

ABSTRACT

Title of Dissertation: THE INFLUENCE OF ZINC STATUS ON AKT SIGNALING PATHWAY IN HUMAN NORMAL PROSTATE EPITHELIAL CELLS AND HUMAN MALIGNANT PROSTATE CELLS

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Akt is known for promoting tumorigenesis through cellular proliferation. *Supra-physiologic* levels of zinc has been shown to stimulate the phosphorylation of Akt (p-Akt), which is frequently detectable in prostate tumors. Zinc content of malignant prostate epithelial cells is substantially lower than that of the surrounding normal epithelial cells. The influence of *physiologic* level of zinc on cell cycle progression via phosphoinositide-3-OH-kinase (PI3K)/Akt signaling pathway was examined in human normal prostate epithelial cells (PrEC) and human prostate malignant LNCaP cells. These cells were selected because of their susceptibility to zinc uptake and ability to express wild-type phosphatase and tensin homolog (PTEN) gene, the tumor suppressor responsible for blocking PI3K/Akt signaling. As a downstream effector of Akt, Mdm2 can be phosphorylated and translocated into the nucleus, subsequently promoting the ubiquitin-dependent degradation of tumor suppressor p53 protein. p-

Akt can also affect cell cycle progression by phosphorylating p21, which restricts p21's nuclear entry to induce cell cycle arrest. Cells were cultured for 6 d in low-zinc growth medium added with 0 (zinc-deficient; ZD), 4 (zinc-normal; ZN), 16 (zinc-adequate; ZA), or 32 (zinc-supplemented; ZS) $\mu\text{mol/L}$ of zinc. The effects of zinc on intracellular zinc status and cell cycle progression were determined by atomic absorption spectrophotometry and flow cytometry, respectively. Cytoplasmic and nuclear levels of p-Akt, p-PTEN, p-Mdm2, p53, and p21 proteins were analyzed by Western blotting. In addition, the dependence of zinc-induced Akt phosphorylation on the modulation of p-Akt, p-Mdm2, p53, and p21 protein levels was ascertained by using a PI3K/Akt inhibitor LY294002. Cellular zinc status of PrEC was more readily altered in a dose-dependent manner than LNCaP cells. In both cells, p-Akt was higher in ZD than ZN cells and both levels were normalized to that of ZN cells by LY294002. p-PTEN was higher in ZD than ZN-PrEC. Nuclear p-Mdm2 was higher in PrEC, while nuclear p53 was depressed in both PrEC and LNCaP cells by zinc deficiency. Nuclear p21 was unaffected in ZD-PrEC, but it was depressed in ZD-LNCaP cells. Nuclear p21 was higher in ZA and ZS than ZN-PrEC which coincided with faster G2/M progression. With LY294002, nuclear p21 protein was elevated in all groups, which correlated with an inhibition of G1/S cell cycle progression. Hence, zinc may affect cell cycle through Akt-Mdm2-p53 signaling axis in normal versus Akt-p21 in malignant prostate cells.

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HUMAN NORMAL PROSTATE EPITHELIAL CELLS AND HUMAN
MALIGNANT PROSTATE CELLS**

By

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CHAPTER 1.

INTRODUCTION

The overall objective of this dissertation was to test the hypotheses that whether the Akt-Mdm2-p53 and Akt-p21 signaling axes could be influenced by zinc status in human prostate normal versus cancerous cells. The **first objective** was to establish whether gain or loss of phosphoinositide-3-OH-kinase (PI3K)/Akt function can alter the ability of cells to survive and/or proliferate under *physiological* conditions of zinc deficiency and supplementation. The **second objective** was to examine the effect of zinc-responsive PI3K/Akt signaling on the phosphorylation of its downstream effector Mdm2 and its subsequent degradation of tumor suppressor protein p53. The cytoplasmic and nuclear levels of p21 were also determined. Moreover, the dependence of zinc on PI3K/Akt signaling cascade was ascertained by using a pharmacological inhibitor LY294002. The above objectives were partitioned into the following five specific aims:

A. Specific Aims:

- 1) To characterize the effect of zinc treatment on cellular zinc uptake
- 2) To determine the influence of zinc status on cell cycle progression
- 3) To examine the effect of zinc status on Akt and PTEN phosphorylation
- 4) To study whether the cytoplasmic and nuclear levels of p-Mdm2, Mdm2, p53, and p21 can be altered by zinc treatments
- 5) To establish the dependence of zinc-induced Akt phosphorylation on the modulation of p-Akt, p-Mdm2, Mdm2, p53, and p21 protein levels by using a PI3K/Akt inhibitor LY294002.

B. Hypotheses: Since cellular zinc is low in prostate cancer, we hypothesized that zinc-deficient cells would keep proliferating in part through the Akt signal transduction pathway. Similarly, Mdm2 would be highly phosphorylated in zinc deficiency. Subsequently, enhanced translocation of p-Mdm2 into the nucleus would reduce p53 protein level through degradation. Nuclear entry of p21 would be inhibited by Akt phosphorylation. Treatment with PI3K/Akt inhibitor LY294002 would suppress zinc-induced phosphorylation of Akt and Mdm2, and sensitize cells to p53- or p21-mediated growth arrest.

The primary goal of this dissertation was to investigate the effect of zinc deficiency/supplementation on molecular changes in prostate cells. Specifically, to explore whether the action of zinc was mediated by a signaling cascade that would involve the activation of PI3K/Akt pathway. Furthermore, the transient inhibition of PI3K signaling would prevent zinc-induced cellular processes. Data generated would contribute to the elucidation of mechanism(s) by which zinc exerts its effect on cell growth and proliferation via a signaling cascade that involves the activation of PI3K/Akt and that transient inhibition of PI3K/Akt by LY294002 treatment prevents zinc-altered cellular processes.

CHAPTER 2.

LITERATURE REVIEW

Prostate cancer is the most frequently diagnosed malignancy and second leading cause of cancer death in men (Jemal et al. 2006). Since zinc is depleted in prostate cancer (Zaichick et al. 1997), zinc may play a significant role in the development and/or progression of prostate cancer. Furthermore, this reduction of prostatic zinc may be accentuated in subjects with marginal dietary zinc deficiency, which involves 10% of the U.S. population (Wakimoto and Block 2001).

A. Zinc and Prostate

The prostate is comprised of branching glands, with ducts that are lined by secretory epithelial cells and basal cells (McNeal 1988). The major function of a normal prostate is to produce seminal fluid and facilitate sperm motility. Within the normal prostate, there are four major zones: the peripheral zone (70% of glandular tissue), the central zone (20% of glandular tissue), the transition zone (5% of glandular tissue), and the anterior fibromuscular stroma (5% of glandular tissue) (McNeal 1981). The peripheral zone represents the most common site in the prostate for developing prostate carcinomas (McNeal et al. 1988).

A.1) Functions of zinc in normal prostate

As early as the 1920s, the remarkably high zinc content was discovered in the human prostate by Bertrand and Vladesco (1921). The ability of normal prostate glands to accumulate 10-fold higher zinc as compared to other soft tissues, such as liver and kidney is well established (**Table 2.1**) (Mawson and Fischer 1952, Rosoff 1981, Costello and Franklin 1998). Accumulation of cellular zinc and secretion of zinc into the prostatic fluid by the prostate glands are essential functions of the prostate secretory epithelial cells (Sorensen et al. 1997). Low level of zinc in seminal plasma may affect the mobility of sperm which can result in infertility in men (Koca et al. 2003, Pant and Srivastava 2003, Caldamone et al. 1979).

This zinc-accumulating function of secretory epithelial cells has also been tied to the inhibitory effect of high cellular zinc on net citrate production via Krebs cycle (Costello et al. 1997). Zinc was shown to inhibit the m-aconitase activity, which prevents the oxidation of citrate that is accumulated in mitochondria and secreted into prostatic fluid. In contrast, the inhibitions of m-aconitase activity and citrate oxidation in other mammalian cells are lethal. Therefore, this zinc-accumulating function of prostate epithelial cells has a major effect on the bioenergetics of the cell.

Table 2.1. Zinc content of human prostate and other tissues ¹

	Zinc <i>μg/g wet tissue</i>
Prostate	
Normal (mixed tissue)	209
Normal (peripheral zone)	295
Normal (central zone)	121
BPH ²	589
PCa ³ (mixed tissue)	55
PCa (malignant tissue)	-
Prostatic fluid	590
Other soft tissue	30
Liver	58
Blood plasma	1

¹ Adapted from Costello et al. 2004, Suzuki et al. 1991.

² BPH, benign prostatic hypertrophy.

³ PCa, prostate cancer.

A.2) Status of zinc in normal prostate and prostate cancer

The glandular epithelial cells in the peripheral zone of the prostate accumulate the highest levels of intracellular zinc (**Table 2.1**) (Costello et al. 2004, Suzuki et al. 1991). This is also the area where most prostate cancers occur (Wong et al. 2000). Studies have indicated that the intracellular zinc levels in malignant prostate epithelial cells are 70-80% lower than the surrounding normal epithelial cells (Zaichick et al. 1997, Feustel and Wennrich 1984). This finding contrasts with data derived from benign prostatic hyperplasia (BPH) in which the epithelial cells accumulate normal or higher levels of zinc (Zaichick et al. 1997, Feustel and Wennrich 1984). Moreover, there appears to be a strong, inverse correlation between plasma zinc levels and various prostatic diseases, with 83% rise in BPH patients and 37% fall in patients with malignancy as compared to normal patients (Goel and Sankhwar 2006). In accordance with plasma level, the whole

blood zinc levels in patients with prostate cancer were 25% lower than controls (Picurelli et al. 1991, Ozmen et al. 2006).

Although zinc is an important factor in prostate biology and pathology, the exact roles of zinc and its homeostatic regulation in the normal versus malignant prostate glands are not well understood. Human zinc transporter ZIP1 has been localized to plasma membranes of prostate cells (Costello et al. 1999). ZIP1 is a major zinc uptake transporter for the accumulation of zinc in the prostate gland (Franklin et al. 2003, 2005). Overexpression of human ZIP1 in transfected PC-3 cells enhances zinc uptake and inhibits growth. In contrast, antisense oligonucleotide inhibition of human ZIP1 expression markedly reduces zinc uptake in PC-3 cells (Franklin et al. 2003). Altogether, the relationship between low zinc status and prostate cancer development and/or progression appears to be significant.

A.3) Effect of zinc on cell cycle progression

Zinc is an essential mineral for many cellular processes and is critical in maintaining the normal function of the prostate (Falchuk 1998). *Supra-physiologic* levels of zinc were shown to inhibit prostate cancer initiation and/or progression via cell cycle arrest, programmed cell death, or necrosis (Uzzo et al. 2002, Iguchi et al. 1998, Bostwick et al. 2000). G2/M arrest observed in zinc supplemented human prostate carcinoma cell lines, LNCaP (p53^{+/+}) and PC-3 (p53^{-/-}) cells, indicated that the impaired cell cycle progression might be independent of p53 (Liang et al. 1999). Zinc administered by osmotic pump has been shown to suppress tumor growth of PC-3 cells in nude mice (Feng et al. 2003). Growth of LNCaP and PC-3 cells has shown to be suppressed by 200 and 100 $\mu\text{mol/L}$ of

zinc, respectively (Hasumi et al. 2003). These studies have demonstrated that *in vivo* or *in vitro* treatment with *supra-physiologic* level of zinc increases zinc accumulation in tumor tissues and inhibits tumor growth or inhibits cancer cell growth. The inhibitory effect of zinc appears to result from zinc-induced apoptosis. Nonetheless, the ability of *physiologic* level of zinc (<32 $\mu\text{mol/L}$) in altering cell cycle progression in prostate cancer cells has not been established.

On the contrary, studies have established an association of low intracellular zinc concentrations with the induction of apoptosis. Both p53 and NF- κ B are known pathways that negatively and positively, respectively, affecting cell proliferation in various cell types. Observations from our laboratory have shown that zinc deficiency amplifies nuclear p53 accumulation in human hepatoblastoma HepG2 cells (Alshatwi et al. 2006, Reaves et al. 2000), as well as in normal human bronchial epithelial cells (Fanzo et al. 2001) and human aortic endothelial cells (Fanzo et al. 2002) in the absence of apoptosis. Others have found that zinc deficiency inhibits NF- κ B promoter activities in human LNCaP, PC-3 and DU-145 prostate cancer cells (Iguchi et al. 1998, Uzzo et al. 2002), human lung fibroblasts (Ho et al. 2003), rat glioma cells (Ho and Ames 2002), mouse 3T3 fibroblasts (Chou et al. 2004, Clegg et al. 2005), and human neuroblastoma IMR-32 cells (Mackenzie et al. 2002) in the presence of apoptosis. But whether low zinc status would block cell cycle progression in human normal prostate epthelial cells (PrEC) cells is unknown.

A.4) Effect of zinc supplementation on prostate cancer risk

Zinc is abundant in many food sources, e.g. red meat, poultry, beans, nuts, seafood, whole grains, and fortified breakfast cereals. The average person usually obtains sufficient zinc through a well-balanced diet and the Recommended Dietary Allowance (RDA) is set at 11 and 8 mg/day for adult male and female, respectively (Institute of Medicine 2001). From a review of cohort and cross-sectional data, 10% of the U.S. population consumes less than half of the RDA (Wakimoto and Block 2001).

Nevertheless, as a standard component in multivitamins/mineral supplements zinc is taken by approximately 15% of the U.S. population (Briefel et al. 2000). About 10% of men who take zinc supplements have an average daily zinc intake that is 2-3 times the RDA (Moss et al. 1989). A large cohort study, the Health Professionals Follow-Up Study, found men who took zinc supplements of more than 100 mg/day for 10 or more years were more than twice as likely to develop advanced prostate cancer compared with those who did not take supplemental zinc (Leitzmann et al. 2003). In this cohort, 32% of the total zinc intake was from dietary supplements, which represented the largest source of zinc. Thus, excessive intake of zinc, especially from supplements, may potentiate the transition of BPH to cancer. Currently, it is an area of debate whether clinicians should discourage or immediately discontinue zinc supplementation for most individuals (Jarrard 2005). Further mechanistic studies are warranted to untangle this controversy through cellular, clinical, and animal models.

B. Prostate Cancer and PI3K/Akt Signaling Pathway

Cellular phosphorylation is a reversible, covalent modification of a protein or lipid. It serves to modify the activity of the phosphorylated molecule by inducing conformational changes within the molecule. This modification occurs either through the addition of phosphate groups, e.g. action of ATP on an amino acid residue (regulated by kinases) and/or by their removal (regulated by phosphatases). The functions of these post-translational modifications include the alteration of the activity of a substrate, its subcellular localization, binding properties or association with other proteins.

B.1) Inhibitors of PI3K/Akt signaling pathway

Akt is a cytoplasmic serine/threonine kinase that controls programmed cell death by phosphorylating substrates that regulate both apoptosis and cell cycle progression, including inactivating BAD, p21, and activating Mdm2. Uncontrolled activation of Akt, due to activation of upstream of PI3K, leads to the development of cancer (Testa and Bellacosa 2001). Thus, pharmacological inhibition of PI3K/Akt activation has been an attractive strategy for cancer treatment. Two compounds, wortmannin and LY294002, have been widely used as small molecule inhibitors of the PI3K/Akt pathway in *in vitro* studies. LY294002 is a flavonoid derivative that competitively and reversibly inhibits the ATP binding site of PI3K, with an IC_{50} of 1.4 μ M, which is about 500-fold higher than that of wortmannin (Sanchez-Margalet et al. 1994)^a. Wortmannin is more selective and more potent, but forms covalent associations with the kinases and is, therefore, irreversible. LY294002 has been shown to block proliferation of cultured cells without inducing apoptosis.

Footnote ^a IC_{50} , the half maximal inhibitory concentration; represents the concentration of an inhibitor that is required for 50% inhibition of an enzyme *in vitro*

As an endogenous inhibitor of the PI3K/Akt signaling, phosphatase and tensin homolog (PTEN) functions as a key signal regulator and tumor suppressor (Cantley and Neel 1999, Stambolic et al. 1998). PTEN uses the phosphoinositide second messenger, phosphatidylinositol 3,4,5-triphosphate (PIP₃), as its physiological substrate. To block the PI3K signaling, PTEN removes a phosphate from PIP₃. Loss or mutation of PTEN in a cell leads to increased concentration of PIP₃ and Akt phosphorylation. Mammalian cells lacking PTEN have increased proliferation and reduced apoptosis – both phenotypes that favor tumorigenesis.

PTEN consists of an N-terminal phosphatase domain, a lipid binding C2 domain, and a 50-amino acid C-terminal tail containing a PDZ binding sequence. Phosphorylation of three residues (Ser380, Thr382, and Thr 383) of the PTEN tail negatively regulates PTEN activity and also suppresses the activity by controlling the recruitment of PTEN into the PTEN-associated complex (Vazquez et al. 2000, Vazquez et al. 2001).

B.2) Hyper-phosphorylation of Akt in prostate cancer

No gene mutation of Akt has been reported in human cancer. However, there have been reports of the gene amplification of Akt. Overexpression of Akt3 mRNA was demonstrated in prostate cancer (Nakatani et al. 1999).

Immunohistochemical studies using specific antibodies against p-Akt at Ser473 (active Akt) have shown that Akt activity is detectable in prostate cancer specimens (Malik et al. 2002, Kremer et al. 2006) but not in normal prostate cells (Chen et al. 2001). P-Akt at Ser473 has been identified as a biomarker for prostate cancer and has been correlated with a higher probability of disease recurrence (Ayala et al. 2004, Kreisberg et

al. 2004). Goswami et al. (2005) have shown that suppression of Akt activation by the PI3K inhibitor LY294002, Akt expression by RNA-interference, or Akt function by dominant-negative Akt caused apoptosis in prostate cancer cells. In studies with prostate cells, LY294002-treated LNCaP cells showed a dose-dependent apoptotic response, as assessed by DNA fragmentation, while PrEC were relatively insensitive to LY294002 (Lin et al. 1999).

Somatic inactivation of PTEN through either gene deletion or point mutation is very common in localized and metastatic prostate cancers (Ittmann 1998). The loss of PTEN and subsequent activation of Akt is becoming recognized as a critical event in human prostate cancer. At least one quarter of men with prostate cancer has tumors that are missing PTEN, and these tend to be more aggressive tumors (Suzuki et al. 1998). PTEN inactivation is therefore another line of evidence linking the activation of PI3K/Akt signaling with prostate cancer. In summary, the inhibition of the PI3K/Akt pathway may have therapeutic value for patients with prostate cancer.

B.3) Akt-Mdm2-p53 signaling axis

Akt can indirectly regulate the tumor suppressor p53 protein, which acts as sensor of cellular stress, and transduces stress signals into apoptotic signals (Evan and Vousden 2001). As illustrated in **Figure 2.1**, Akt can phosphorylate Mdm2 and promote its translocation to the nucleus (Mayo and Donner 2001). In the nucleus, p-Mdm2 can form a complex with p53 and p300 to promote the ubiquitin-dependent degradation of p53 (Mayo et al. 2002, Zhou et al 2001). As a result, p-Akt may indirectly hinder p53-

dependent growth suppression and apoptosis, leading to cell survival (Ogawara et al. 2002).

The Mdm2 gene is amplified in a variety of human tumors (Momand et al. 1998), including prostate cancer (Ittmann et al. 1994, Gao et al. 1997, Osman et al. 1999, Zhang et al. 2003). Mdm2 has been a target for prostate cancer therapy as established by several antisense oligonucleotide studies (Mu et al. 2004, Zhang et al. 2003, Wang et al. 2002). Therefore, whether stabilization of Mdm2 is a result of Akt hyper-phosphorylation in prostate cancer would be intriguing to study.

B.4) Akt-p21 signaling axis

The subcellular localization of p21 has been proposed to be critical for the regulation of p21 function (Vivanco and Sawyers 2002). In the nucleus, p21 is a cyclin-dependent kinases (CDK) inhibitor that can arrest the cell cycle in response to DNA damage or stress. More specifically, p21 can interact with proliferating cell nuclear antigen (PCNA) and block DNA synthesis required for progression through the S phase of the cell cycle. As opposed to its anti-proliferative functions, p21 can be pro-proliferative when localized in the cytoplasm. Akt has been shown to phosphorylate p21 at Thr145 residue, which lies near the nuclear localization signal (NLS) domain (Zhou et al. 2001). Phosphorylation near this NLS site prevents p21's entry into the nucleus (**Figure 2.1**). Growth-inhibiting activity of p21 is therefore suppressed by this cytoplasmically retained p21 through facilitating the assembly and activity of cyclin D-CDK 4 complex. Therefore, the cell cycle can be progressed through G1 phase into S phase (Boonstr 2003).

