the non-supplemented cells is shown in (C). Each value is expressed as the mean \pm SEM of three independent experiments. ^a $P < 0.05$ (paired T -test), compared with control cells at the respective time-point. M_r , protein marker (NEB, Beverly, USA).

after Zn^{2+} was chelated with TPEN (Fig. 4A, lane 5 and Fig. 4B). These reductions were lower when compared to the marked reduction (45, 64, 53 and 69%, respectively) observed in cells treated with H_2O_2 , a known inhibitor of phosphatases (Fig. 4A, lane 4 and Fig. 4B). These findings suggested that the restoration of Zn^{2+} to LNCaP cells following continuous supplementation with a supraphysiological concentration of Zn^{2+} resulted in an increased abundance of LNCaP prostate cancer cell phosphatases, including VHR, PTP1B and PTP1C.

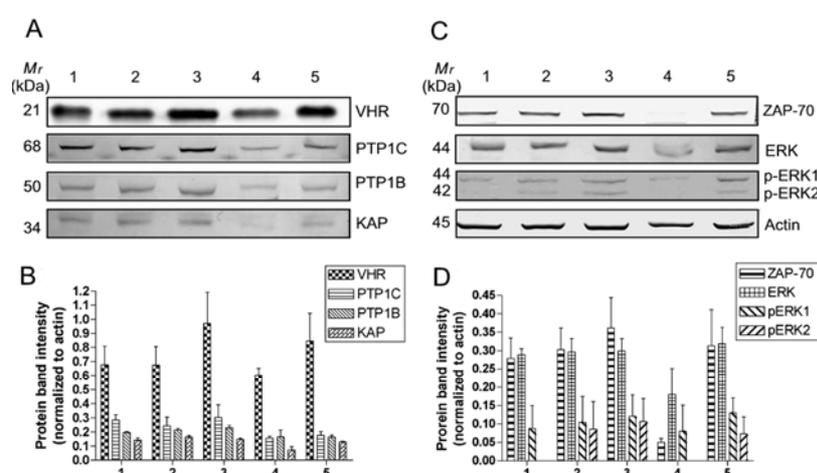


Fig. 4. The effects of Zn^{2+} on the expressions of tyrosine phosphatases and ERKs. The expression levels of: VHR, PTP1B, PTP1C, KAP (A); and the ZAP-70 kinase, ERK and phosphorylated ERK1 and 2 (C) for: the control non-supplemented cells (1); and the cells supplemented with $1 \mu\text{g/ml } Zn^{2+}$ (2), $10 \mu\text{g/ml } Zn^{2+}$ (3), or $10 \mu\text{g/ml } Zn^{2+}$ and treated either with H_2O_2 (4) or TPEN (5) are shown. The protein band intensity of each sample was normalized to the actin level (B, D). A representative gel from three independently performed experiments is shown.

Prolonged Zn^{2+} supplementation increases ZAP-70 kinase and ERK activities in LNCaP cells

The abundance of ZAP-70, a kinase that phosphorylates VHR and participates in the activation of ERK, increased by 32% in cells supplemented with $10 \mu\text{g/ml } Zn^{2+}$ when compared to the non-supplemented control (Fig. 4C, lanes 1 and 3 and Fig. 4D). The abundance of ZAP-70 kinase in these cells decreased to 23% when the Zn^{2+} was chelated with TPEN (Fig. 4C, lane 5 and Fig. 4D). This observation suggests that ZAP-70 kinase is responsive to changes in the intracellular Zn^{2+} levels. The abundance of phosphorylated ERK1 (p-ERK1; 44 kDa) and p-ERK2 (42 kDa), which were the substrates for VHR dephosphorylation, also increased in LNCaP cells supplemented with Zn^{2+} $1 \mu\text{g/ml}$ (36 and 2%, respectively; Fig. 4C, lane 2 and Fig. 4D) and with Zn^{2+} $10 \mu\text{g/ml}$ (140 and 335%, respectively; Fig. 4C, lane 3 and Fig. 4D). By contrast,

only the p-ERK1 protein band was detectable in the untreated control cells (Fig. 4C, lane 1). The abundance of p-ERK1 increased by 45% when the Zn^{2+} was chelated with TPEN, but the abundance of p-ERK2 decreased by 38% relative to the non-chelated cells (Fig. 4C, lanes 3 and 5). The level of unphosphorylated ERK in all the cells remained unchanged before and after the Zn^{2+} was chelated. By contrast, there was a marked reduction of the unphosphorylated ERK, p-ERK1 and p-ERK2 in the H_2O_2 -treated cells (Fig. 4C, lane 4; Fig. 4D). Taken together, these findings suggest that the sustained presence of high intracellular Zn^{2+} in the LNCaP prostate cancer cells resulted in an elevation in the activities of ZAP-70 kinase and VHR phosphatase, and in the activation of the ERKs.