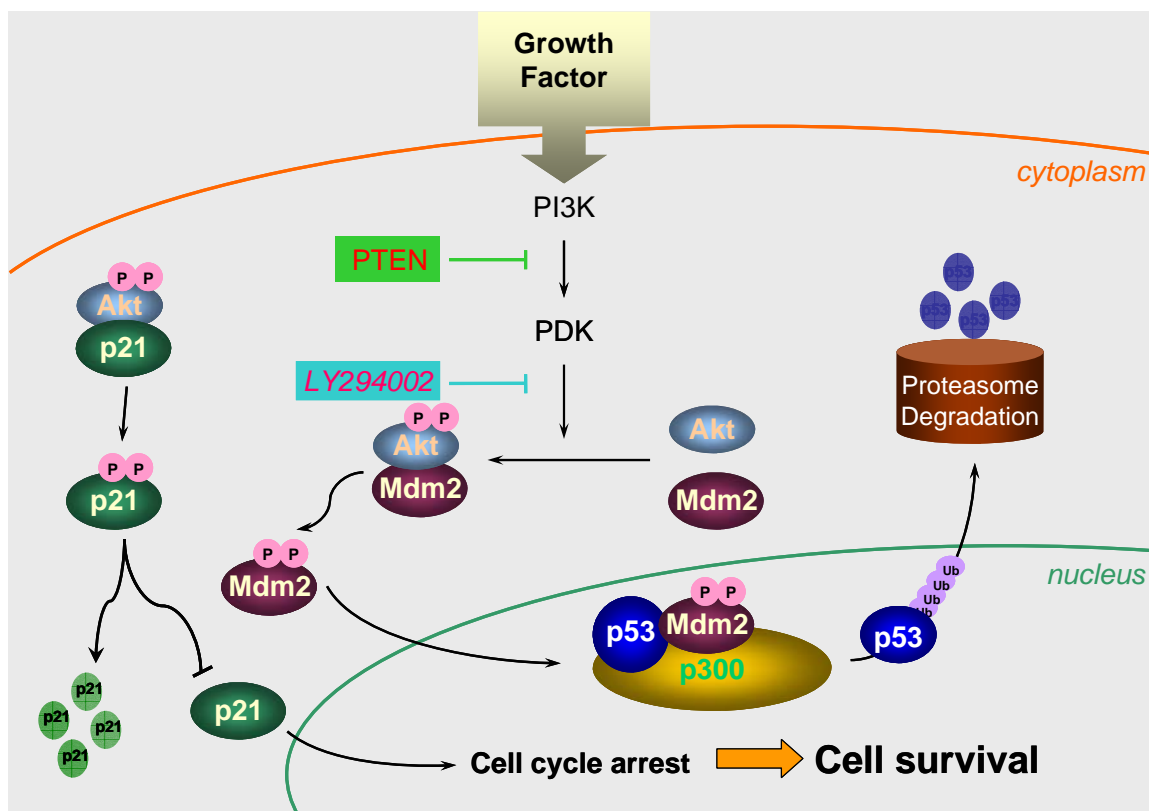


Figure 2.1. Akt signaling pathway

C. Proposed Mechanism Responsible for Zinc-Induced PI3K/Akt Signaling Pathway

Studies have shown that *supra-physiologic* levels of zinc (>50 $\mu\text{mol/L}$) can induce PI3K/Akt signaling in fibroblasts, adipocytes, breast, and lung epithelial cells (Tang and Shay 2001, Kim et al. 2000, Ostrakhovitch and Cherian 2004, Wu et al. 2003, 2005, Bao and Knoell 2006). Several studies have proposed that tyrosine kinases are the mechanism involved in the activation of PI3K/Akt signaling pathway by zinc. Kim et al. (2000) demonstrated that PI3K activities associated with anti-phosphotyrosine immunoprecipitates were raised significantly following *in vivo* zinc stimulation. May and Contoreggi (1982) have found that zinc exerted insulin-like growth factor-1 (IGF-1)

effects by generating H_2O_2 in isolated rat adipocytes. Intracellular reactive oxygen species (ROS) can also induce the tyrosine phosphorylation of numerous cytosolic proteins and activation of growth factor receptor tyrosine kinases such as epidermal growth factor receptor (Wang et al. 2000). Furthermore, oxidative stress activates PI3K and causes the accumulation of phosphatidylinositol 3,4-bisphosphate, which recruits Akt to the plasma membrane and activates it (Van der Kaay et al. 1999). From these studies, we can postulate a model that zinc deficiency induces the generation of ROS such as H_2O_2 in the cell and consequently activates growth factor receptor tyrosine kinases to stimulate the PI3K signaling pathway.

Alternatively, zinc may activate non-receptor tyrosine kinases associated with PI3K. For example, focal adhesion kinase (FAK), a non-receptor tyrosine kinase, was found to mediate PI3K activation in T98 glioblastoma cells (Sonoda et al. 1999). In addition, FAK and Src family tyrosine kinases have been found to be tyrosine-phosphorylated and their association with PI3K enhanced in response to H_2O_2 treatment (Sonoda et al. 1999, Lander et al. 1995, Abe et al. 1997). Thus, these non-receptor tyrosine kinases may also be involved in the zinc-mediated activation of PI3K. Since zinc deficiency induces the generation of H_2O_2 (Mackenzie et al. 2006), the ability of low zinc status to signal through the activation of PI3K pathway via H_2O_2 has not been established yet.

CHAPTER 3.

THE INFLUENCE OF ZINC STATUS ON AKT-MDM2-P53 SIGNALING AXIS IN HUMAN NORMAL PROSTATE EPITHELIAL CELLS

ABSTRACT

The phosphorylated form of Akt (p-Akt) can indirectly hinder p53-dependent growth suppression and apoptosis through Mdm2 phosphorylation. With prostate being the highest zinc-accumulating tissue before the onset of cancer, the effects of physiologic levels of zinc on Akt-Mdm2-p53 signaling axes in human normal prostate epithelial cells (PrEC) were examined herein. Cells were cultured for 6 d in low-zinc growth medium added with 0 (zinc-deficient; ZD), 4 (zinc-normal; ZN), 16 (zinc-adequate; ZA), or 32 (zinc-supplemented; ZS) $\mu\text{mol/L}$ of zinc. Cellular zinc status of PrEC was readily altered in a dose-dependent manner. Zinc had no effect on G0/G1 and S phases of the cell cycle. However, ZA and ZS groups had fewer cells at G2/M phase of the cell cycle than ZN group. p-Akt was higher in ZD than ZN group and both levels were normalized to that of ZN group by LY294002. Similarly, p-PTEN was higher in ZD than ZN group. Nuclear p-Mdm2 was higher, while nuclear p53 was depressed by zinc deficiency. Nuclear p21 and p300 was unaffected by zinc deficiency. Nuclear p21 was higher in ZA and ZS than ZN group, which coincided with slower G2/M progression. Hence, zinc deficiency-induced Akt phosphorylation was a result of inactive PTEN, which led to Mdm2 phosphorylation and less nuclear accumulation of p53, thus exerting less of the tumor-suppressive effect

of p53. As a result, zinc may promote cell cycle progression through Akt-Mdm2-p53 signaling axis in normal prostate cells.

INTRODUCTION

Normal prostate is unique in their ability to accumulate high levels of zinc (Rosoff 1981, Costello and Franklin 1998). However, this ability is lost during malignancy (Zaichick et al. 1997, Feustel and Wennrich 1984). Some have found that zinc deficiency leads to apoptosis in fibroblasts (Clegg et al. 2005, Chou et al. 2004) and neuroblastoma cells (Mackenzie et al. 2006), while others found that high zinc treatment inhibits prostate cancer initiation and/or progression via cell cycle arrest, programmed cell death, or necrosis (Costello and Franklin 1998, Liang et al. 1999, Uzzo et al. 2002, Iguchi et al. 1998, Bostwick et al. 2000).

Akt or protein kinase B is known for promoting tumorigenesis through phosphorylating proteins involved in pathways regulating apoptosis and proliferation via phosphoinositide-3-OH-kinase (PI3K) signaling (Testa and Bellacosa 2001). Phosphatase and tensin homologue (PTEN) can dephosphorylate phosphatidylinositol 3,4-bisphosphate (PIP2) and phosphatidylinositol 3,4,5-triphosphate (PIP3), thus antagonizing PI3K activity and inhibiting Akt phosphorylation. Phosphorylation of three residues (Ser380, Thr382, and Thr383) of the PTEN tail inactivates its phosphatase activity, thus unphosphorylated PTEN is the active form. Hence, PTEN activity is regulated not only in expressed protein levels but also in the ratio of phosphorylated and unphosphorylated form of the protein (Torres and Pulido 2001, Vazquez et al. 2000, Vazquez et al. 2001).

Phosphorylated form of Akt (p-Akt) has been shown to interact with Mdm2 and phosphorylate Mdm2 at Ser166 and Ser186 residues (Gottlieb et al. 2002). These Akt phosphorylation sites in Mdm2 are close to its nuclear localization signal and nuclear export signal domains, which are critical in determining the shuttling of Mdm2 between nucleus and cytoplasm. Therefore, Akt-mediated phosphorylation of Mdm2 (p-Mdm2) can lead to its nuclear localization (Mayo and Donner 2001, Zhou et al. 2001, Ogawara et al. 2002).

Mdm2, consists of an ubiquitin E3 ligase, is known for its role in controlling the levels and activity of p53 (Momand et al. 2000). Once Mdm2 is phosphorylated by p-Akt, p-Mdm2 translocates to the nucleus and forms a complex with p53 and p300 to promote the ubiquitin-dependent degradation of p53 (Mayo and Donner 2001, Mayo et al. 2002, Zhou et al. 2001). As a result, p-Akt may indirectly hinder p53-dependent growth suppression and apoptosis, thus leading to cell survival (Ogawara et al. 2002).

Apart from the role of Akt in Mdm2-p53 signaling axis, Akt can also promote cell survival through p21, a p53 responsive gene. P-Akt phosphorylates p21 within its nuclear localization signal domain and thus restricting it to the cytoplasm for degradation through a p53-independent pathway (Zhou et al. 2001). Furthermore, p-Akt has been shown to promote cell cycle progression through limiting nuclear p21 protein levels to bind and activate cyclin D-CDK4 complex (Li et al. 2002).

P-Akt has been suggested as a biomarker for prostate cancer (Ayala et al. 2004, Kreisberg et al. 2004). *Supra-physiologic* levels of zinc (50-200 $\mu\text{mol/L}$) have been shown to stimulate the phosphorylation of Akt in fibroblasts, adipocytes, and lung epithelial cells (Tang and Shay 2001, Kim et al. 2000, Wu et al. 2003, 2005, Bao and

Knoell 2006). However, only a few studies have examined the effect of *physiologic* levels of zinc (<32 $\mu\text{mol/L}$) on Akt phosphorylation in breast cancer cells and adipocytes (Ostrakhovitch and Cherian 2004, Clegg et al. 2005). Yet, as a zinc-accumulating tissue, the effects of zinc on prostate cells have never been investigated in the aspect of Akt phosphorylation. Previous studies have shown a zinc-induced activation of PI3K/Akt signaling and PTEN degradation (Kim et al. 2000, Wu et al. 2003), suggesting that zinc may affect PTEN phosphorylation. Because PTEN, the negative regulator of Akt, is functionally inactivated in prostate cancer, we explored whether zinc-altered Akt phosphorylation was a result of inactivated PTEN.

Epidemiological evidence showed that men on high doses of zinc supplements had increased risk for prostate cancer (Leitzmann et al. 2003). In view of the prevalence of dietary zinc deficiency and supplementation and the clinical significance of low zinc status in prostate cancer, we designed the current study to examine the influence of low and high zinc levels on cell cycle progression via PI3K/Akt signaling pathway in human normal prostate epithelial cells (PrEC). These cells were selected because of their responsiveness to extracellular zinc uptake and ability to express wild-type PTEN gene. The dependence of zinc-influenced Akt phosphorylation on the modulation of nuclear and cytoplasmic p-Mdm2, p53, p21 protein levels was ascertained by using a PI3K/Akt inhibitor LY294002. The results of this study might contribute to the elucidation of mechanism(s) by which zinc exerts its effect on cell cycle progression via PI3K/Akt signaling cascade and that transient inhibition of PI3K/Akt prevents zinc-induced cellular processes.

METHODS

PrEC Maintenance

PrEC (Lots 3F0854 and 5F1199) were obtained at second passage from Cambrex (Walkersville, MD) and expanded in serum-free prostate epithelial cell growth media (PrEGM). PrEGM was made from prostate epithelial cell basal medium (PrEBM) supplemented with growth factors, cytokines, and nutrients, namely bovine pituitary extract, hydrocortisone, human recombinant epidermal growth factor, epinephrine, insulin, tri-iodothyronine, transferrin, gentamicin/amphotericin-B, and retinoic acid. Zinc-free PrEBM was specifically formulated without the addition of zinc sulfate heptahydrate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ abbreviated as ZnSO_4) by Cambrex (Walkersville, MD). This zinc-free PrEGM, the basal medium, was used as the zinc-deficient treatment (**ZD**).

Zinc and Inhibitor Treatment

Besides ZD treatment, zinc was added to the zinc-free PrEGM in the form of ZnSO_4 (Sigma-Aldrich, St. Louis, MO). Therefore, the only difference between these media was the zinc concentration. For the zinc-normal (**ZN**) medium, 4 $\mu\text{mol/L}$ ZnSO_4 was added to the ZD medium; the zinc-adequate (**ZA**) medium contained 16 $\mu\text{mol/L}$ ZnSO_4 ; and the zinc-supplemented (**ZS**) medium contained 32 $\mu\text{mol/L}$ ZnSO_4 . The ZN medium was used as the control group for all experiments. The ZA treatment was used as a representative of human plasma zinc levels. The ZS group was used to represent the high end of plasma zinc levels attainable by oral supplementation in humans. After one passage in ZN media, PrEC were sub-cultured and stabilized overnight in ZN medium prior to changing into their respective medium, namely ZD, ZN, ZA, or ZS medium, for another 6 days (1

passage). One subgroup of ZD cells (zinc-repleted: **ZR**) was depleted with ZD medium for 5 days and then repleted with ZS for 1 day. Cells were then harvested for assays. The dependence of zinc-induced Akt phosphorylation was ascertained by using a PI3K inhibitor LY294002 (Calbiochem, San Diego, CA). Cells were treated with either 5 $\mu\text{mol/L}$ of LY294002 or 0.05% DMSO (as vehicle control) for 16 hours before harvesting.

DNA Content and Zinc Status

The effect of zinc treatment on cellular zinc uptake in PrEC cultured for one passage in medium containing different levels of zinc was determined by atomic absorption spectrophotometry. Cells were collected by scraping from 100-mm Petri dishes and centrifugation. Cell pellets were then washed twice with phosphate-buffered saline (PBS). Cells were resuspended into 1.5 ml PBS and sonicated for two 30-second intervals on ice. An aliquot of the sonicated cell suspension was used for the determination of cellular zinc content by a flame atomic absorption spectrophotometer (PerkinElmer, Boston, MA). Zinc standard solutions ranging from 0.05 to 1.0 parts per million (ppm) were used to generate a linear standard curve. The zinc content of the cells was calculated based on these zinc standard solutions. From the same sample, another aliquot of the sonicated cell suspension was used for the determination of cellular DNA content by the diphenylamine method (Gendimenico et al. 1988), in which diphenylamine is used to develop a blue color with the carbohydrate moiety from the purine nucleotides of the nucleic acid. Since there is a linear relationship between cellular DNA and cell number as previously

established by Wu et al. (1997), data were expressed as nanogram of cellular zinc per microgram of DNA.

Cell Cycle Analysis

The influence of zinc status on cell cycle progression was analyzed by flow cytometry. Cells were collected by trypsinization and centrifugation. Cell pellet was washed twice with PBS and reconstituted to 1 ml. Then, 1×10^6 cells were fixed with 70% ethanol, treated with RNase to remove any double-stranded RNA, and then labeled with propidium iodide, which binds to double-stranded DNA (Gomez-Angelats et al. 2000). A total of 10,000 cell events were collected for DNA analyses on a FACScalibur cytometer with CELLQuest program (Becton Dickinson, San Jose, CA). Percentages of stained nuclei, distributed among cell cycle phases, were calculated using ModFit LT (Version 3.0, Verity Software House, Topsham, ME). Calibration standards, LinearFlow Green (Molecular Probes, Carlsbad, CA) and DNA QC Particle Kit (Becton Dickinson, San Jose, CA), were used to verify instrument performance. The DNA content of a cell population analyzed by this assay would determine the proportion of cells that are in G0/G1, S, or G2/M phase of the cell cycle.

Nuclear and Cytoplasmic Extractions

Nuclear and cytoplasmic extracts were obtained by using NE-PER™ Nuclear and Cytoplasmic Extraction Kit (Pierce Biotechnology, Rockford, IL). Briefly, cells were washed with PBS, scraped off the dish, and collected by centrifugation at $1,500 \times g$ for 5 minutes. The pellet was resuspended in Cytoplasmic Extraction Reagent I (CERI) and

incubated on ice. Cytoplasmic Extraction Reagent II (CERII) was then added and the extracts were further incubated on ice. The extracts were centrifuged for 15 minutes, and the supernatant (cytoplasmic fraction) was transferred to a new tube. Nuclear Extraction Reagent (NER) was added to the pellet and the nuclear pellet was incubated on ice for 40 minutes while vortexing every 10 minutes. The extract was centrifuged for 15 minutes and the supernatant (nuclear fraction) was transferred to a new tube. In addition to the standard HALT™ protease inhibitor cocktail (Pierce Biotechnology, Rockford, IL), a phosphatase inhibitor cocktail (Pierce Biotechnology, Rockford, IL) was added to the CERII and NER to prevent de-phosphorylation. The isolated fractions were stored at -80°C until analysis. Protein concentration was determined by using Bicinchoninic Acid Protein Assay Reagents (Pierce Biotechnology, Rockford, IL) with bovine serum albumin as the standard.

Western Blot Analysis

Approximately 40 µg protein of cell extracts were mixed with equal volume of Laemmli sample loading buffer, which consists of 62.5 mmol/L Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% Bromophenol Blue, and 35.5 mmol/L β-mercaptoethanol. These extracts were size-separated by 10% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis under reduced conditions, followed by electrotransfer onto a ECL nitrocellulose membrane (Amersham Life Science, Arlington Heights, IL). A blocking buffer consisting 5% (w/v) nonfat dry milk in 10 mmol/L Tris-HCl, pH 7.4 with 0.1% Tween-20 (TBS-T) were used to block non-specific proteins on the membrane for 1 hour at room temperature. After several washes with TBS-T, the membrane was probed with

p-Akt (Ser473) antibody (Cell Signaling Technology, Beverly, MA) diluted in 1% (w/v) bovine serum albumin with 0.1% Tween-20 overnight at 4°C. Bound primary antibody was detected with horseradish peroxidase conjugated secondary antibody and chemiluminescent substrate SuperSignal WestPico Chemiluminescence reagent (Pierce Biotechnology, Rockford, IL). Immunoreactive bands were visualized by exposing membranes to X-OMAT film (Eastman Kodak, Rochester, NY), which was developed by an X-Ray Film Processor (AFP Imaging, Elmsford, NY). Membranes were then stripped and re-probed with Akt antibody (Cell Signaling Technology, Beverly, MA) to verify equal protein loading. Relative band intensities were digitally quantified by using an imaging system FluorChem 8900 with AlphaEase® FC Stand Alone Software (Alpha Innotech, San Leandro, CA). Other primary antibodies used were: p-PTEN (Ser380), PTEN, p-Mdm2 (Ser166) (Cell Signaling Technology, Beverly, MA), Mdm2 (C-18), p53 (DO-1), p300 (Santa Cruz Biotechnology, Santa Cruz, CA), and p21 (Ab-1) (Calbiochem, San Diego, CA). Membranes were re-probed with either nucleoporin (p62) or GAPDH (FL-335) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) to ensure equal loading of nuclear or cytoplasmic protein, respectively. Cross contaminations of nuclear and cytoplasmic extracts as determined by probing with GAPDH and nucleoporin, respectively, have been routinely established to be less than 5%.

Statistical Analysis

Data represent means \pm SEM from three independent experiments. Statistical analyses were performed using SPSS (Version 10.0, Chicago, IL). Data was analyzed using one-

way ANOVA, and the means were further compared by least significant differences (LSD) *post-hoc* test with p values < 0.05 regarded as significant.

RESULTS

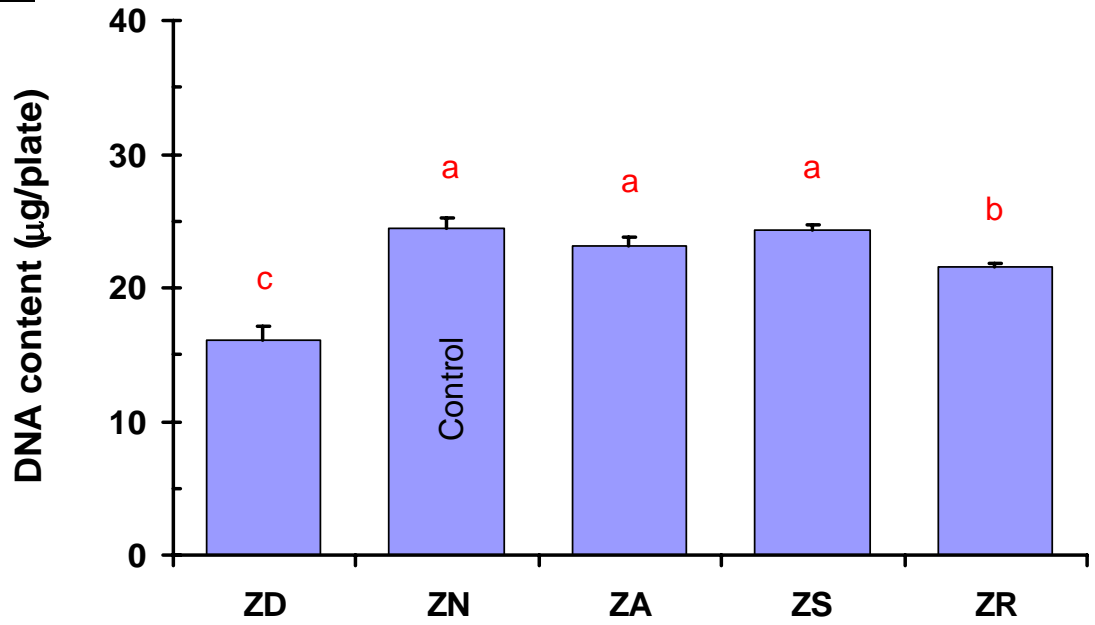
PrEC were susceptible to zinc treatments dose-dependently.