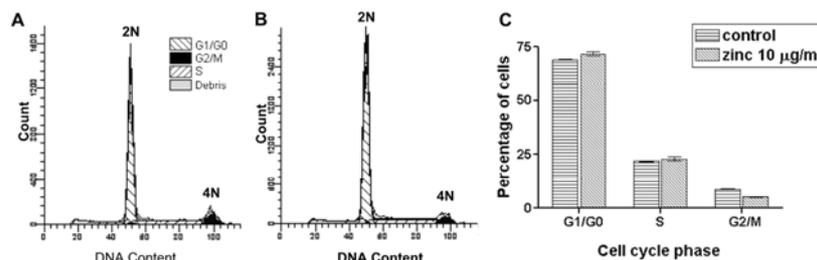


Fig. 5. The effects of continuous Zn^{2+} supplementation on the LNCaP cell cycle distributions. The cells were harvested after five weeks of culture in $10 \mu\text{g/ml } Zn^{2+}$, and analysed for their DNA contents by propidium iodide staining and flow cytometry. The DNA distribution of the cell cycle of the cells cultured continuously without additional Zn^{2+} (A) and with $10 \mu\text{g/ml } Zn^{2+}$ supplementation (B) were compared. The percentage of cells in different phases of the cell cycle is shown in (C). The DNA distribution of the cell cycle was analyzed using BD Cell Quest Pro Version 5.1.1 (BD Biosciences, San Diego, USA) and ModFit LT 3.1 (Verity Software House, Topsham, USA).

Prolonged Zn^{2+} supplementation retards LNCaP cell proliferation

At week 5 post-initiation of the Zn^{2+} treatment, slight increases in the population of cells in the G1/G0 and S phases in the Zn^{2+} -supplemented cell cultures relative to the control non-supplemented cell culture were observed (Fig. 5). In the Zn^{2+} -supplemented cell cultures, the percentage of cells in the G2/M phase markedly decreased from $8.8 \pm 0.2\%$ to $5.2 \pm 0.1\%$ following long-term culture in $10 \mu\text{g/ml } Zn^{2+}$ (Fig. 5C). This indicated that continuous culture of the LNCaP prostate cancer cells in a supraphysiological concentration of Zn^{2+} reduced the proportion of mitotic cells but was otherwise not cytotoxic. A significantly lower number of cells was obtained for the LNCaP prostate cancer cells maintained in long-term culture with $10 \mu\text{g/ml } Zn^{2+}$ than for the cells supplemented with only $1 \mu\text{g/ml } Zn^{2+}$ or without additional Zn^{2+} when the cells were stimulated to proliferate by passing the cells into fresh 10% FBS-supplemented growth medium on week six post-initiation of the Zn^{2+} treatment (Fig. 6A; $P < 0.05$, ANOVA). In these cells, the inhibition of cell proliferation was $42.2 \pm 7.4\%$ and

76.7 ± 2.0% on days 3 and 6, respectively (Fig. 6B). These results suggest that the continuous cultivation of LNCaP cells in 10 µg/ml Zn²⁺ resulted in a marked retardation of the ability of the LNCaP cells to proliferate. The proliferation rate was restored when cells supplemented with 10 µg/ml Zn²⁺ were treated with TPEN on day 6 when compared to the non-TPEN-treated Zn²⁺-supplemented cells and cells cultured without additional Zn²⁺ (Fig. 6C; *P* < 0.05, ANOVA). The cell proliferation rate of these cells increased by 94.5 ± 45.0% and 427.2 ± 55.1% and on day 3 and 6, respectively, when Zn²⁺ was chelated from the cell cultures (Fig. 6D). These findings suggest that removing the high intracellular Zn²⁺ from the cell cultures restored the LNCaP prostate cancer cells' cancerous proliferation rate.