Normal primary prostate epithelial cells (PrEC) were cultured for one passage (6 days) in the basal PrEC growth medium supplementing with different levels of zinc. This basal PrEC growth medium was specifically formulated by Clonetics without the normal addition of zinc. After adding the growth factors, cytokines, and nutrient supplements, zinc concentration of the basal medium increased to $0.9 \mu\text{mol/L}$ as determined by atomic absorption spectrophotometry (data not shown). When the PrEC were cultured directly in this basal medium containing $0.9 \mu\text{mol/L}$ of zinc, the rate of these ZD cells reaching confluency was slightly lower than cells cultured in the regular medium of $4 \mu\text{mol/L}$ of zinc. Cell growth, measured by DNA content per plate, was significantly lower in ZD group by 34% as compared to ZN group (**Figure 3.1A**). However, no differences in cell growth were observed among ZA, ZS, and ZN groups. One day of zinc repletion to ZD cells with ZS medium (ZR group) increased DNA content by 23% but did not reach the level of ZN group. Thus, cell growth appeared to be adversely affected by zinc deficiency. Higher than adequate levels of zinc (16 and $32 \mu\text{mol/L}$), however, had no further effect on cell growth. Zinc repletion was able to increase cell growth, but one day of zinc supplementation was insufficient to totally correct for the zinc deficiency-induced growth reduction.

The uptake of zinc, from media with various zinc levels, by PrEC was determined by cellular zinc status. This was expressed as per DNA content to correct for any differences in cell numbers between plates. As shown in **Figure 3.1B**, cellular zinc status was dose-dependently increased as the level of zinc increased in the media. ZD treatment was able to deplete cellular zinc status to 59% of ZN controls. Cells cultured in ZA and ZS media had significantly higher zinc status than ZN cells by 86% and 137%, respectively. Moreover, cellular zinc levels between ZA and ZS groups were significantly different from each other. The zinc repletion response demonstrated that the zinc status of ZD cells could be normalized to the level of ZN cells by merely one day of ZS treatment. These data suggested that cellular zinc status was highly susceptible to the availability of zinc in the medium in a dose-dependent manner. PrEC showed ability for zinc uptake even at the highest level of zinc treatment (32 $\mu\text{mol/L}$). Correction of zinc deficiency by a day of zinc supplementation appeared to be effective in the enhancement of cellular zinc status to that of ZN cells.

Figure 3.1

A



B

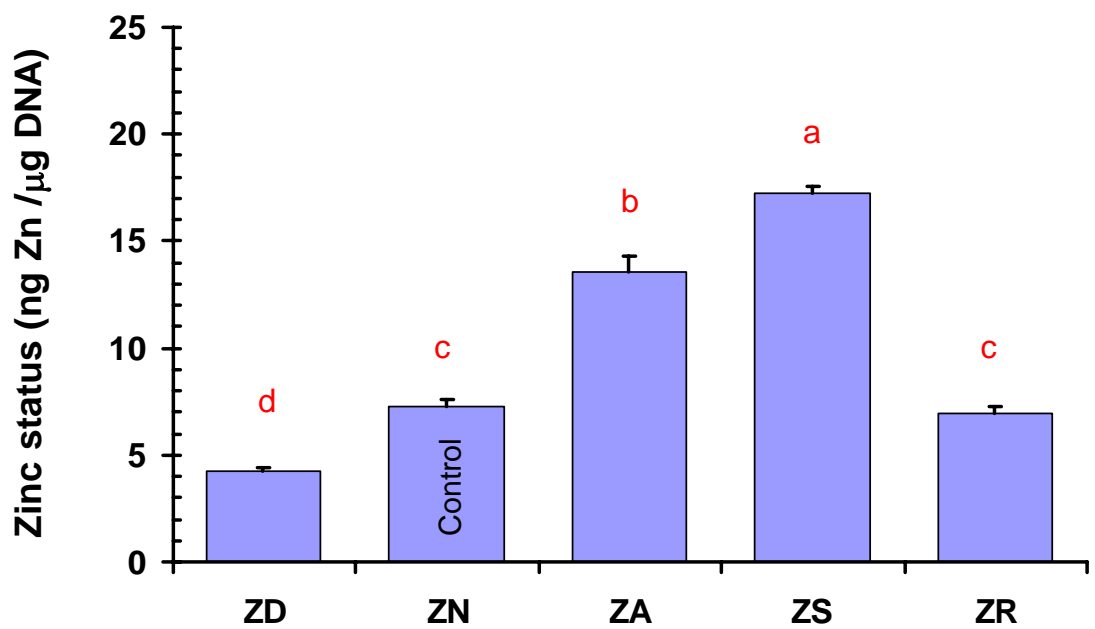


Figure 3.1. Effect of zinc on the (A) DNA content and (B) cellular zinc status of human normal prostate epithelial cells (PrEC). Cells were treated with zinc-deficient (ZD; 0.9 $\mu\text{mol/L}$ zinc), zinc-normal (ZN; 4 $\mu\text{mol/L}$ zinc), zinc-adequate (ZA; 16 $\mu\text{mol/L}$ zinc), and zinc-supplemented (ZS; 32 $\mu\text{mol/L}$ zinc) media for 6 days. One subgroup of ZD cells (zinc-repleted; ZR) was depleted with ZD for 5 days and then repleted with ZS for 1 day. DNA content was measured by diphenylamine method and expressed as microgram per plate. Cellular zinc status was determined by atomic absorption spectrophotometry. Nanogram of zinc per microgram of DNA was expressed to account for any differences in cell numbers between plates. Data are means \pm SEM, which represent an average of triplicates from two different donors of PrEC. Different letters indicate significant differences among groups ($p < 0.05$); treatments with the same letters indicate no significant difference.

Effect of zinc on cell cycle distribution.

Previous observations have indicated that zinc-deficient hepatoblastoma HepG2 cells and zinc-supplemented human normal bronchial epithelial cells had depressed G1/S and G2/M progression, respectively (Cui et al. 2002, Shih et al. 2006). However, the effect of zinc status on cell cycle progression in PrEC has never been investigated. As shown in **Figure 3.2**, the flow cytometric analysis of PrEC indicated that low- and high-zinc treatments exerted little or no change in the G0/G1 and S phases of the cell cycle. However, zinc repletion (ZR group) resulted in 5% less but 6% more cells retained in G0/G1 and S phases, respectively, than ZN controls (**Figure 3.2B**). Both ZA and ZS groups had 2% fewer cells progressing through G2/M phase of the cell cycle than ZN group (**Figure 3.2B**). Fraction of ZS cells in G2/M phase was significantly different from ZD, ZN, and ZR cells, but not from ZA cells. These results suggested that G2/M progression of the cell cycle was depressed by high-zinc treatments.

At all zinc treatments, the percentage distributions of LY294002-treated cells in G0/G1, S, and G2/M phases were all significantly altered from their untreated counterparts (vehicle control, VC). Across the zinc groups, LY294002 treatment significantly increased 12-18% of cells retained at G0/G1 phase to nearly similar level, with ZD group being the highest (**Figure 3.2A**). LY294002 treatment significantly reduced 6-14% of cells retained at S phase. In particular, the percentages of LY294002-treated ZD and ZR cells in S phase were only 57% and 50% of their untreated counterparts, respectively (**Figure 3.2B**). Moreover, LY294002 treatment resulted in a 4-7% reduction at G2/M phase across the zinc groups (**Figure 3.2B**). Overall, LY294002

markedly increased the fractions of cells retained at G0/G1 phase. Thus, a reduction of cells in S and G2/M phases was concomitantly observed.

A large blockage of G1 to S progression (as expressed by G1/S ratio) was only seen in LY294002 treated zinc-deficient PrEC (**Figure 3.2A**). The G1/S ratios were 127%, 60%, 63%, 71%, and 93% higher in LY294002-treated ZD, ZN, ZA, ZS, and ZR groups, respectively, than LY294002-untreated ZN group (**Figure 3.2A**). Thus, only zinc-deficient and zinc-repleted PrEC were sensitive to PI3K inhibitor LY294002 treatment during G1-S progression.

Figure 3.2

A

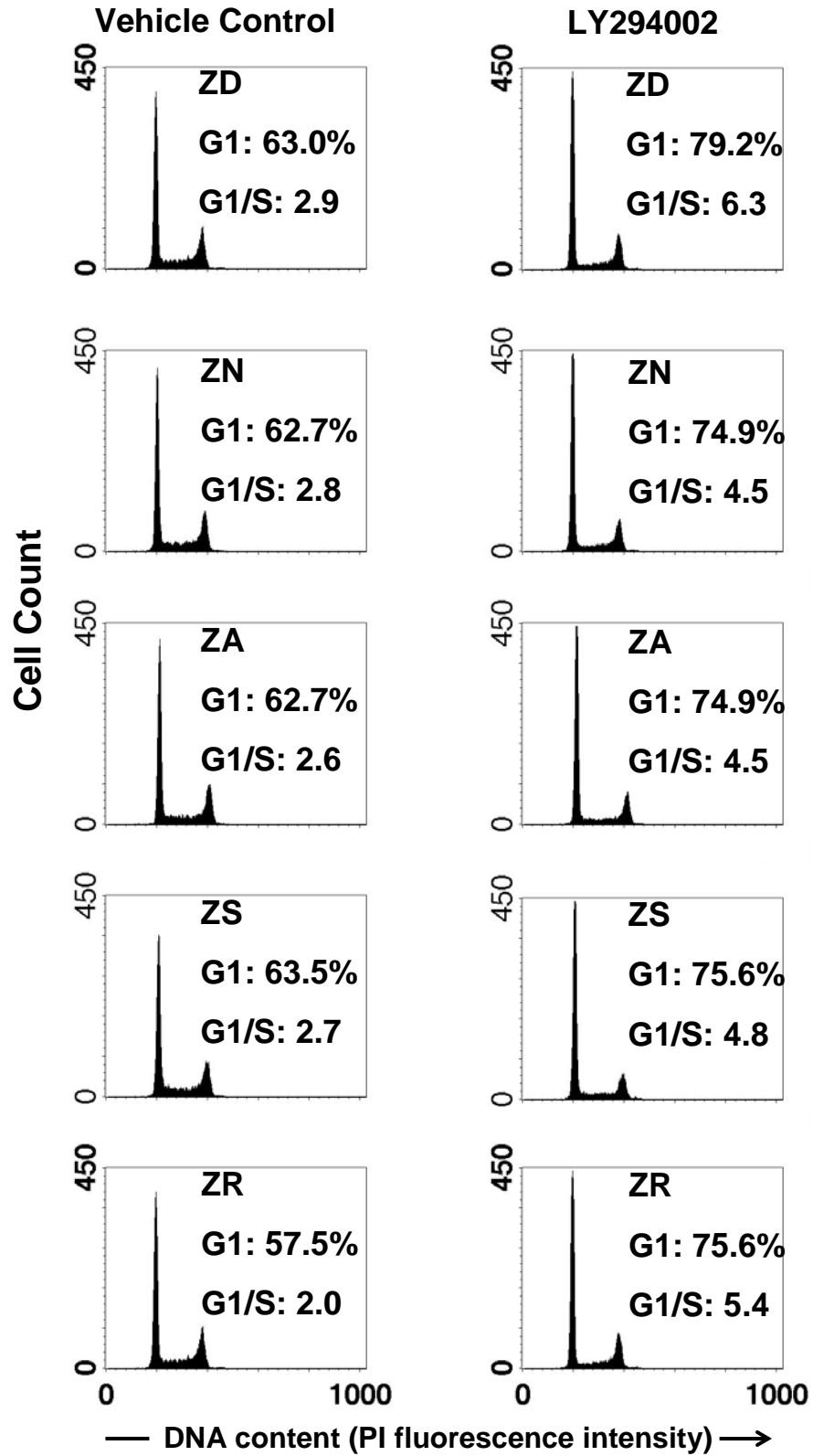


Figure 3.2 (con't)

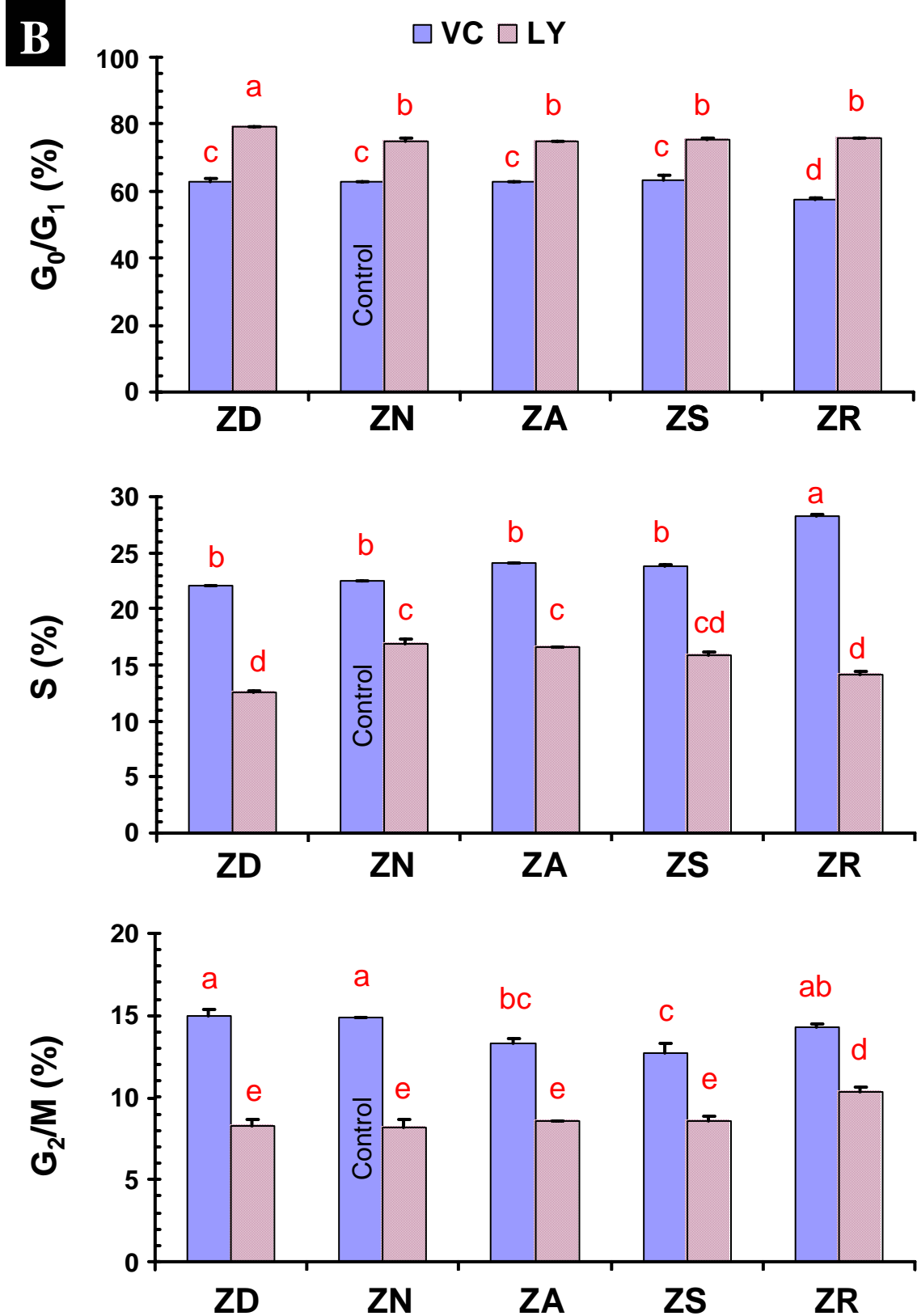


Figure 3.2. Effect of zinc on the cell cycle progression of PrEC. Cells were treated with zinc-deficient (ZD; 0.9 $\mu\text{mol/L}$ zinc), zinc-normal (ZN; 4 $\mu\text{mol/L}$ zinc), zinc-adequate (ZA; 16 $\mu\text{mol/L}$ zinc), and zinc-supplemented (ZS; 32 $\mu\text{mol/L}$ zinc) media for 3 days. One subgroup of ZD cells (zinc-repleted; ZR) was depleted with ZD for 2 days and then repleted with ZS for 1 day. Cells were treated with vehicle control (■, VC) or LY294002 (▨, LY) for 16 hours before harvesting. **(A)** Histograms are representative of two independent experiments. The proportions of cells in G1 phase and the G1-to-S ratios (G1/S) are indicated for each zinc treatment. **(B)** Bar graph shows three phases of the cell cycle: G0/G1 phase, S phase, and G2/M phase. Values are means \pm SEM from 2 separate experiments of one donor. Data obtained from another donor of PrEC exhibiting similar trend. Different letters indicate significant differences among groups ($p < 0.05$); treatments with the same letters indicate no significant difference.

Hyper-phosphorylation of Akt was induced by zinc deficiency and reversed by LY294002.

The effect of *supra-physiological* levels of zinc (50-200 $\mu\text{mol/L}$) on PI3K/Akt signaling has been elucidated in fibroblasts, adipocytes, breast, and lung epithelial cells (Tang and Shay 2001, Kim et al. 2000, Ostrakhovitch and Cherian 2004, Wu et al. 2003, 2005, Bao and Knoell 2006). However, the effect of *physiologic* levels of zinc ($<32 \mu\text{mol/L}$) on Akt phosphorylation has never been examined, in particular in prostate cells. Moreover, phosphorylated Akt (p-Akt) has been suggested as a biomarker for prostate cancer (Ayala et al. 2004). To explore the influence of zinc on the phosphorylation of Akt, we used Western blot analysis with anti-phospho Akt (Ser473) antibody to detect phospho-specific protein expression level in various zinc-treated PrEC. Data was normalized to that of total-Akt. As shown in **Figure 3.3A**, cytoplasmic protein levels of phosphorylated Akt (p-Akt) were 42% and 36% higher in ZD and ZR, respectively, than ZN groups. However, the levels of p-Akt were similar among ZN, ZA, and ZS groups. Since the phosphorylation of Akt is dependent on PI3K (Datta et al. 1996), its inhibitor LY294002 was used to examine the role of PI3K in zinc deficiency-induced Akt phosphorylation. The elevated p-Akt levels observed in ZD and ZR cells were normalized to that of ZN, ZA, and ZS cells after treating with LY294002 for 16 hours (**Figure 3.3A**). For ZD and ZR groups, LY294002 treatment significantly reduced p-Akt level 55% and 52% from their untreated counterparts, respectively. These results suggested that zinc deficiency induced hyper-phosphorylation of Akt and sensitized cells to LY294002. Moreover, zinc repletion with ZS medium for one day was unable to correct for zinc deficiency-induced Akt phosphorylation. Therefore, the present data indicated that PI3K signaling pathway

appeared to be a key mediator in the activation of Akt by zinc, rather than zinc acting on Akt directly.

Hyper-phosphorylation of PTEN in zinc deficiency.

PTEN can physiologically dephosphorylate PIP2 and PIP3, thus antagonizing PI3K activity and inhibiting its downstream effector Akt phosphorylation. PTEN activity is regulated not only in expressed protein levels but also in the ratio of phosphorylated and unphosphorylated form of the protein (Torres and Pulido 2001, Vazquez et al. 2000, Vazquez et al. 2001). Phosphorylation of three residues (Ser380, Thr382, and Thr 383) of the PTEN tail inactivates its phosphatase activity, and unphosphorylated PTEN is the active form. To prevent an untimely or unregulated activation of PTEN, dephosphorylation of the tail would result in an increase in PTEN activity and in its rapid degradation by proteasome. Since the magnitude of PTEN phosphorylation reflects the capacity of PI3K/Akt signaling, whether PTEN plays a role in zinc deficiency-induced Akt phosphorylation can be determined by the ratio of phosphorylated (inactive) and unphosphorylated (active) form of PTEN. Because PTEN, the negative regulator of Akt, is functionally inactivated in prostate cancer, we explored whether increased Akt phosphorylation by zinc deficiency was a result of inactivated PTEN. Moreover, previous studies showed that *supra-physiologic* levels of zinc induced PI3K/Akt signaling pathway and PTEN degradation (Kim et al. 2000, Wu et al. 2003), suggesting that zinc may affect PTEN phosphorylation.

To test this assumption, phospho-specific and total PTEN protein levels were measured in PrEC exposed to various concentrations of zinc using Western blotting. A

phospho-specific PTEN antibody to Ser380, one of the three residues that can be phosphorylated on the tail, was used to measure the extent of phosphorylation. Data were expressed as a ratio of phosphorylated PTEN (p-PTEN) to total-PTEN to indicate PTEN activity. As shown in **Figure 3.3B**, cytoplasmic levels of p-PTEN were 41% and 53% higher in ZD and ZR, respectively, than ZN groups. However, the levels of p-PTEN were similar among ZN, ZA, and ZS groups. Zinc repletion with ZS medium for one day was inadequate to depress zinc deficiency-induced PTEN phosphorylation to the level of ZN group. Since PTEN antagonizes the PI3K/Akt pathway, these data suggested that zinc deficiency-induced PI3K/Akt signaling was due to PTEN inactivation.

Figure 3.3

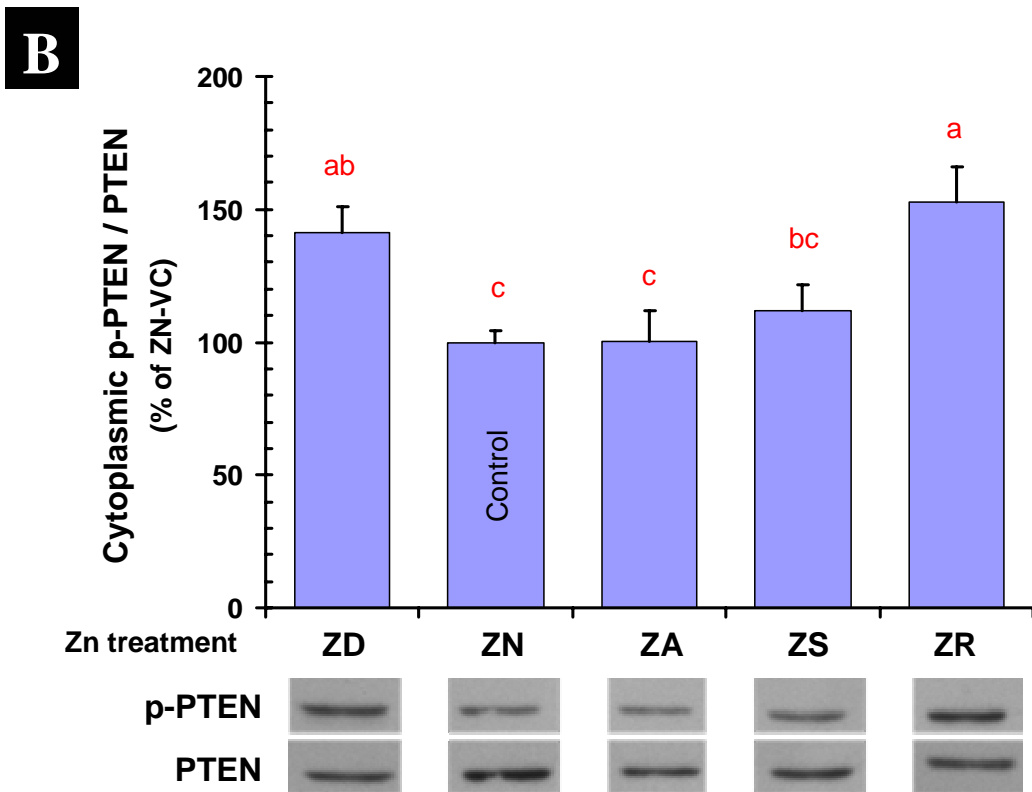
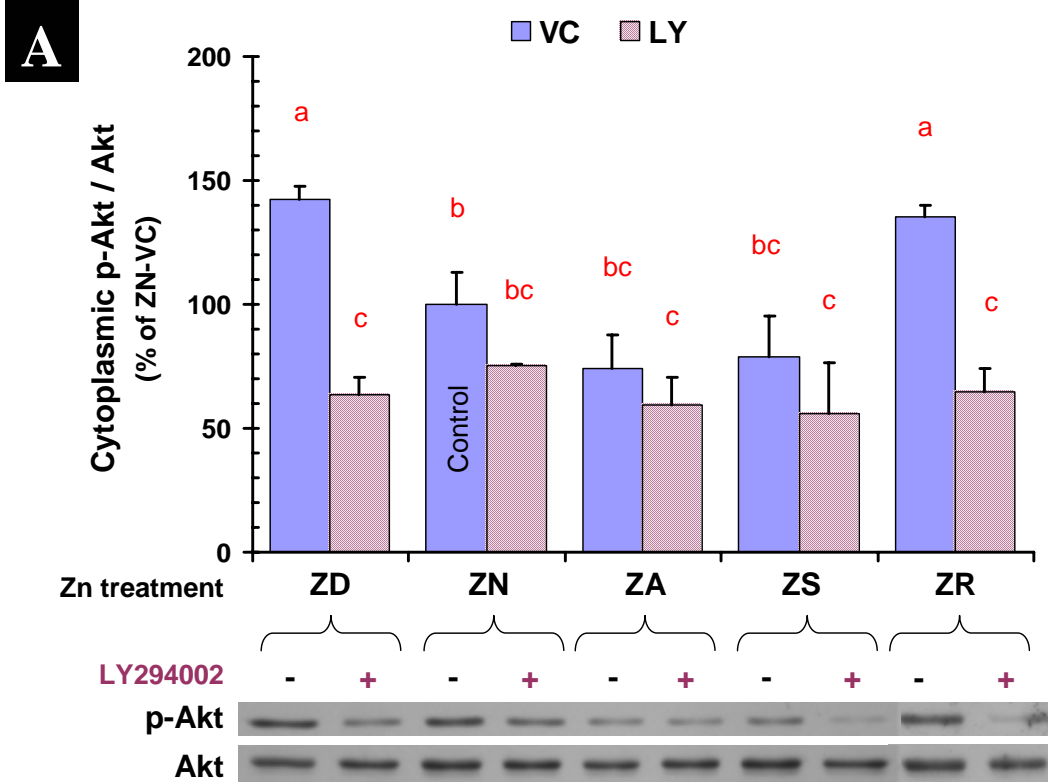


Figure 3.3. Effect of zinc on the phosphorylation of (A) Akt and (B) PTEN

proteins. PrEC were treated with zinc-deficient (ZD; 0.9 $\mu\text{mol/L}$ zinc), zinc-normal (ZN; 4 $\mu\text{mol/L}$ zinc), zinc-adequate (ZA; 16 $\mu\text{mol/L}$ zinc), and zinc-supplemented (ZS; 32 $\mu\text{mol/L}$ zinc) media for 6 days. One subgroup of ZD cells (zinc-repleted; ZR) was depleted with ZD for 5 days and then repleted with ZS for 1 day. Cells were treated with vehicle control (■, VC) or LY294002 (▨, LY) for 16 hours before harvesting. Cytoplasmic extracts were subjected to SDS-PAGE and immunoblotting using anti-phospho-Akt (Ser473) or anti-phospho-PTEN (Ser380) antibody (upper). To control equal loading, the blot was reprobbed with anti-Akt or anti-PTEN antibody (lower). Protein bands were detected using enhanced chemiluminescence reagents and quantified using densitometry. Bar represents means \pm SEM, which is an average of triplicate from two different donors of PrEC. Blot shown is a representative of three independent experiments. All three experiments showed similar results. Different letters indicate significant differences among groups ($p < 0.05$); treatments with the same letters indicate no significant difference.

Hyper-phosphorylation of Mdm2 was induced by zinc deficiency and abrogated by LY294002.

Phosphorylated Akt has been shown to interact with Mdm2 and phosphorylate Mdm2 at Ser166 and Ser186 residues (Gottlieb et al. 2002). These Akt phosphorylation sites in Mdm2 are close to its nuclear localization signal and nuclear export signal domains, which are critical in determining Mdm2 to shuttle between the nucleus and the cytoplasm. Therefore, Akt-mediated phosphorylation of Mdm2 (p-Mdm2) leads to its nuclear localization (Mayo et al. 2001, Zhou et al. 2001, Ogawara et al. 2002). In the present study, we investigated whether zinc deficiency-induced Akt phosphorylation would phosphorylate its downstream effector Mdm2 and mediate nuclear translocation of Mdm2.

Using a phospho-specific Mdm2 antibody, Mdm2 phosphorylation at Ser166 was detectable in the nuclear fraction. As shown in **Figure 3.4A**, the levels of p-Mdm2 were significantly higher by 47% and 52% in ZD and ZR, respectively, than ZN groups. However, there was no difference among ZN, ZA, and ZS groups. With LY294220 treatment, hyper-phosphorylation of Mdm2 induced by zinc deficiency was normalized to that of ZN untreated group. This significant inhibition by LY294002 lowered p-Mdm2 level of ZD group to 67% of LY294002-untreated ZD group. Surprisingly, LY294002 had no inhibitory effect on ZN, ZA, ZS, and ZR groups. Replenishing ZD cells with ZS medium for one day did not minimize the phosphorylation of Mdm2. Unlike ZD group, LY294002 could not reverse this phosphorylation in ZR group. As shown in **Figure 3.4B**, cytoplasmic levels of Mdm2 were unchanged by zinc and/or LY294002 treatments.

These data indicated that Mdm2 was susceptible to phosphorylation by Akt in zinc deficiency. This might result in an accumulation of p-Mdm2 in the nucleus. Since Mdm2 is specifically phosphorylated by Akt at Ser166, inhibitor of PI3K/Akt pathway LY294002 was able to prevent zinc deficiency-induced Mdm2 phosphorylation at Ser166 and nuclear accumulation. However, phosphorylation of Mdm2 was not influenced by LY294002 in cells with higher zinc status. Overall, only nuclear p-Mdm2 was affected by zinc-deficient and LY294002 treatment, but not for cytoplasmic Mdm2.

Figure 3.4

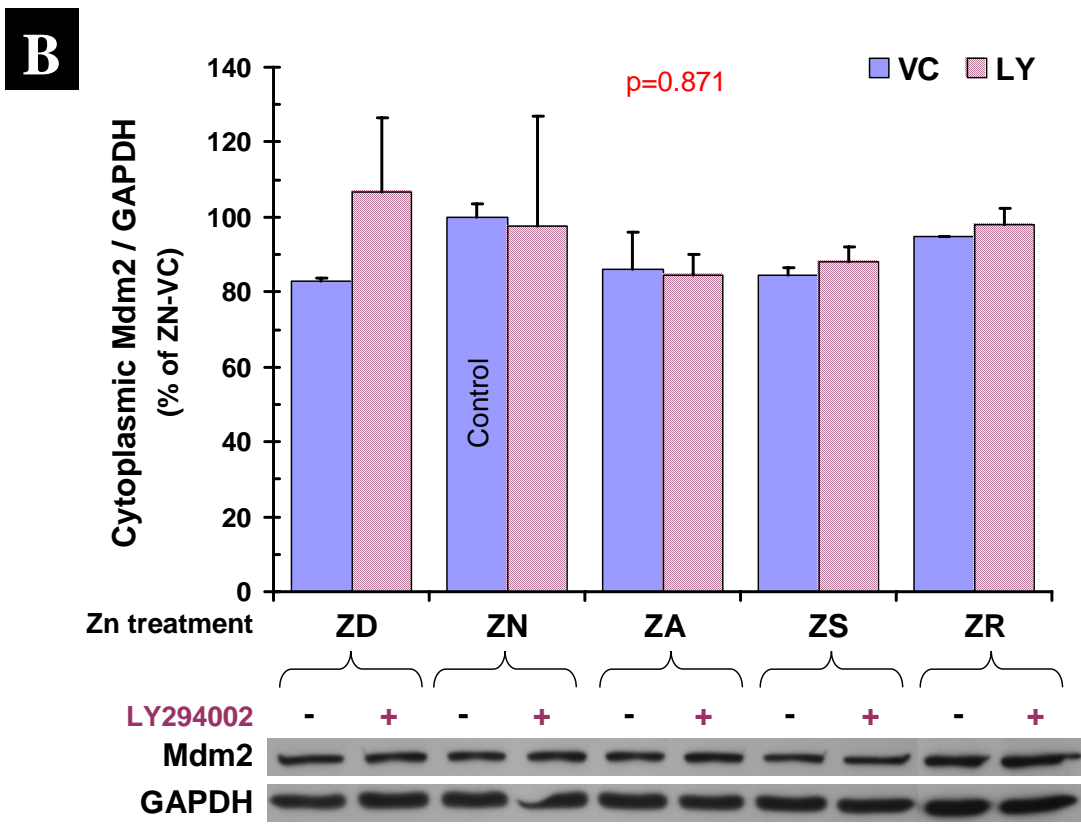
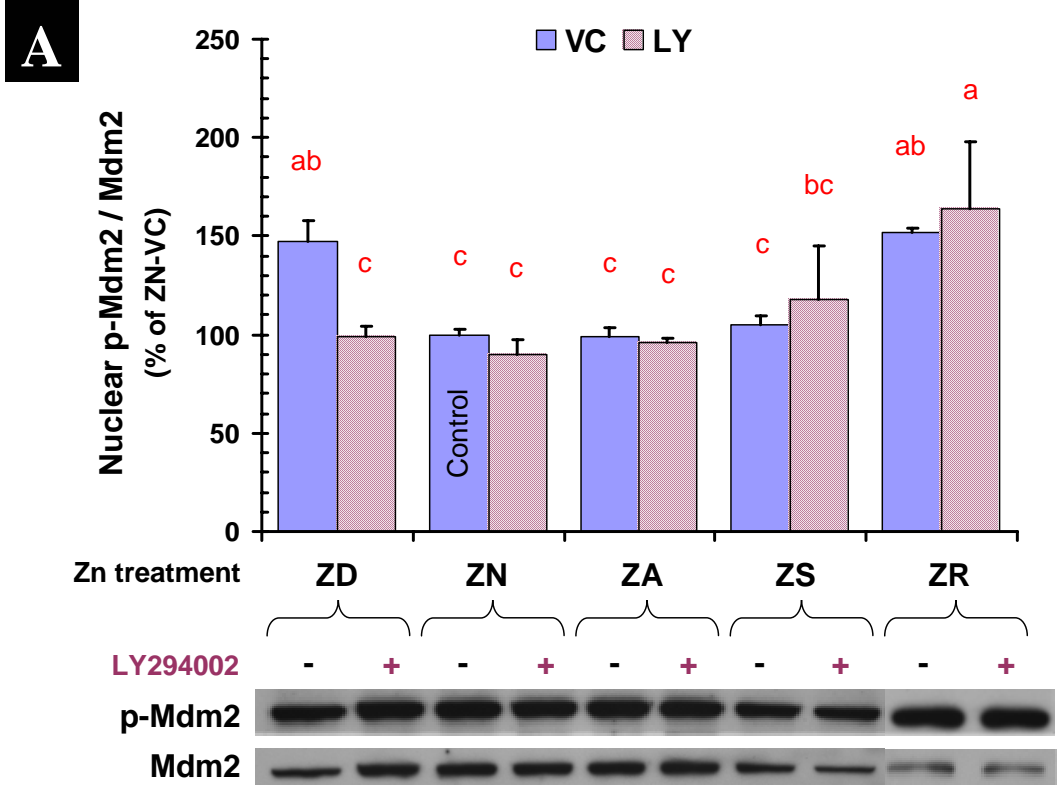


Figure 3.4. Effect of zinc on the (A) nuclear and (B) cytoplasmic levels of Mdm2

protein. PrEC were cultured in zinc-deficient (ZD; 0.9 $\mu\text{mol/L}$ zinc), zinc-normal (ZN; 4 $\mu\text{mol/L}$ zinc), zinc-adequate (ZA; 16 $\mu\text{mol/L}$ zinc), and zinc-supplemented (ZS; 32 $\mu\text{mol/L}$ zinc) media for 6 days. One subgroup of ZD cells (zinc-repleted; ZR) was depleted with ZD for 5 days and then repleted with ZS for 1 day. Cells were treated with vehicle control (■, VC) or LY294002 (▨, LY) for 16 hours before harvesting. Nuclear extracts were subjected to SDS-PAGE and immunoblotting using anti-phospho-Mdm2 (Ser166) antibody and reprobbed with anti-Mdm2 (C-18) antibody for controlling equal loading. Similarly, cytoplasmic protein extracts were probed with anti-Mdm2 (C-18) antibody and reprobbed with anti-GAPDH (FL-335) antibody. Protein bands were detected using enhanced chemiluminescence reagents and quantified using densitometry. Data represent means \pm SEM of triplicates from two different donors of PrEC. Blot shown is a representative of three independent experiments. All three experiments showed similar results. Different letters indicate significant differences among groups ($p < 0.05$); treatments with the same letters indicate no significant difference.

Nuclear and cytoplasmic accumulations of p53 were suppressed by zinc deficiency.

Mdm2, consists of an ubiquitin E3 ligase, is known for its role in controlling the levels and activity of p53 (Momand et al. 2000). Once Mdm2 is phosphorylated by p-Akt, p-Mdm2 translocates to the nucleus and forms a complex with p53 and p300 to promote the ubiquitin-dependent degradation of p53 (Mayo and Donner 2001, Mayo et al. 2002, Zhou et al 2001). As a result, p-Akt may indirectly hinder p53-dependent growth suppression and apoptosis, leading to cell survival (Ogawara et al. 2002). Since we have observed hyper-phosphorylations of Akt and Mdm2 in zinc deficiency, we next investigated whether p53 was affected through this signaling pathway. As shown in **Figure 3.5**, nuclear and cytoplasmic accumulations of p53 were significantly lower by 32% and 39%, respectively, in ZD than ZN groups. There was no difference among ZN, ZA, ZS, and ZR groups. Zinc repletion normalized the depressed levels of nuclear and cytoplasmic p53 seen in ZD group to the levels that were similar to ZN, ZA, and ZS groups. LY294002 treatment lowered nuclear p53 accumulation significantly by 38% and 32% in LY294002-treated ZN and ZA groups, respectively, as compared to their untreated counterparts (**Figure 3.5A**). However, LY294002 had no effect on p53 nuclear accumulation in ZD, ZS, and ZR groups. On the contrary, cytoplasmic p53 levels were significantly lower in LY294002-treated ZN and ZS groups by 45% and 32%, respectively, than their untreated counterparts (**Figure 3.5B**). These data indicated that zinc deficiency had suppressive effect on nuclear and cytoplasmic accumulations of p53. Repleting zinc-deficient cells with ZS medium for one day significantly increased p53 nuclear and cytoplasmic accumulations. Unlike zinc-deficient, zinc-supplemented, and zinc-repleted cells, nuclear p53 accumulation of zinc-normal and zinc-adequate cells was

sensitive to the dose of LY294002 used in this experiment. For cytoplasmic p53 accumulation, only zinc-normal and zinc-supplemented cells were sensitive to the dose of LY294002 chosen for this experiment. For both nuclear and cytoplasmic p53 accumulations, a lack of response of zinc-deficient cells to LY294002 might be due to the treatment dose chosen was not high enough to induce apoptosis. All together, these results suggested that p53 level, either in the nucleus or cytoplasm, might be inhibited through Akt-Mdm2 signaling axis in zinc deficiency.