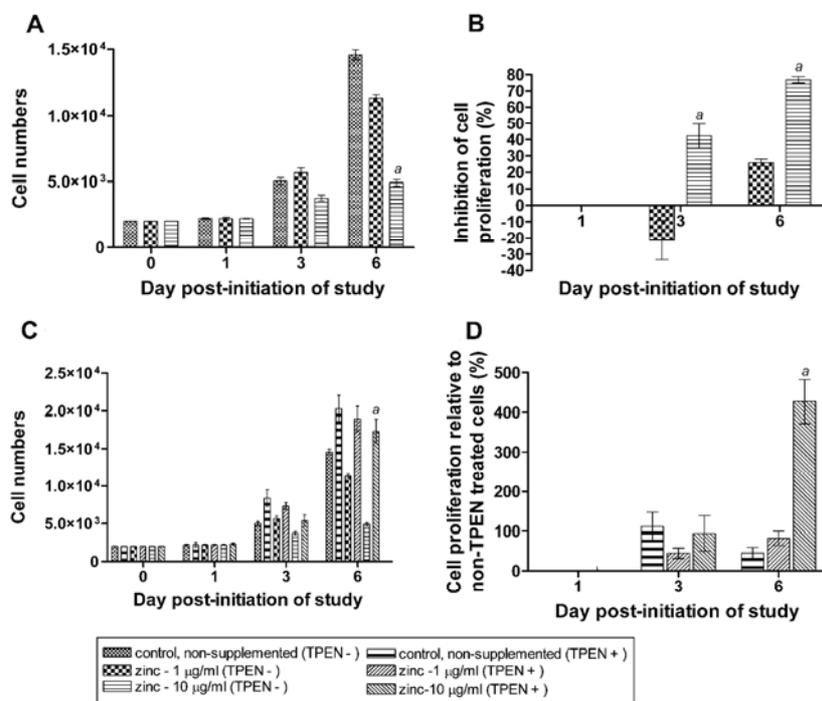


Fig. 6. The effects of continuous supplementation with Zn²⁺ on LNCaP cell proliferation. The rate of cell proliferation was determined over a period of 6 days in week 6 post-initiation of the Zn²⁺ treatment, and the percentage of viable cells was determined at selected intervals post-stimulation of cell proliferation. The cell numbers obtained for cells cultured continuously in growth medium without Zn²⁺, with only 1 µg/ml Zn²⁺ and with 10 µg/ml Zn²⁺ were compared (A). The relative percentage of cell growth inhibition in comparison to control untreated cells is shown in (B). The cell numbers were also determined when Zn²⁺ was chelated with TPEN (C). The relative percentage of cell growth in comparison to the non-TPEN-treated cell cultures is shown in (D). Each value is expressed as the mean ± SEM of 5 independent experiments. ^a*P* < 0.05 (ANOVA), compared with control untreated cells.

DISCUSSION

The importance of Zn^{2+} in prostate health was suggested by the findings that the Zn^{2+} concentration is highest in the prostate gland but tends to be very low in cancerous prostates. Analyses of malignant prostate tissues showed a 60-70% reduction in Zn^{2+} levels in comparison to those of the normal peripheral zone tissues [3]. A decrease in Zn^{2+} uptake is also observed in the RWPE2 prostate cancer cell line when compared to its non-tumorigenic equivalent, RWPE1, even though initially, the normal RWPE1 cells had a slightly higher Zn^{2+} concentration than the cancerous RWPE2 cells [21].