Figure 3.5

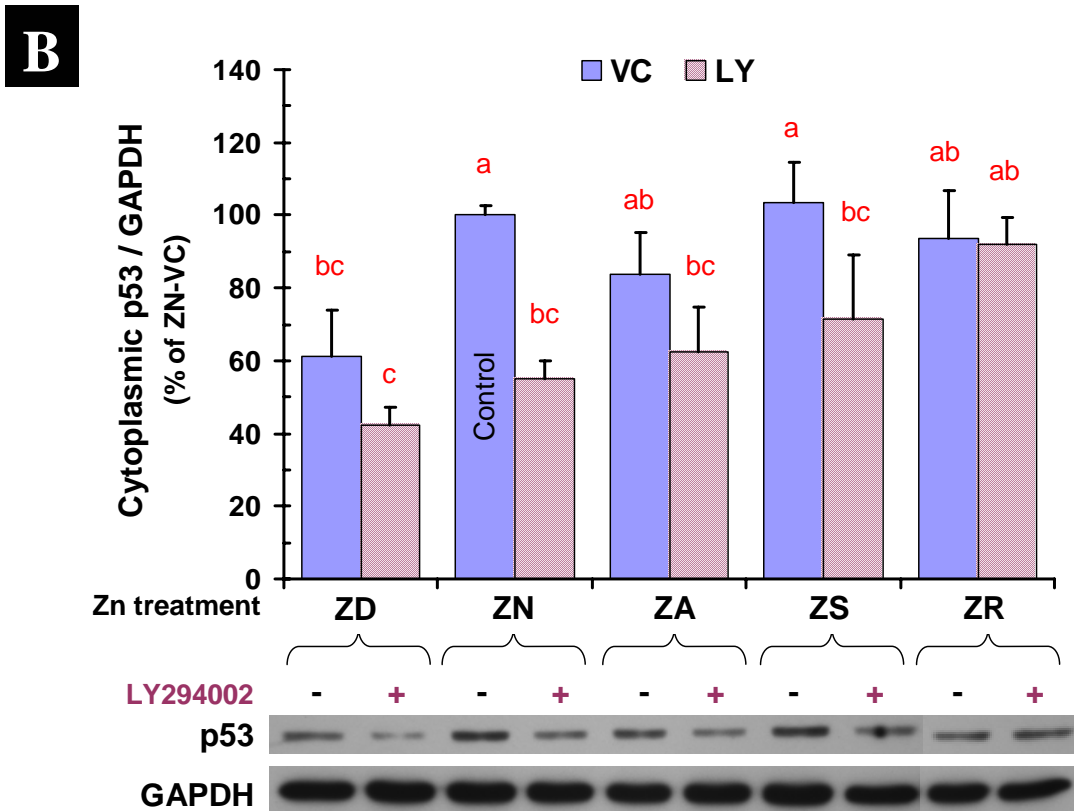
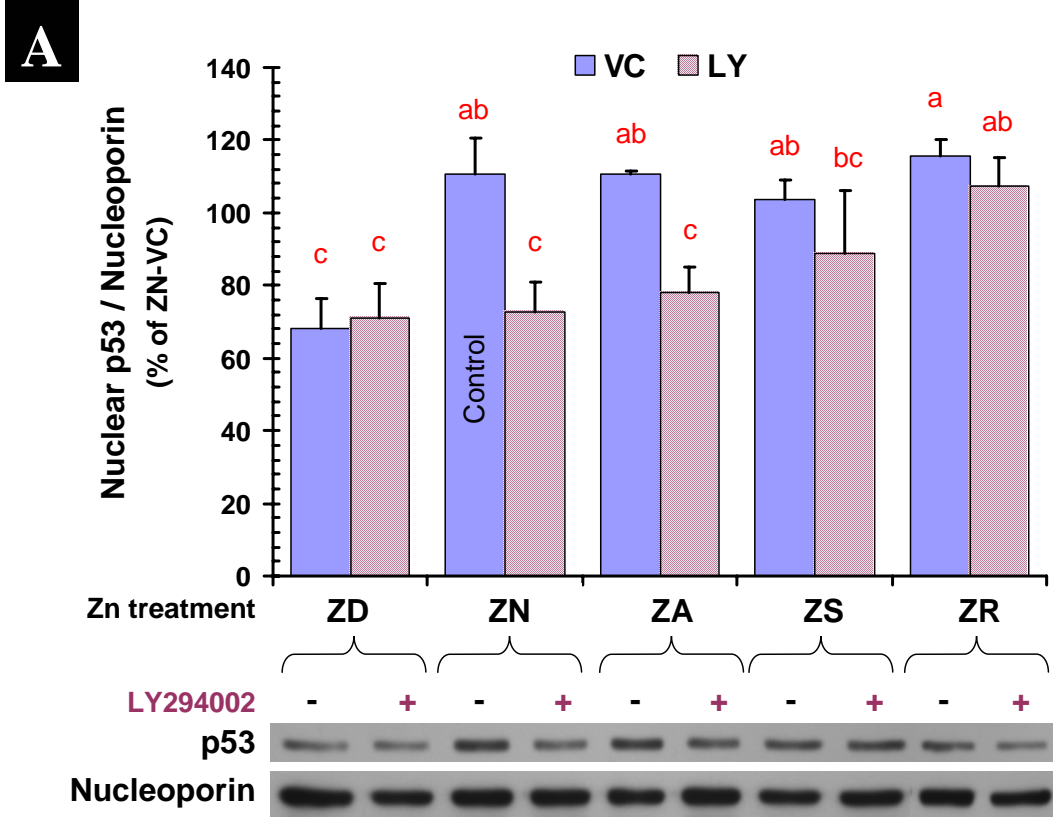


Figure 3.5. Effect of zinc on the (A) nuclear and (B) cytoplasmic levels of p53

protein. PrEC were cultured in zinc-deficient (ZD; 0.9 $\mu\text{mol/L}$ zinc), zinc-normal (ZN; 4 $\mu\text{mol/L}$ zinc), zinc-adequate (ZA; 16 $\mu\text{mol/L}$ zinc), and zinc-supplemented (ZS; 32 $\mu\text{mol/L}$ zinc) media for 6 days. One subgroup of ZD cells (zinc-repleted; ZR) was depleted with ZD for 5 days and then repleted with ZS for 1 day. Cells were treated with vehicle control (■, VC) or LY294002 (▨, LY) for 16 hours before harvesting. Nuclear and cytoplasmic extracts were subjected to SDS-PAGE and immunoblotting using anti-p53 (DO-1) antibody. To control equal loading, blots of nuclear and cytoplasmic extract were reprobated with anti-nucleoporin (N-19) or anti-GAPDH (FL-335) antibody, respectively. Protein bands were detected using enhanced chemiluminescence reagents and quantified using densitometry. Data represent means \pm SEM, which is an average of triplicate experiments from two different donors of PrEC. Blot shown is a representative of three independent experiments. All three experiments showed similar results. Different letters indicate significant differences among groups ($p < 0.05$); treatments with the same letters indicate no significant difference.

Effects of zinc on nuclear p300 level.

The ability of Mdm2 to degrade p53 depends on its ubiquitin E3 ligase activity and its nuclear localization and nuclear export signals. Yet, a transcriptional activator p300 also plays a role in the efficient degradation of p53. In the nucleus, p300 forms a complex with Mdm2 to provide a platform for the assembly of the protein complex necessary for the Mdm2-mediated ubiquitination and degradation of p53 (Grossman et al. 1998, Lill et al. 1997). The two Akt consensus phosphorylation sites in Mdm2 are in the regions that control shuttling of the Mdm2 between nucleus and cytoplasm, which overlap with the regions required for p300 binding (Zhou et al. 2001). To determine whether depressed levels of nuclear and cytoplasmic p53 observed in zinc deficiency were a result of Akt-mediated Mdm2 phosphorylation and p53 degradation, next we examined the availability of nuclear p300 protein that was critical in forming a complex with Mdm2 and p53. As shown in **Figure 3.6**, nuclear p300 protein levels were not significantly different among groups. After treating with LY294002, a two-fold increase was only observed in ZR group as compared to untreated group. These data suggested that the availability of nuclear p300 protein was not affected by various zinc treatments. However, nuclear p300 level was significantly elevated by LY294002 in once zinc-deficient, then repleted cells.

Figure 3.6

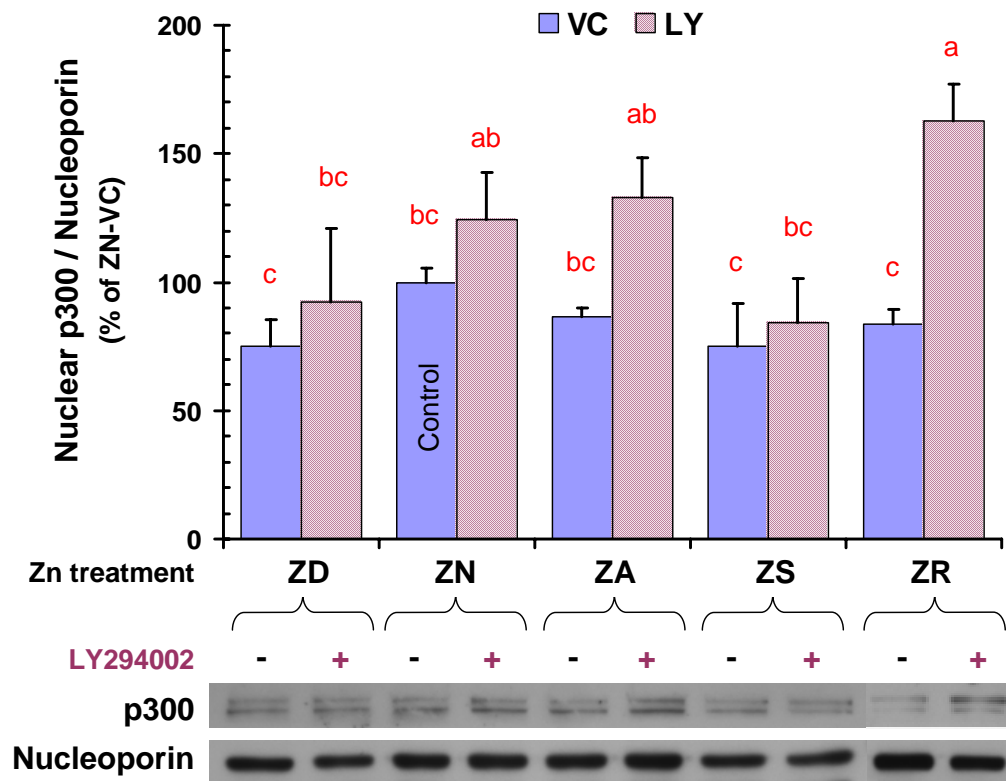


Figure 3.6. Effect of zinc on the nuclear level of p300 protein. PrEC were cultured in zinc-deficient (ZD; 0.9 μ M zinc), zinc-normal (ZN; 4 μ M zinc), zinc-adequate (ZA; 16 μ M zinc), and zinc-supplemented (ZS; 32 μ M zinc) media for 6 days. One subgroup of ZD cells (zinc-repleted; ZR) was depleted with ZD medium for 5 days and then repleted with ZS medium for one day. Cells were treated with vehicle control (■, VC) or LY294002 (▨, LY) for 16 hours before harvesting. Nuclear extracts were subjected to SDS-PAGE and immunoblotting using anti-p300 (C-20) antibody. Blot was reprobbed with anti-nucleoporin (N-19) antibody to correct for equal loading. Protein bands were detected using enhanced chemiluminescence reagents and quantified using densitometry. Data represent an average of duplicate experiments from two different donors of PrEC. Bar represents means \pm SEM from two separate experiments. Blot shown is a representative of two independent experiments. Both experiments showed similar results. Different letters indicate significant differences among groups ($p < 0.05$); treatments with the same letters indicate no significant difference.

Nuclear p21 level was suppressed in zinc deficiency and reversed by LY294002.

Akt can phosphorylate p21 within its nuclear localization signal domain and thus restricting it to the cytoplasm for degradation (Zhou et al. 2001). Furthermore, phosphorylated Akt has been shown to promote cell cycle progression through limiting nuclear p21 protein levels to bind and activate cyclin D-CDK4 complex (Li et al. 2002). Since Akt was hyper-phosphorylated in zinc deficiency, we tested whether the activation of Akt could affect cellular localization of p21. As shown in **Figure 3.7A**, nuclear level of p21 protein was significantly higher in ZA and ZS by 72% and 87%, respectively, than ZN and ZD cells. When compared to ZD group, p21 nuclear protein level of ZR group rose 24%. However, this difference was not statistically significant. LY294002 treatment significantly elevated nuclear p21 protein level across the groups, with LY294002-treated ZN group showing the largest increase (1.5-fold) from untreated ZN group. LY294002-treated ZD and ZR groups had a 71% and 94% increase from their untreated counterparts, respectively. LY294002-treated ZA and ZS groups had a 71% and 79% increase, respectively, as compared to their untreated counterparts. In contrast, cytoplasmic level of p21 protein was indifferent among groups, except LY294002-treated ZR group (**Figure 3.7B**). These data suggested that zinc deficiency had no effect on nuclear p21 protein level. Zinc repletion resulted in higher nuclear p21 protein level, which was similar to the levels observed in zinc-adequate and zinc-supplemented groups.

Figure 3.7

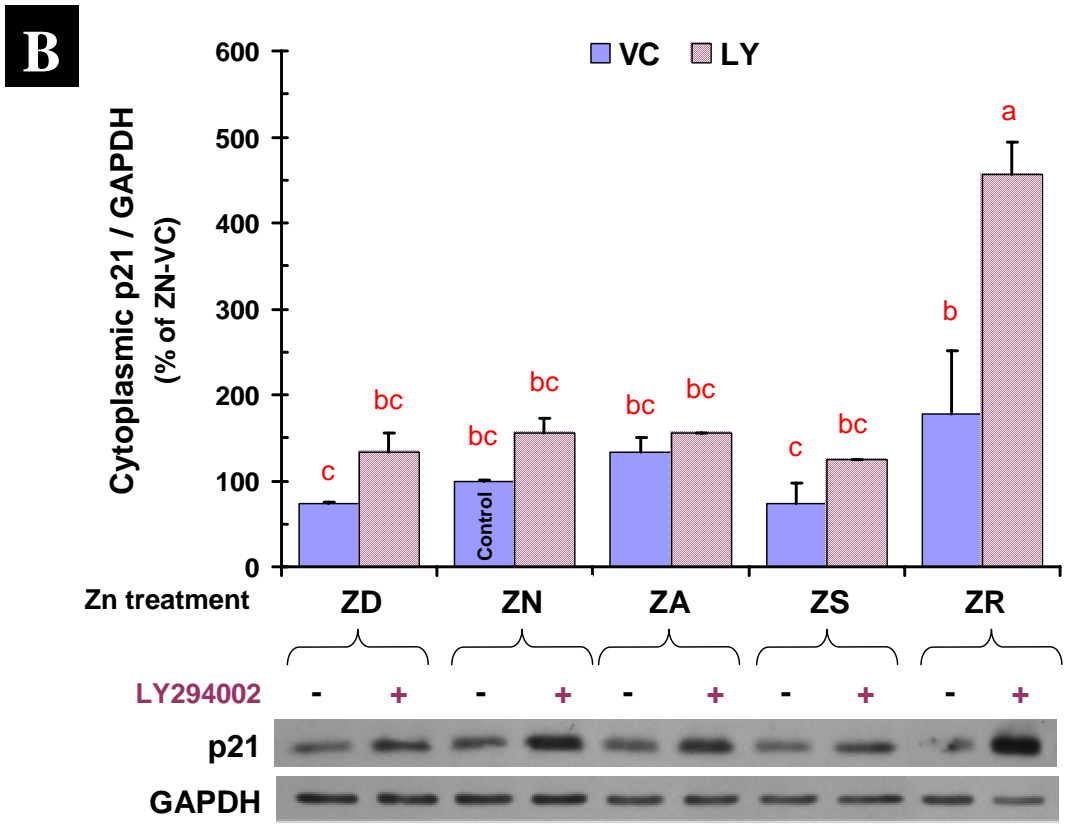
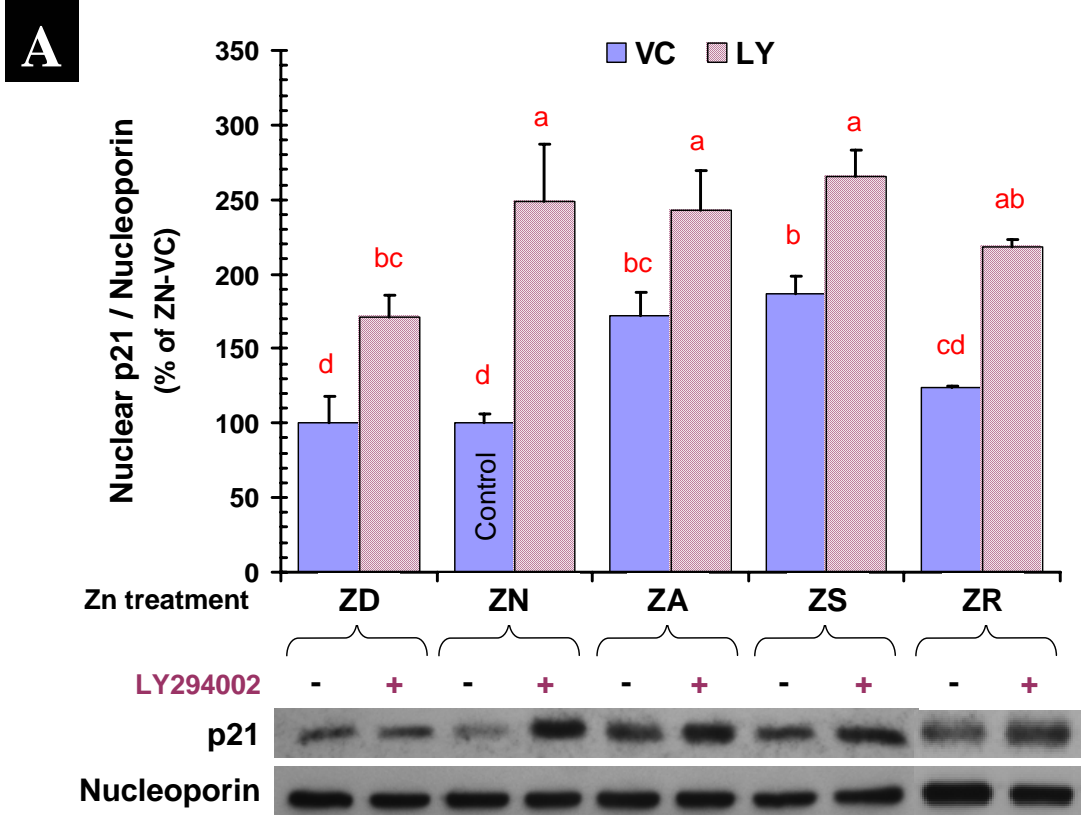


Figure 3.7. Effect of zinc on the (A) nuclear and (B) cytoplasmic levels of p21

protein. PrEC were cultured in zinc-deficient (ZD; 0.9 $\mu\text{mol/L}$ zinc), zinc-normal (ZN; 4 $\mu\text{mol/L}$ zinc), zinc-adequate (ZA; 16 $\mu\text{mol/L}$ zinc), and zinc-supplemented (ZS; 32 $\mu\text{mol/L}$ zinc) media for 6 days. One subgroup of ZD cells (zinc-repleted; ZR) was depleted with ZD for 5 days and then repleted with ZS for 1 day. Cells were treated with vehicle control (■, VC) or LY294002 (▨, LY) for 16 hours before harvesting. Nuclear and cytoplasmic extracts were subjected to SDS-PAGE and immunoblotting using anti-p21 (Ab-1) antibody. To control equal loading, blots of nuclear and cytoplasmic extract were reprobated with anti-nucleoporin (N-19) or anti-GAPDH (FL-335) antibody, respectively. Protein bands were detected using enhanced chemiluminescence reagents and quantified using densitometry. Bar represents means \pm SEM, which is an average from two different donors of PrEC. Blot shown is a representative of two independent experiments. Both experiments showed similar results. Different letters indicate significant differences among groups ($p < 0.05$); treatments with the same letters indicate no significant difference.

DISCUSSION

Normal prostate is unique in their ability to accumulate high levels of cellular zinc. However, this zinc-accumulating ability of cells is lost during malignancy as evident in prostate cancer (Zaichick et al. 1997, Feustel and Wennrich 1984). Some researchers have reported that zinc deficiency led to apoptosis in fibroblasts (Clegg et al. 2005, Chou et al. 2004) and neuroblastoma cells (Mackenzie et al. 2006), while others found that high zinc treatment inhibited malignant prostate cell growth (Liang et al. 1999). In view of the prevalence of dietary zinc deficiency and supplementation as well as the clinical significance of low zinc status in prostate cancer, the current study was designed to examine the influence of zinc on cellular processes mediated via a signaling cascade in the aspects of cellular zinc status, cell growth, and cell cycle progression. Since *supra-physiologic* levels of zinc have been shown to stimulate Akt phosphorylation (Tang and Shay 2001, Kim et al. 2000, Ostrakhovitch and Cherian 2004, Wu et al. 2003, 2005, Bao and Knoell 2006), which is known for signaling cell survival, we specifically investigated whether *physiologic* levels of zinc had an effect on Akt signaling pathway in normal primary prostate epithelial cells (PrEC).

PrEC were selected as our model system because: a) they represent normal prostate tissue; b) they contain wild-type PTEN to maintain a low basal activity of Akt phosphorylation (Whang et al. 1998, Uzgaré and Isaacs 2004); c) they express endogenous and wild-type Mdm2 and p53; and d) they accumulate high levels of zinc. As expected, PrEC were responsive to zinc concentration in the medium dose-dependently. Unlike the zinc status of LNCaP cells, which tended to reach a plateau beyond 4 $\mu\text{mol/L}$ (Han et al. 2006), the zinc uptake of PrEC is evident even at the highest concentration

tested (32 $\mu\text{mol/L}$) (**Figure 3.1B**). This highlights the propensity of normal prostate cells (PrEC) towards zinc and suggests the down-regulation of zinc uptake transporter ZIP during cancer initiation and/or progression as the responsible mechanism (Hasumi et al. 2003). Zinc-deficient treatment was able to deplete zinc status of PrEC to 59% of their zinc-normal control (**Figure 3.1B**). Thus, the zinc status of PrEC was readily modified according to zinc concentration in the media. Similarly, cell growth was inhibited by zinc deficiency, while remained unchanged with zinc-normal, zinc-adequate, and zinc-supplemented treatments.

Studies have established an association of low intracellular zinc concentrations with the induction of apoptosis. Both p53 and NF- κ B are known pathways that negatively and positively, respectively, affecting cell proliferation in various cell types. Observations from our laboratory have shown that zinc deficiency amplifies nuclear p53 accumulation in human hepatoblastoma HepG2 cells (Alshatwi et al. 2006, Reaves et al. 2000), as well as in normal human bronchial epithelial cells (Fanzo et al. 2001) and human aortic endothelial cells (Fanzo et al. 2002) in the absence of apoptosis. Others have found that zinc deficiency inhibits NF- κ B promoter activities in human LNCaP, PC-3 and DU-145 prostate cancer cells (Uzzo et al. 2002, Iguchi et al. 1998), human lung fibroblasts (Ho et al. 2003), rat glioma cells (Ho and Ames et al. 2002), mouse 3T3 fibroblasts (Clegg et al. 2005, Chou et al. 2004), and human neuroblastoma IMR-32 cells (Mackenzie et al. 2002) in the presence of apoptosis. Despite these contradictory findings on zinc deficiency-induced apoptosis, our zinc-deficient PrEC did not undergo apoptosis as no accumulation exhibited at sub-G0 phase of the cell cycle (**Figure 3.2A**). They progressed through G0/G1 and S phases of the cell cycle in similar fashion as the other

zinc-treated groups. This might be a result from hyper-phosphorylation of Akt, which is known for its pro-survival activity, as suggested by our findings of zinc deficiency-induced phosphorylation of Akt in PrEC.

Indifferent from previous findings on zinc deficiency-induced p53 nuclear accumulation in HepG2 (Alshatwi et al. 2006, Reaves et al. 2000), normal human bronchial epithelial cells (Fanzo et al. 2001) and human aortic endothelial cells (Fanzo et al. 2002), we did not observe this accumulation in PrEC. In contrast, nuclear p53 level was 32 % lower in zinc-deficient PrEC (**Figure 3.5A**). Under cellular stress, p21 expression is increased through p53-dependent and –independent pathways. Previously, our laboratory demonstrated that nuclear p21 protein level was lower in zinc-deficient than zinc-normal HepG2 cells despite of p53 nuclear accumulation (Alshawti et al. 2006). However, zinc deficiency-suppressed p53 nuclear accumulation did not seem to correlate with the nuclear p21 protein level in PrEC (**Figures 3.5A and 3.7A**). Similarly, our laboratory found that p21 mRNA level remained unaffected by zinc deficiency in normal primary aortic endothelial cells (Fanzo et al. 2002). The discrepancy of p21 protein level between HepG2 and PrEC might be due to the zinc status of zinc deficient-PrEC being not sufficiently depleted in these originally zinc-accumulating cells, to exert a suppressive effect on nuclear p21 protein level. For HepG2 cell culture, we used chelex to remove the zinc from fetal bovine serum (FBS) (Reaves et al. 2000). Thus, the growth medium containing 10% of FBS had a concentration of <0.2 $\mu\text{mol/L}$ zinc. In contrast for PrEC culture, we omitted the zinc during the formulation of basal medium. After adding growth factors, cytokines, and nutrient supplements to the basal medium, this growth medium contained 0.9 $\mu\text{mol/L}$ of zinc. Consequently, the zinc status of zinc-deficient

PrEC might not have been as depleted as in zinc-deficient HepG2 cells. Therefore, we did not observe a depressive effect on nuclear p21 protein level by zinc deficiency in PrEC. Alternatively, it might be due to the fact that zinc deficiency-enhanced Akt phosphorylation mediates the phosphorylation, retention, and degradation of p21 in the cytoplasm, thus inhibiting translocation of p21 into the nucleus. As a result, zinc-deficient PrEC were able to progress through all three phases of the cell cycle. Treating PrEC with LY294002 was able to increase nuclear p21 levels, which is consistent with findings on malignant prostate DU-145 and PC3 cells (Gao et al. 2003).

Lower percentage of PrEC was detected at G2/M phase with our zinc-adequate and zinc-supplemented treatments (**Figure 3.2B**). This observation is coincided with the elevated nuclear p21 protein levels seen in zinc-adequate and zinc-supplemented PrEC (**Figure 3.7A**). In contrast, previous study has shown that zinc-induced G2/M phase arrest was accompanied by increased mRNA levels of p21 in malignant prostate LNCaP (p53^{+/+}) and PC3 (p53^{-/-}) cells, which indicated that the impaired cell cycle progression might be independent of p53 (Liang et al. 1999). However, the protein level of p21 was not reported in their study. Also, their study was done in serum-starved cells prior to and during zinc treatment. This discrepancy in culture condition and zinc treatment might have caused the G2/M arrest in their study. Furthermore, growth of LNCaP and PC-3 cells has been shown to be suppressed by 200 and 100 $\mu\text{mol/L}$ of zinc, respectively, which are *supra-physiologic* (Hasumi et al. 2003). Although we did not observe growth inhibition in PrEC with the highest zinc treatment at 32 $\mu\text{mol/L}$, the concentration chosen was based on physiological relevance. Since p21 plays a role in growth arrest through inhibiting interactions between cyclins and cyclin-dependent kinases (CDKs), further

study needs to determine whether zinc-induced nuclear p21 accumulation has any effect on the expression of cyclins and the activity of CDKs in PrEC.

Treatment with PI3K inhibitor LY294002 induced a higher percentage of PrEC being retained at G0/G1 phase. A large blockage of G1 to S progression (as expressed by G1/S ratio in **Figure 3.2A**) was only seen in LY294002 treated-zinc-deficient PrEC. This led us to believe that zinc deficient-cells are susceptible to Akt phosphorylation, thus makes them more vulnerable to its antagonist LY294002.

Previous studies have shown that higher than 50 $\mu\text{mol/L}$ of zinc could induce Akt phosphorylation in fibroblasts, adipocytes, breast cancer cells, and lung epithelial cells (Tang and Shay 2001, Kim et al. 2000, Ostrakhovitch and Cherian 2004, Wu et al. 2003, 2005, Bao and Knoell 2006), while zinc deficiency lead to hypo-phosphorylation of Akt in fibroblasts (Clegg et al. 2005). However, no study to date has used normal human prostate cells or prostate cancer cells to examine this zinc effect on Akt phosphorylation. Since 16 $\mu\text{mol/L}$ is the normal zinc levels found in the human plasma and 32 $\mu\text{mol/L}$ of zinc is the highest attainable level by oral supplementation in humans, we used them as zinc-adequate and zinc-supplemented groups, respectively, in our study. Surprisingly, we found that Akt phosphorylation was 42% higher in zinc deficient cells (0.9 $\mu\text{mol/L}$), while Akt phosphorylation status of zinc-adequate (16 $\mu\text{mol/L}$) and zinc-supplemented (32 $\mu\text{mol/L}$) cells were indifferent from zinc-normal (4 $\mu\text{mol/L}$) PrEC (**Figure 3.3A**). A specific inhibitor of PI3K/Akt pathway, LY294002, was able to normalize the zinc deficiency-induced Akt phosphorylation. This suggested that the dependency of zinc deficiency on Akt phosphorylation was via PI3K signaling. The mechanisms by which zinc activate PI3K signaling can be explained through 1) receptor tyrosine kinase; or 2)

non-receptor tyrosine kinase signaling. First, Kim et al. (2000) demonstrated that PI3K activities associated with anti-phosphotyrosine immunoprecipitates were raised significantly following zinc stimulation in Swiss 3T3 cells. This result suggested that tyrosine kinases were involved in the activation of the PI3K signaling pathway by zinc. Insulin-like growth factor-1 (IGF-1) receptor is another example of receptor tyrosine kinases. MacDonald (2000) highlighted the effects of zinc deficiency on membrane signaling systems and intracellular second messengers that coordinate cell proliferation in response to IGF-1. Moreover, May and Contoreggi (1982) have found that $>250 \mu\text{mol/L}$ zinc exerted the insulin-like effects by generating hydrogen peroxide in isolated rat adipocytes. Intracellular reactive oxygen species (ROS) have been shown to induce the tyrosine phosphorylation of cytosolic proteins and activation of growth factor receptor tyrosine kinases such as epidermal growth factor receptor (Wang et al. 2000). Furthermore, oxidative stress activates PI3K and causes the accumulation of phosphatidylinositol 3,4-bisphosphate, which recruits Akt to the plasma membrane and activates it (Van der Kaay et al. 1999). From these results, we can postulate a model that zinc deficiency induces the generation of ROS such as hydrogen peroxide in the cell and consequently activates growth factor receptor tyrosine kinases to stimulate the PI3K signaling pathway.

Alternatively, zinc may activate non-receptor tyrosine kinases associated with PI3K. For example, a study done by Sonoda et al. (1999) implicated that focal adhesion kinase (FAK), a non-receptor tyrosine kinase, functions as the upstream mediator of PI3K activation in T98 glioblastoma cells. In addition, FAK and Src family tyrosine kinases were tyrosine-phosphorylated and induced to associate with PI3K in response to

hydrogen peroxide treatment (Sonoda et al. 1999, Lander et al. 1995, Abe et al. 1997). Thus, these non-receptor tyrosine kinases may also be possibly involved in the zinc deficiency-mediated activation of PI3K in primary cells.

According to previous observations, low zinc concentrations provided a condition that was favorable to the production of reactive oxygen/reactive nitrogen species (Oteiza et al. 2000, Cui et al. 1997, Ho and Ames 2002). Smet et al. (2003) demonstrated that zinc depletion by copper treatment led to the production of superoxide dismutase and oxidative stress in HepG2 cells. Nair et al. (2005) showed that short-term zinc deficient diet increased sensitivity to oxidative stress in testes and epididymis of rats, as a consequence of increased ROS generation and/or decreased zinc-dependent antioxidant processes. Therefore, it could be possible that our zinc-deficient PrEC were undergoing oxidative stress, thus activating the PI3K signaling to hyper-phosphorylate Akt.

Since PrEC contain wild-type PTEN, which is an endogenous negative regulator of Akt signaling pathway, the present study also examined the extent of PTEN phosphorylation because that is indicative of how much inactive PTEN which is unable to prevent Akt phosphorylation (Vazquez et al. 2000). Again, PTEN phosphorylation was higher in zinc deficiency (**Figure 3.3B**). This validated our assumptions that zinc deficiency-induced Akt phosphorylation was due to inhibited PTEN activity.

Following the Akt-Mdm2-p53 signaling axis, we examined the degree of Mdm2 phosphorylation and p53 accumulation in the nucleus. In accordance with zinc deficiency-induced Akt phosphorylation, Mdm2 phosphorylation was higher and p53 accumulation was lower in the zinc-deficient PrEC's nucleus than all other treatment groups (**Figures 3.4A and 3.5A**). Treatment with inhibitor LY294002 was able to reverse

zinc deficiency-induced Mdm2 phosphorylation. This finding agreed with the notion that phosphorylated Akt could subsequently phosphorylate Mdm2 and promote its nuclear translocation (Mayo et al. 2001). Consequently, nuclear Mdm2 could complex with p53 and p300 to form poly-ubiquitinated p53, which was then shuttled to the proteasome for degradation (Mayo and Donner 2001, Grossman et al. 2003). Thus, cytoplasmic level of p53 was also lower in zinc-deficient PrEC than all other treatment groups. Despite our previous data showed that nuclear p300 level was markedly depressed by zinc deficiency in HepG2 cells (Alshatwi et al. 2006), it was unchanged in PrEC and treatment conditions of the present study (**Figure 3.6**). This suggested that in zinc-deficient PrEC, p300 was maintained in the nucleus at the normal level capable forming functional complex with p53 and Mdm2, necessary for the normal p53 ubiquitination and degradation. As a result, Akt may exert an indirect effect on inhibiting apoptotic function of p53 tumor suppressor protein through Mdm2 in zinc deficiency.

In additional to phosphorylating Mdm2, Akt has also been established to mediate the phosphorylation of p300 at Ser1834 which is essential for its transcriptional activity (Liu et al. 2006, Huang and Chen 2005). Specifically, phosphorylation of p300 by Akt induced the recruitment of p300 to its target gene's promoter, leading to the acetylation of histones in chromatin and association with the basal transcriptional machinery RNA polymerase II. With inhibitor LY294002 treatment, nuclear p300 and both nuclear and cytoplasmic p21 protein levels increased substantially in zinc-repleted PrEC as compared to their untreated counterparts (**Figures 3.6 and 3.7B**). Since p300 was shown to collaborate with transcriptional factor Sp1 to enhance the promoter activity of p21 (Xiao

et al. 2000), we postulated that p300 might be involved in the transcription and synthesis of p21 in response to LY294002.

Together, the results of this study suggested that the zinc deficiency-induced Akt phosphorylation in PrEC was a result of PTEN phosphorylation, which led to Mdm2 phosphorylation and less nuclear accumulation of p53, thus exerting less of the tumor-suppressive effect of p53 and promoting cell survival. Therefore, the progression of the cell cycle was maintained through an Akt-Mdm2-p53 signaling axis in zinc-deficient PrEC.

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CHAPTER 4.

THE INFLUENCE OF ZINC STATUS ON AKT-P21 SIGNALING AXIS IN HUMAN MALIGNANT PROSTATE CELLS

ABSTRACT

The phosphorylated form of Akt (p-Akt), a phosphoinositide-3-OH-kinase (PI3K) activated protein kinase, is highly expressed in prostate tumors. P-Akt can affect cell cycle progression by phosphorylating p21 and restricting it to the cytoplasm for degradation. Also, p-Akt can phosphorylate Mdm2 and enhance its nuclear entry, subsequently promoting the ubiquitin-dependent degradation of tumor suppressor p53. As a result, Akt can indirectly hinder p53-dependent growth suppression and apoptosis. *Supra-physiologic* level of zinc has been shown to stimulate the phosphorylation of Akt in cell culture. Conversely, zinc has been established to be depleted in prostate cancer cells. The objective of this study was to determine whether *physiologic* level of zinc influences the cell cycle progression and apoptosis via Akt signaling pathway. Human malignant prostate LNCaP cells was cultured in RPMI containing 10% chelex-FBS (< 0.1 $\mu\text{mol/L}$ zinc) added with 0 (zinc-deficient; ZD), 4 (zinc-normal; ZN), 16 (zinc-adequate; ZA), or 32 (zinc-supplemented; ZS) $\mu\text{mol/L}$ of zinc sulfate. One subgroup of ZD cells (zinc-repleted; ZR) was depleted with ZD for 5 d and then repleted with ZS for 1 d. ZD treatment lowered cellular zinc level to 25% below ZN control. The level of p-Akt was higher and cytoplasmic p21 was lower in ZD, ZA, ZS, and ZR than ZN cells. When treated with a specific inhibitor of PI3K/Akt pathway, LY294002, both levels were

depressed to similar extent. Nuclear p21 and p53 levels were lower in ZD than ZN cells. However, ZA and ZS treatments had no effect on nuclear p21 and p53 levels. Nuclear phosphorylated Mdm2 was unaffected by zinc treatments. Higher fraction of ZD, ZA, ZS, and ZR cells was retained at G0/G1 phase of the cell cycle than ZN cells, with proportionally less at S and G2/M phases. In summary, zinc deficiency might induce cellular proliferation through Akt signaling pathway in LNCaP cells by promoting p21 phosphorylation and degradation, thus restricting p21 nuclear entry to induce cell cycle arrest. Since p53 is also depressed in zinc deficiency, whether zinc deficiency-inhibited nuclear p21 is p53-dependent requires further investigation.

INTRODUCTION

Prostate cancer is the most frequently diagnosed malignancy and second leading cause of cancer death in men (Jemal et al. 2006). Known as a zinc-accumulating organ, normal prostate glands accumulate 10-fold higher zinc as compared to other soft tissues, such as liver and kidney (Rosoff 1981, Costello and Franklin 1998). The capability to accumulate zinc is retained in benign prostatic hyperplasia (BPH), whereas the zinc levels of malignant prostate epithelial cells are 70-80% lower than the surrounding normal epithelial cells (Zaichick et al. 1997, Feustel and Wennrich 1984). Such changes in zinc accumulation may implicate a significant role in the development and/or progression of prostate malignancy.

The requirement of zinc in cellular processes, including cell growth and proliferation, has been well-established (MacDonald 2000). Studies have found that zinc deficiency leads to apoptosis in fibroblasts (Clegg et al. 2005, Chou et al. 2004) and

neuroblastoma cells (Mackenzie et al. 2006). However, the effect of zinc deficiency on prostate cancer cell cycle progression is unknown. On the other hand, *in vivo* or *in vitro* treatment of *supra-physiologic* zinc level has shown an inhibitory effect on tumor or cancer cell growth. Zinc administered by osmotic pump was shown to increase zinc accumulation in tumor tissues and suppress tumor growth of PC-3 cells in nude mice (Feng et al. 2003). Growth of LNCaP and PC-3 cells was shown to be suppressed by 100-500 $\mu\text{mol/L}$ of zinc (Hasumi et al. 2003, Iguchi et al. 1998). This inhibitory effect of zinc appears to result from zinc-induced apoptosis or necrosis. Nonetheless, the ability of *physiologic* level of zinc in altering cell cycle progression in prostate cancer cells has not been established.

Phosphorylated form of Akt (p-Akt) has been frequently detected to be elevated in prostate cancer (Ayala et al. 2004, Kreisberg et al. 2004). This is a malignancy in which mutation and deletion of the phosphatase and tensin (PTEN) gene are especially common in metastatic tumors and result in elevated phosphatidylinositol-3-OH kinase (PI3K)/Akt signaling. The PI3K/Akt pathway is an important signaling cascade in promoting tumorigenesis (Testa and Bellacosa 2001). Akt, a serine/threonine kinase, can phosphorylate substrates, such as p21 (Zhou et al. 2001) and Mdm2 (Mayo and Donner 2001), to promote cell proliferation and prevent cells from undergoing apoptosis.

The subcellular localization of p21 has been proposed to be critical for the regulation of p21 function (Vivanco and Sawyers 2002). In the nucleus, p21 is a cyclin-dependent kinases (CDK) inhibitor that can arrest the cell cycle in response to DNA damage or stress. More specifically, p21 can interact with proliferating cell nuclear antigen (PCNA) and block DNA synthesis required for S phase of the cell cycle. As

opposed to its anti-proliferative functions, p21 can be pro-proliferative when localized in the cytoplasm. Akt has been shown to phosphorylate p21 at Thr145 residue, which lies near the nuclear localization signal (NLS) domain (Zhou et al. 2001). Phosphorylation near this NLS site prevents p21's entry into the nucleus. Growth-inhibiting activity of p21 is therefore suppressed by this cytoplasmically retained p21 through facilitating the assembly and activity of cyclin D-CDK 4 complex. Therefore, the cell cycle can be progressed through G1 phase into S phase (Boonstra 2003).

On the contrary, Akt promotes the nuclear entry of Mdm2 after phosphorylating the latter on Ser166 and Ser186 residues (Mayo and Donner 2001). Phosphorylation at sites proximal to Mdm2's NLS domain is necessary for translocation of Mdm2 from the cytoplasm into the nucleus. This phosphorylation also stabilizes the protein by inhibiting self-ubiquitination and increasing its association with p300 (Feng et al. 2004). Consequently, the complex of Mdm2 and p300 promotes the subsequent poly-ubiquitination and degradation of p53 (Grossman et al. 2003). This provides a mechanism by which Akt can hinder p53-dependent apoptosis in cells.

In studies exploring the effect of zinc on PI3K/Akt signaling pathway, *supra-physiologic* levels of zinc (50-200 $\mu\text{mol/L}$) have been shown to induce hyper-phosphorylation of Akt in fibroblasts, adipocytes, and lung epithelial cells (Tang and Shay 2001, Kim et al. 2000, Wu et al. 2003, 2005, Bao et al. 2006). However, only one study has examined the effect of *physiologic* levels of zinc (10-25 $\mu\text{mol/L}$) on Akt phosphorylation in breast cancer cells (Ostrakhovitch and Cherian 2004). Nevertheless, hypo-phosphorylation of Akt was shown to occur in zinc-deficient fibroblasts (Clegg et al. 2005). Yet, as a zinc-enriched tissue, the effects of zinc on prostate cells have never

been investigated in the aspect of Akt phosphorylation. Therefore, the objective of the current is to determine whether *physiologic* levels of zinc can influence the cell cycle progression and apoptosis via PI3K/Akt signaling pathway. Following the downstream signaling events of Akt, nuclear and cytoplasmic levels of p21, Mdm2, and p53 were measured. The dependence of any zinc-altered Akt phosphorylation via PI3K signaling was ascertained by using a specific PI3K/Akt inhibitor LY294002.

METHODS

LNCaP Maintenance

Human prostate malignant LNCaP cells were purchased from the American Type Culture Collection (Manassas, VA). Cell culture reagents were obtained from Invitrogen (Carlsbad, CA). Cells were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, 0.1 U/L penicillin, and 0.05 µg/L streptomycin sulfate, in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. Medium was replaced every other day, and 6 days of culture constitute one passage. Nearly confluent (<85%) cells were subcultured using trypsin-EDTA for the initiation of experimental treatment. Chelex-100 resin (Bio-Rad, Hercules, CA), a divalent ion-chelating resin, was used to remove zinc from FBS before the addition of FBS to RPMI (Reaves et al. 2000, Suhy et al. 1999). The resin was first neutralized to physiological pH with 0.25 mol/L HEPES, pH 7.4, and then mixed with FBS at a 1:4 ratio (w/v) at 4°C for 2 hours. The Chelex-100 resin was separated from FBS by centrifugation, followed by filtration through a 0.22 µm filter for sterilization and

removal of residual Chelex-100 resin. The RPMI with 10% chelexed-FBS, containing <math><0.1 \mu\text{mol/L}</math> zinc, was termed the zinc-deficient (**ZD**) medium.