In this study, we simulated the effects of progressive long-term intracellular Zn^{2+} accumulation in LNCaP prostate cancer cells. LNCaP cells were used because they have been shown to exhibit the characteristics of the lateral prostate cells of the rat, which is homologous to the lateral lobes of the peripheral zone of the human prostate [22], the dominant region for the origin of prostate malignancy. Furthermore, LNCaP cells, unlike the *in situ* prostate cancer cells, retain the ability to accumulate high Zn^{2+} levels through a rapid-uptake Zn^{2+} transporter like hZIP1 (SLC39A1) [23], and hence, would better facilitate a Zn^{2+} uptake study. The results from this study showed that all the Zn^{2+} -treated LNCaP cells exhibited the presence of a high number of zincosomes, which are membrane-bound vesicles with a high amount of labile Zn^{2+} and a high total intracellular Zn^{2+} concentration. This is in contrast to the much lesser number of zincin-staining zincosomes and lower total intracellular Zn^{2+} concentration in the non- Zn^{2+} -treated LNCaP cells and the PNT2 prostate normal cells. The zincosomes were found to be present throughout the five-week treatment period, but absent when the cells were treated with TPEN, a known Zn^{2+} chelator. These findings strongly suggest that it is possible to restore a high intracellular Zn^{2+} content to prostate cancer cells and that it can be achieved *in vitro* by prolonged the culture of the cells in supraphysiological Zn^{2+} concentrations.

Restoring a high intracellular Zn^{2+} level affected the LNCaP prostate cancer cell proliferation. This is consistent with the assertion that the high intracellular Zn^{2+} level found in normal prostate tissues plays an important role in the regulation of the prostate cells [4-6]. It was shown here that the removal of Zn^{2+} through chelation with TPEN restored the LNCaP cell proliferation rate, which further substantiates the importance of Zn^{2+} in maintaining a healthy prostate. Similar effects were observed in the PC3 cell induced tumors in nude mice, where diet supplementation with Zn^{2+} for 5 weeks resulted in an increased plasma Zn^{2+} level and subsequent reduction of the tumor growth [24]. We showed that these effects could be achieved in LNCaP prostate cancer cells *in vitro*, and that enabled the identification of the signaling pathways affected by high intracellular Zn^{2+} in prostate cancer cells.

How Zn^{2+} reduces the proportion of mitotic LNCaP cells or exerts its effects on the cell proliferation rate is not clearly understood. Even though the role of certain cell cycle components has not been excluded in this study, several earlier

studies suggested that Zn^{2+} affects the MAPK, NF- κ B and PI3K pathways [25-27]. Zn^{2+} also increases the phosphorylation of ERK1/2, JNK and p38 MAPK in prostate cancer cells and in other cell lines [6, 16, 17]. Furthermore, Zn^{2+} mediates cell proliferation or cell death differentially through the ERK signaling pathway [28, 29]. In this study, a high level of protein tyrosine phosphorylation activities is observed in LNCaP cells treated for a prolonged period with a supraphysiological concentration of Zn^{2+} . The ZAP-70 kinase, VHR and ERK levels are also high in the Zn^{2+} -treated cells. The increase in VHR is perhaps directly associated with the increase in ZAP-70 kinase, as the latter uses VHR as its phosphorylation substrate [30]. The ZAP-70/VHR/ERK-associated pathways could be the mechanism by which Zn^{2+} exerts its effects on LNCaP prostate cancer cells. This suggestion is supported by the findings that TPEN treatment not only abrogates the inhibition of Zn^{2+} -induced cell proliferation, but also reduces the abundance of ZAP-70 kinase and VHR. Alternatively, the increase in VHR could also be due to the accumulation of phosphorylated ERKs, although other MAP kinase phosphatases (MKPs) present in the cells may be competing to inactivate ERKs [31, 32]. It is noted from this study that a decrease in VHR following the chelation of Zn^{2+} from the cells using TPEN only increased the abundance of p-ERK1 and not p-ERK2. ERK1 could be a specific target of dephosphorylation by VHR in the Zn^{2+} -treated LNCaP prostate cancer cells. How the ZAP-70/VHR/ERK-associated pathways modulate the prostate cancer cell growth is currently unclear, but an earlier study reported that ZAP-70 activates SLP76 and its associated proteins downstream for the activation of Ras and subsequently ERKs in T cells [33], and, in several studies, sustained activation of ERK has been reported to promote cell growth inhibition [34-36]. However, further study will be necessary to examine these possibilities in Zn^{2+} -treated LNCaP prostate cancer cells.

In summary, the results of this study suggest that high intracellular Zn^{2+} could be restored to prostate cancer cells *in vitro* by prolonged supplementation with high Zn^{2+} . It is likely that high intracellular Zn^{2+} affects the cells' proliferation rate through activation of the ERK via the VHR/ZAP-70 pathway.

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