Zinc and Inhibitor Treatment

Besides ZD treatment, zinc was added to chelexed-RPMI media in the form of zinc sulfate hepta-hydrate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ abbreviated as ZnSO_4 , Sigma-Aldrich, St. Louis, MO) for LNCaP cell culture. Therefore, the only difference between these media was the zinc concentration. For the zinc-normal (**ZN**) media, $4 \mu\text{mol/L}$ ZnSO_4 was added to the ZD media; the zinc-adequate (**ZA**) media contained $16 \mu\text{mol/L}$ ZnSO_4 ; and the zinc-supplemented (**ZS**) media contained $32 \mu\text{mol/L}$ ZnSO_4 . The ZN media was used as the control group for all experiments. The ZA treatment was used as a representative of human plasma zinc levels. The ZS group was used to represent the high end of plasma zinc levels attainable by oral supplementation in humans. After one passage in ZN media, LNCaP cells were sub-cultured and stabilized overnight in ZN medium prior changing into their respective media, namely ZD, ZN, ZA, or ZS media, for another 6 days (1 passage). One subgroup of ZD cells was depleted with ZD medium for 5 days and then repleted with ZS medium for 1 day to provide the zinc-repleted (**ZR**) cells. Cells were then harvested for assays. The dependence of zinc-induced Akt phosphorylation was ascertained by using a PI3K inhibitor LY294002 (Calbiochem, San Diego, CA). Cells were treated with either $5 \mu\text{mol/L}$ of LY294002 or 0.05% DMSO (as vehicle control) for 16 hours before harvest.

DNA Content and Zinc Status

The effect of zinc treatment on cellular zinc uptake in LNCaP cells cultured for one passage in medium containing different levels of zinc was determined by atomic absorption spectrophotometry. Cells were collected by scraping from 100-mm Petri dishes and centrifugation. Cell pellets were then washed twice with phosphate-buffered saline (PBS). Cells were resuspended into 1.5 ml PBS and sonicated for two 30-second intervals on ice. An aliquot of the sonicated cell suspension was used for the determination of cellular zinc content by a flame atomic absorption spectrophotometer (PerkinElmer, Boston, MA). Zinc standard solutions ranging from 0.05 to 1.0 parts per million (ppm) were used to generate a linear standard curve. The zinc content of the cells was calculated based on these zinc standard solutions. From the same sample, another aliquot of the sonicated cell suspension was used for the determination of cellular DNA content by the diphenylamine method (Gendimenico et al. 1988). Since there is a linear relationship between cellular DNA and cell number as previously established by Wu et al. (1997), data were expressed as nanogram of cellular zinc per microgram of DNA.

Cell Cycle Progression

The influence of zinc status on cell cycle progression was analyzed by flow cytometry. Cells were collected by trypsinization and centrifugation. Cell pellet was washed twice with PBS and reconstituted to 1 ml. Then, 1×10^6 cells were fixed with 70% ethanol, treated with RNase to remove any double-stranded RNA, and then labeled with propidium iodide, which binds to double-stranded DNA (Gomez-Angelats et al. 2000). A total of 10,000 cell events were collected for DNA analyses on a FACScalibur cytometer

with CELLQuest program (Becton Dickinson, San Jose, CA). Cell cycle distribution percentages of stained nuclei were calculated using ModFit LT (Version 3.0, Verity Software House, Topsham, ME). Calibration standards, LinearFlow Green (Molecular Probes, Carlsbad, CA) and DNA QC Particle Kit (Becton Dickinson, San Jose, CA), were used to verify instrument performance. The DNA content of a cell population analyzed by this assay was used to determine the proportion of cells that were in G0/G1, S, or G2/M phase of the cell cycle.

Nuclear and Cytoplasmic Extractions

Nuclear and cytoplasmic extracts were obtained by using NE-PER™ Nuclear and Cytoplasmic Extraction Kit (Pierce Biotechnology, Rockford, IL). Briefly, cells were washed with PBS, scraped off the dish, and collected by centrifugation at $1,500 \times g$ for 5 minutes. The pellet was resuspended in Cytoplasmic Extraction Reagent I (CERI) and incubated on ice. Cytoplasmic Extraction Reagent II (CERII) was then added and the extracts were further incubated on ice. The extracts were centrifuged for 15 minutes, and the supernatant (cytoplasmic fraction) was transferred to a new tube. Nuclear Extraction Reagent (NER) was added to the pellet and the mixture was incubated on ice for 40 minutes while vortexing every 10 minutes. The extract was centrifuged for 15 minutes and the supernatant (nuclear fraction) was transferred to a new tube. In addition to the standard HALT™ protease inhibitor cocktail (Pierce Biotechnology, Rockford, IL), a phosphatase inhibitor cocktail (Pierce Biotechnology, Rockford, IL) was added to the CERI and NER to prevent de-phosphorylation. The isolated fractions were stored at -80°C until analysis. Protein concentration was determined by using Bicinchoninic Acid

(BCA) Protein Assay Reagents (Pierce Biotechnology, Rockford, IL) with bovine serum albumin as the standard.

Western Blot Analysis

Approximately 40 µg protein of cell extracts were mixed with equal volume of Laemmli sample loading buffer, which consists 62.5 mmol/L Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% Bromophenol Blue, and 35.5 mmol/L β-mercaptoethanol. These extracts were size-separated by 10% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis under reduced conditions, followed by electrotransfer onto a ECL nitrocellulose membrane (Amersham Life Science, Arlington Heights, IL). A blocking buffer consisting 5% (w/v) nonfat dry milk in 10 mmol/L Tris-HCl, pH 7.4 with 0.1% Tween-20 (TBS-T) were used to block non-specific proteins on the membrane for 1 hour at room temperature. After several washes with TBS-T, the membrane was probed with p-Akt (Ser473) antibody (Cell Signaling Technology, Beverly, MA) diluted in 1% (w/v) bovine serum albumin with 0.1% Tween-20 overnight at 4°C. Bound primary antibody was detected with horseradish peroxidase conjugated secondary antibody and chemiluminescent substrate SuperSignal WestPico Chemiluminescence reagent (Pierce Biotechnology, Rockford, IL). Immunoreactive bands were visualized by exposing membranes to X-OMAT film (Eastman Kodak, Rochester, NY), which was developed by an X-Ray Film Processor (AFP Imaging, Elmsford, NY). Membranes were then stripped and re-probed with Akt antibody (Cell Signaling Technology, Beverly, MA) to verify equal protein loading. Relative band intensities were digitally quantified by using an imaging system FluorChem 8900 with AlphaEase® FC Stand Alone Software (Alpha

Innotech, San Leandro, CA). Other primary antibodies used were: p-Mdm2 (Ser166) (Cell Signaling Technology, Beverly, MA), Mdm2 (C-18), p53 (DO-1) (Santa Cruz Biotechnology, Santa Cruz, CA), and p21 (Ab-1) (Calbiochem, San Diego, CA) antibodies. Membranes were reprobbed with either nucleoporin (p62) or GAPDH (FL-335) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) to ensure equal loading of nuclear or cytoplasmic protein, respectively. Cross contaminations of nuclear and cytoplasmic extracts as determined by probing with GAPDH and nucleoporin, respectively, have been routinely established to be less than 5%.

Statistical analysis

Data represent means \pm SEM from three independent experiments. Statistical analyses were performed using SPSS (Version 10.0, Chicago, IL). Data were analyzed using one-way ANOVA, and the means were further compared by least significant differences (LSD) *post-hoc* test with *p* values < 0.05 regarded as significant.

RESULTS

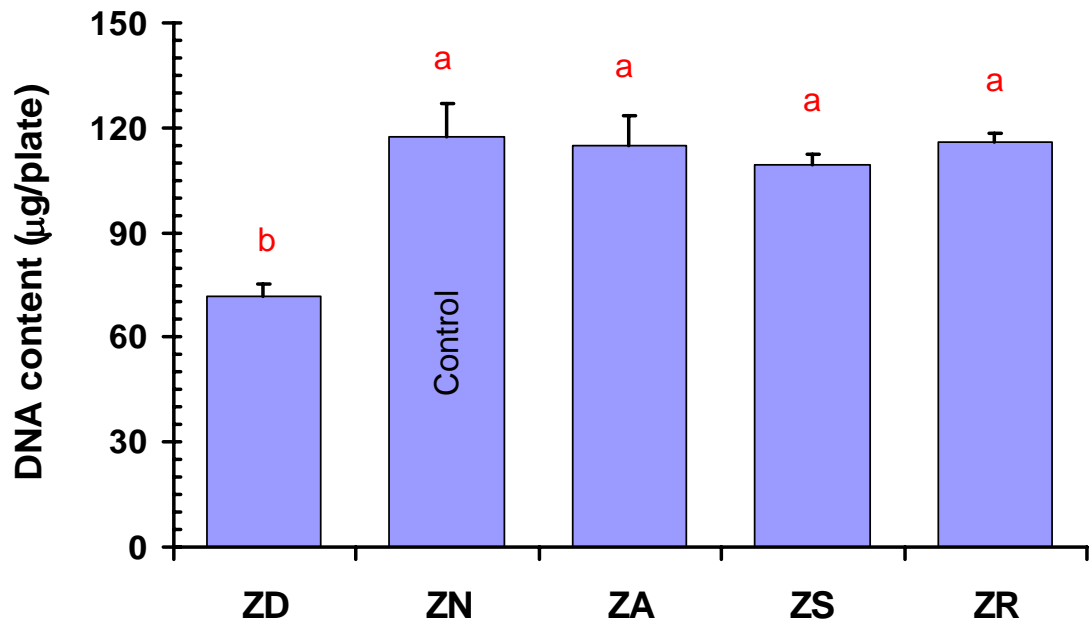
Effect of zinc on cell growth and zinc status.

Since zinc is essential for cell growth and is enriched in the prostate, the influence of zinc on growth of the prostate malignant cells was examined. LNCaP cells were treated with various concentrations of zinc for one passage. After chelexing zinc out of fetal bovine serum (FBS), final zinc concentration of the basal medium consisted of RPMI plus 10% FBS was $< 0.1 \mu\text{mol/L}$ (data not shown). Cell growth was significantly lower in ZD group by 39% as compared to ZN group (**Figure 4.1A**). However, cell growth was indifferent

among ZN, ZA, ZS, and ZR groups. Zinc status of LNCaP cells was shown in **Figure 4.1B**. ZD treatment was able to deplete cellular zinc status to 75% of ZN controls. Cells cultured in ZA medium had no further effect on zinc status, while zinc status of ZS group was only 16% higher than ZN group. The zinc repletion approach showed that the cell growth and zinc status of ZD cells could be normalized to the level of ZN cells by merely one day of ZS treatment. Higher than normal levels of zinc (i.e. 16 and 32 $\mu\text{mol/L}$), however, had no further effect on cell growth. Thus, correction of zinc deficiency by a day of zinc supplementation appeared to be effective in normalizing cell growth and cellular zinc status.

Figure 4.1

A



B

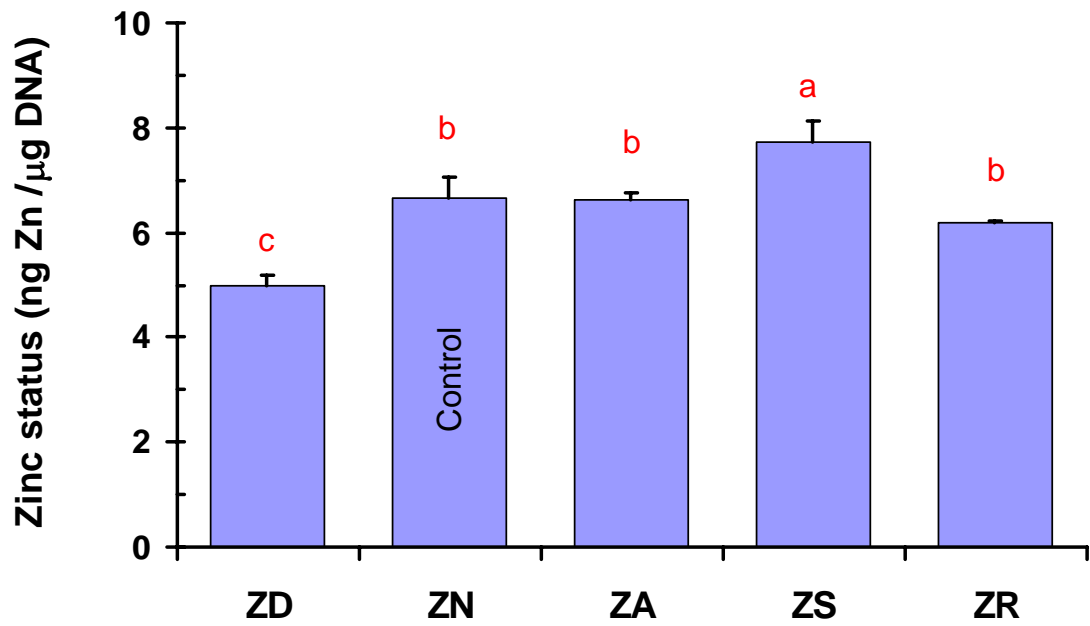


Figure 4.1. Effect of zinc on the (A) DNA content and (B) cellular zinc status of LNCaP cells. Cells were cultured for 6 days in zinc-deficient (ZD; 0 $\mu\text{mol/L}$ zinc), zinc-normal (ZN; 4 $\mu\text{mol/L}$ zinc), zinc-adequate (ZA; 16 $\mu\text{mol/L}$ zinc), and zinc-supplemented (ZS; 32 $\mu\text{mol/L}$ zinc) RPMI medium containing 10% chelex-FBS. One subgroup of ZD cells was depleted with ZD medium for 5 days and then repleted with ZS medium for 1 day to provide the zinc-repleted (ZR) cells. (A) DNA content was measured by diphenylamine method and expressed as microgram per plate. (B) Cellular zinc status was determined by atomic absorption spectrophotometry. Nanogram of zinc per microgram of DNA was expressed to account for any differences in cell numbers between plates. Values are means \pm SEM from three separate experiments. Different letters indicate significant differences among groups ($p < 0.05$); treatments with the same letters indicate no significant difference.

Effect of zinc on cell cycle distribution.

Various studies have demonstrated that zinc plays an important role in regulating cell proliferation (MacDonald 2000). However, little is known about the effect of zinc on cell cycle progression in human prostate malignant cells. LNCaP, which is an androgen sensitive cell line with wild-type p53, was chosen to represent an early cancer progression and a functional p53 to control the cell cycle. All treatments did not induce apoptotic cells as sub-G0 peak is absent (**Figure 4.2A**). **Figure 4.2B** showed that the cell cycle distribution of LNCaP cells after one passage of ZD treatment resulted in 10% more LNCaP cells retained at G0/G1 phase than ZN treatment, while ZA, ZS, and ZR treatments resulted in 6% more. At S phase, 8% less cells was detected in ZD than ZN group; while 4% less was in ZA, ZS, and ZR than ZN group. The effect of zinc was less pronounced at G2/M phase, with 1-2% less cells detected in ZD, ZA, ZS, and ZR than ZN group.

Across the zinc groups, treatment of LNCaP cells with the PI3K inhibitor, LY294002, uniformly affected each phase of the cell cycle to the similar extent (**Figure 4.2B**). As compared to LY294002-untreated cells (vehicle control, VC), there were higher fractions of cells retained at G0/G1 phase, lower at S phase, and about the same at G2/M phase. LY294002 treated-ZD, ZN, ZA, ZS, and ZR groups had 5.3%, 12.5%, 8.5%, 6.7%, and 8.1%, respectively, more cells retained at G0/G1 phase than their untreated counterparts. At the S phase, LY294002 treatment significantly reduced 4.0%, 10.1%, 6.5%, 6.0%, and 6.4% of ZD, ZN, ZA, ZS, and ZR cells, respectively, than their untreated counterparts. ZD, ZA, ZS, and ZR groups all had ~2% less cells at G2/M phase than ZN group. These results suggested that other than normal zinc treatment (ZN),

LNCaP cells responded to either low (ZD) or high zinc treatment (ZA, ZS, ZR) in similar fashion at each stage of the cell cycle.

When expressing the data as G1/S ratio, ZD group was 2.8-fold higher than ZN group, whereas the ratio of ZA and ZS group was about 1.7-fold higher than ZN group (**Figure 4.2A**). Repletion of ZD cells with ZS media for one day resulted in a lower G1/S ratio that was similar to that of ZS cells. Thus, zinc depletion significantly promoted the progression of ZD cells from G1 to S phase.

A significant blockage of G1 to S progression was evident by LY294002 treatment (**Figure 4.2A**). The ratio was highest in LY294002-treated ZD group, followed by ZR, ZS, ZA, and ZN group. Overall, LY294002 had a significant effect on increasing fractions of cells retained at G1 phase, which is consistent with the previous observations that LY294002 caused a blockage at G1-S transition in LNCaP cells (Gottschalk et al. 2005) and other prostate cancer cell lines, DU145 and PC-3 (Gao et al. 2003).

Figure 4.2

A

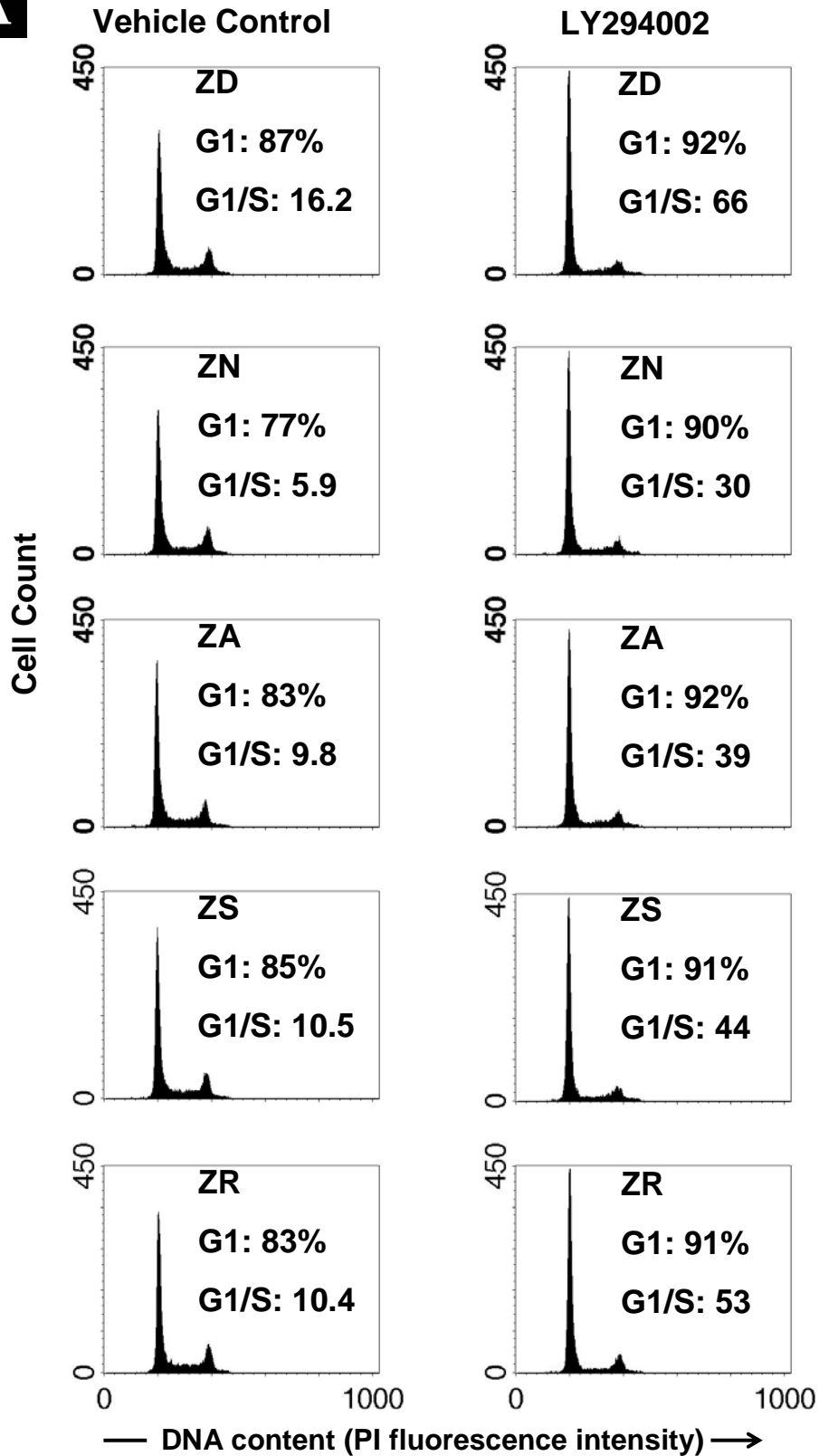


Figure 4.2 (con't)

B

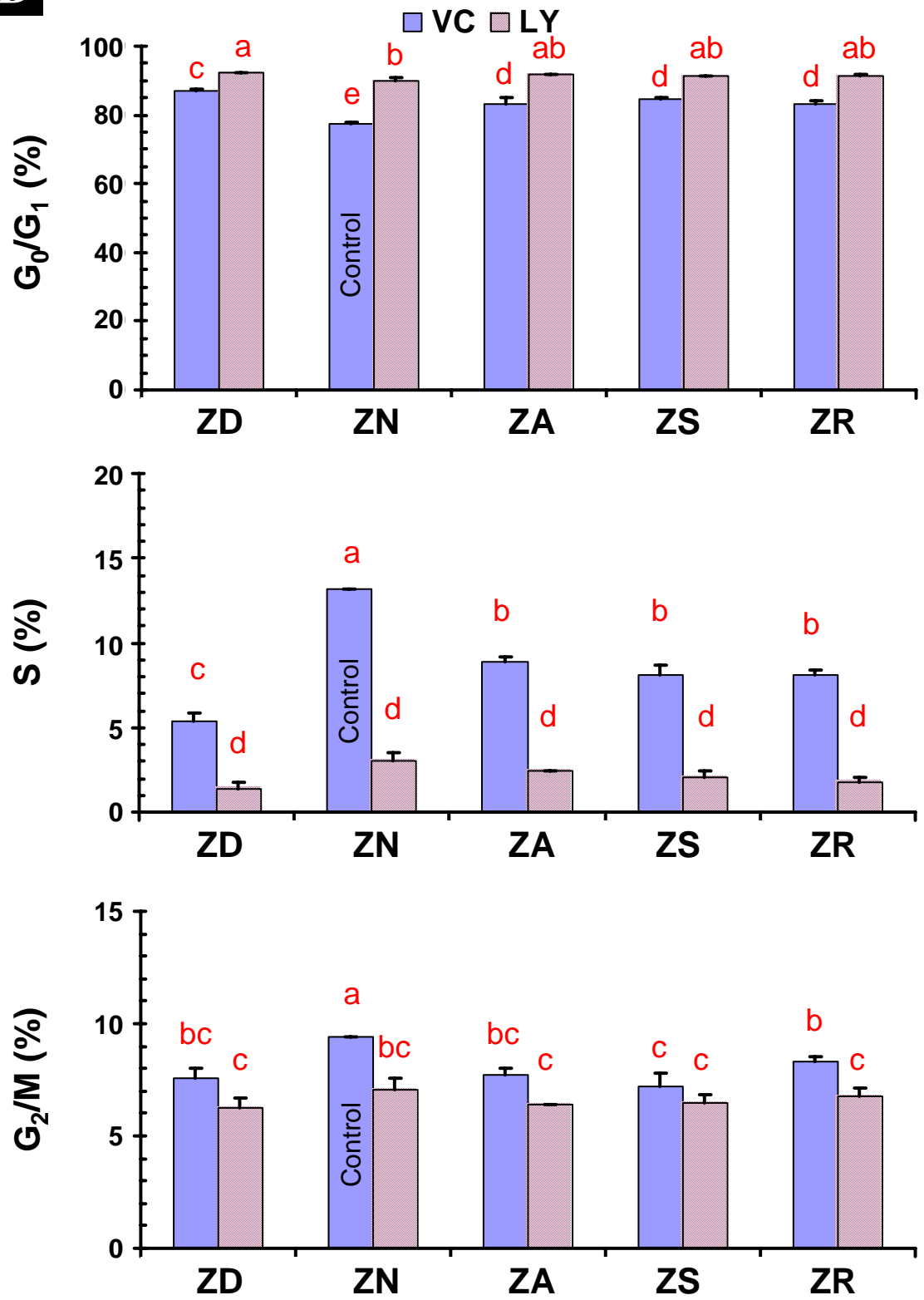


Figure 4.2. Effect of zinc on the cell cycle progression of LNCaP cells. Cells were cultured for 3 days in zinc-deficient (ZD; 0 $\mu\text{mol/L}$ zinc), zinc-normal (ZN; 4 $\mu\text{mol/L}$ zinc), zinc-adequate (ZA; 16 $\mu\text{mol/L}$ zinc), and zinc-supplemented (ZS; 32 $\mu\text{mol/L}$ zinc) RPMI medium containing 10% chelex-FBS. One subgroup of ZD cells was depleted with ZD medium for 2 days and then repleted with ZS medium for 1 day to provide the zinc-repleted (ZR) cells. LNCaP cells were then treated with vehicle control (■, VC) or LY294002 (▨, LY) for 16 hours before harvesting. **(A)** Histograms are representative of two independent experiments. The proportions of cells in G1 phase and the G1-to-S ratios (G1/S) are indicated for each zinc treatment. **(B)** Bar graph shows DNA content at each phase of the cell cycle: G0/G1 phase, S phase, and G2/M phase. Values are means \pm SEM from two separate experiments. Different letters indicate significant differences among groups ($p < 0.05$); treatments with the same letters indicate no significant difference.

Effect of zinc on Akt phosphorylation.

To explore the influence of *physiologic* levels of zinc on the phosphorylation of Akt, we used Western blot analysis with anti-phospho Akt (Ser473) antibody to detect phospho-specific protein expression level in zinc-treated LNCaP cells. Data was normalized to that of total-Akt to control equal loading of proteins. As shown in **Figure 4.3**, cytoplasmic protein levels of phosphorylated Akt (p-Akt) in LNCaP cells were 75%, 81%, 81%, and 115% higher in ZD, ZA, ZS, and ZR, respectively, than ZN groups. Since the phosphorylation of Akt is dependent on PI3K signaling (Datta et al. 1996), its inhibitor LY294002 was used to examine the role of PI3K in zinc-altered Akt phosphorylation. After treating cells with LY294002 for 16 hr, the elevated p-Akt levels observed in ZD, ZA, ZS, and ZR cells were normalized to similar level as in ZN cells (**Figure 4.3**). Yet, the p-Akt level of the LY294002-treated ZN cells was slightly lower than all other LY294002-treated groups. These results suggested that *physiologic* levels of zinc, either lower or higher than normal levels of zinc, would induce hyper-phosphorylation of Akt. Furthermore, these zinc-altered malignant prostate cells were responsive to LY294002 in inhibiting Akt phosphorylation. Since LY294002 antagonizes the PI3K/Akt pathway, these data suggested that zinc-altered Akt phosphorylation was due to PI3K signaling.

Figure 4.3

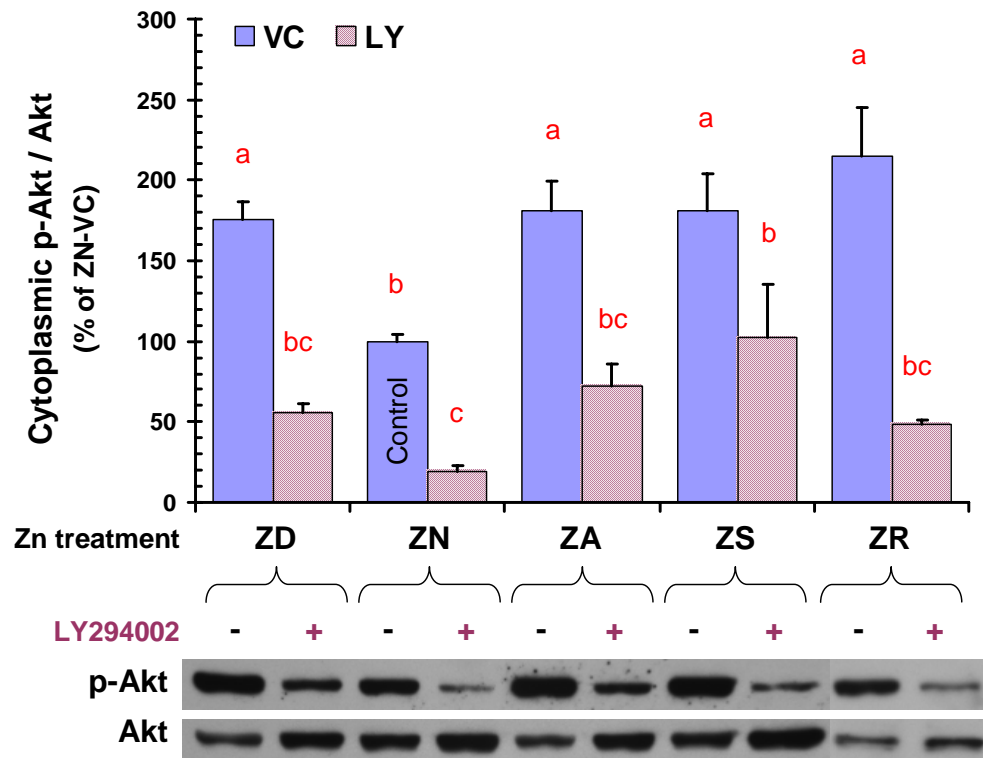


Figure 4.3. Effect of zinc on the phosphorylation of Akt protein. LNCaP cells were cultured in zinc-deficient (ZD; 0 $\mu\text{mol/L}$ zinc), zinc-normal (ZN; 4 $\mu\text{mol/L}$ zinc), zinc-adequate (ZA; 16 $\mu\text{mol/L}$ zinc), and zinc-supplemented (ZS; 32 $\mu\text{mol/L}$ zinc) media for 6 days. One subgroup of ZD cells was depleted with ZD medium for 5 days and then repleted with ZS medium for 1 day to provide the zinc-repleted (ZR) cells. LNCaP cells were then treated with vehicle control (□, VC) or LY294002 (▨, LY) for 16 hours before harvesting. Cytoplasmic extracts were subjected to SDS-PAGE and immunoblotting using anti-phospho-Akt (Ser473) antibody (upper). To control equal loading, the blot was reprobed with anti-Akt (lower). Protein bands were detected using enhanced chemiluminescence reagents and quantified using densitometry. Bar represents means \pm SEM from three separate experiments. Blot shown is a representative of three independent experiments. All three experiments showed similar results. Different letters indicate significant differences among groups ($p < 0.05$); treatments with the same letters indicate no significant difference.

Effect of zinc on p21 nuclear and cytoplasmic levels.

We examined whether zinc-altered Akt phosphorylation has subsequent effect on restricting p21 nuclear entry and lowering cytoplasmic p21 through degradation. Nuclear level of p21 protein in LNCaP cells was 58% lower in ZD than ZN group (**Figure 4.4A**). There was no difference among ZN, ZA, ZS, and ZR groups. Unexpectedly, nuclear p21 protein levels were reduced by LY294002 treatments across the zinc groups. Cytoplasmic p21 level was 45%, 61%, 45%, and 58% lower in ZD, ZA, ZS, and ZR than ZN groups (**Figure 4.4B**). Across the zinc groups, LY294002 treatment depressed cytoplasmic p21 level to similar extent. These observations indicated that zinc deficiency depressed p21 level in the nucleus, while the zinc repletion approach was able to normalize this level to that of ZN cells. Cytoplasmic p21 level was found to be able to respond as expected since p-Akt level was higher in ZD, ZA, ZS, and ZR than ZN groups. Thus, zinc deficiency-induced Akt phosphorylation might have an effect on limiting nuclear p21 and cytoplasmic p21 levels through phosphorylation and degradation of p21. While in zinc-adequate and zinc-supplemented states, only cytoplasmic p21 might be affected via Akt phosphorylation.

Figure 4.4

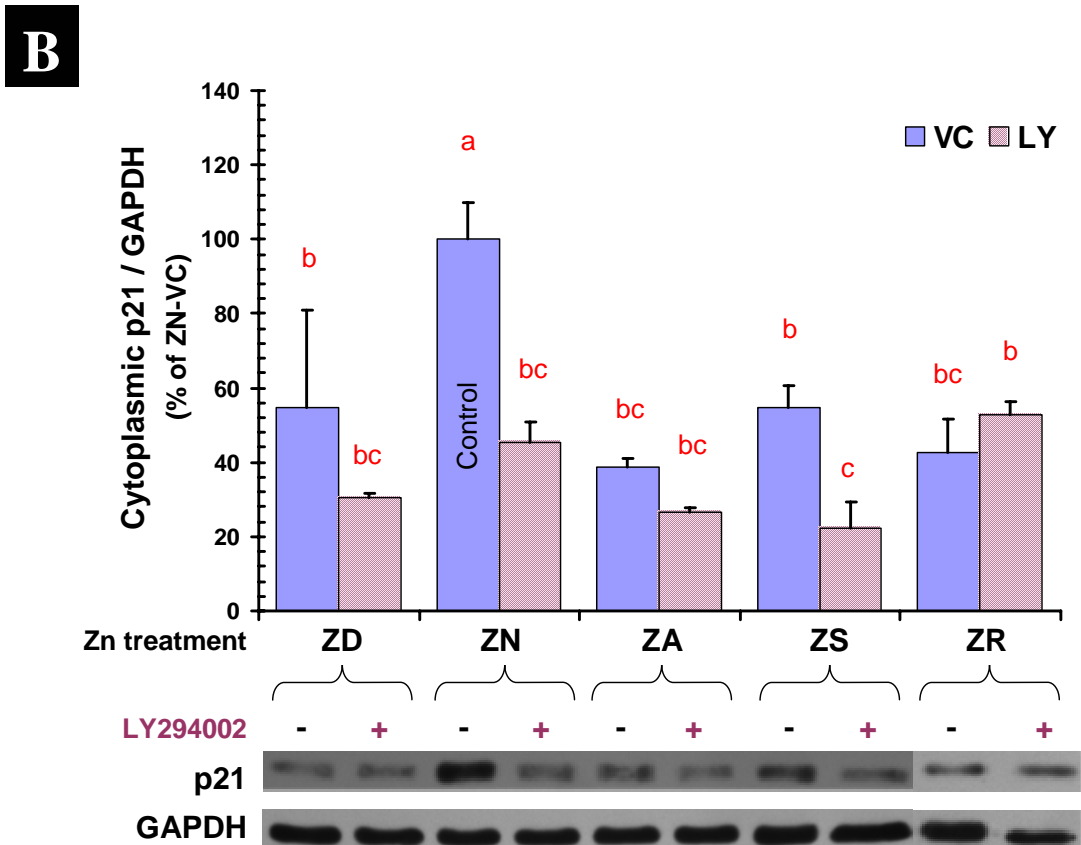
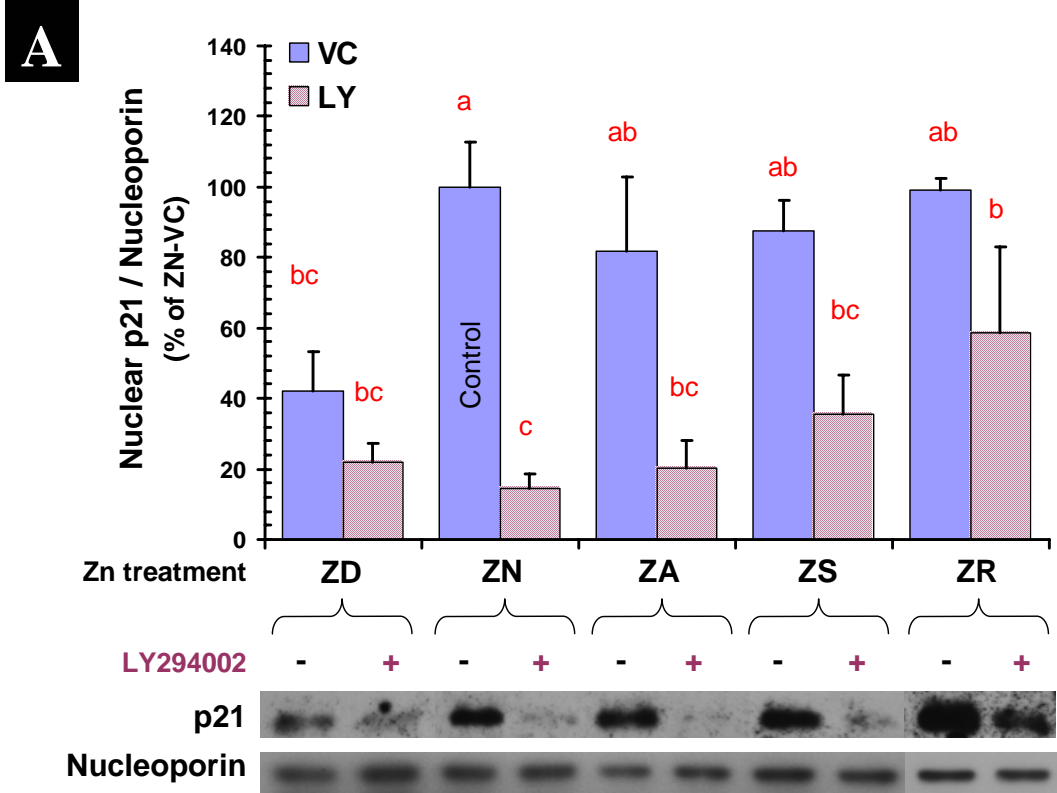


Figure 4.4. Effect of zinc on the (A) nuclear and (B) cytoplasmic levels of p21

protein. LNCaP cells were cultured in zinc-deficient (ZD; 0 $\mu\text{mol/L}$ zinc), zinc-normal (ZN; 4 $\mu\text{mol/L}$ zinc), zinc-adequate (ZA; 16 $\mu\text{mol/L}$ zinc), and zinc-supplemented (ZS; 32 $\mu\text{mol/L}$ zinc) media for 6 days. One subgroup of ZD cells was depleted with ZD medium for 5 days and then repleted with ZS medium for 1 day to provide the zinc-repleted (ZR) cells. Cells were treated with vehicle control (■, VC) or LY294002 (▨, LY) for 16 hours before harvesting. Nuclear and cytoplasmic extracts were subjected to SDS-PAGE and immunoblotting using anti-p21 (Ab-1) antibody. To control equal loading, blots of nuclear and cytoplasmic extract were reprobed with anti-nucleoporin (N-19) or anti-GAPDH (FL-335) antibody, respectively. Protein bands were detected using enhanced chemiluminescence reagents and quantified using densitometry. Bar represents means \pm SEM from two separate experiments. Blot shown is a representative of two independent experiments. Both experiments showed similar results. Different letters indicate significant differences among groups ($p < 0.05$); treatments with the same letters indicate no significant difference.

Effect of zinc on nuclear p53 accumulation.

Nuclear level of p53 in LNCaP cells was 41% and 43% lower in ZD and ZR than ZN groups (**Figure 4.5A**). However, this level was indifferent among ZN, ZA, and ZS groups. LY294002 treatment exerted little or no effect on the nuclear p53 levels as compared to untreated groups (VC), except there was a 30% reduction in ZA group. Cytoplasmic levels of p53 was unaffected by zinc and/or LY294002 treatments (**Figure 4.5B**).

Effect of zinc on Mdm2 phosphorylation.

We examined whether the zinc deficiency-suppressed nuclear p53 level was affected through an Akt-Mdm2 signaling axis. Using a phospho-specific Mdm2 antibody, Mdm2 phosphorylation at Ser166 was only detectable in the nuclear fraction. As shown in **Figure 4.6A**, nuclear levels of p-Mdm2 were indifferent among ZD, ZN, ZA, ZS, and ZR groups. Neither the PI3K inhibitor LY294002 treatment had any effect on this protein level. Cytoplasmic levels of Mdm2 were also unaffected by zinc and/or LY294002 treatments in LNCaP cells (**Figure 4.6B**). However, there was an elevation of cytoplasmic Mdm2 in ZS cells with or without the LY294002 treatment. These data indicated that zinc altered-Akt phosphorylation had no subsequent effect on nuclear Mdm2 phosphorylation or the cytoplasmic level. The second line of evidence of the independence of Mdm2 on Akt signaling was that cells were unresponsive to inhibitor of the PI3K/Akt pathway, LY294002.

Figure 4.5

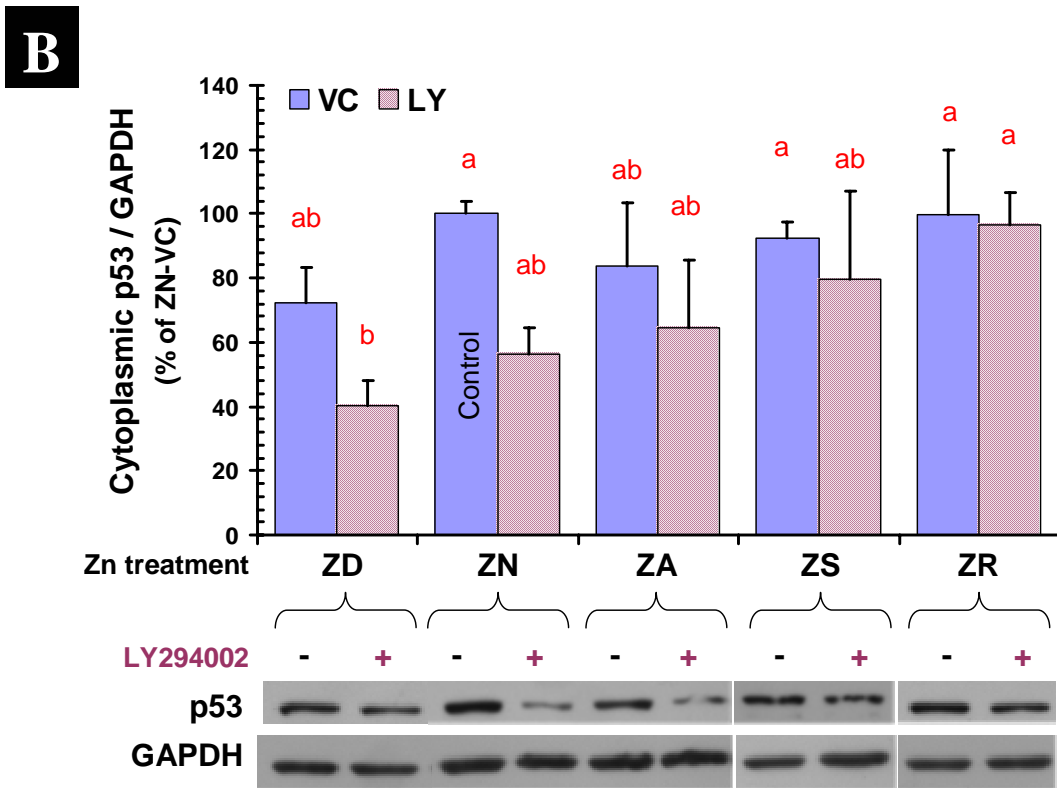
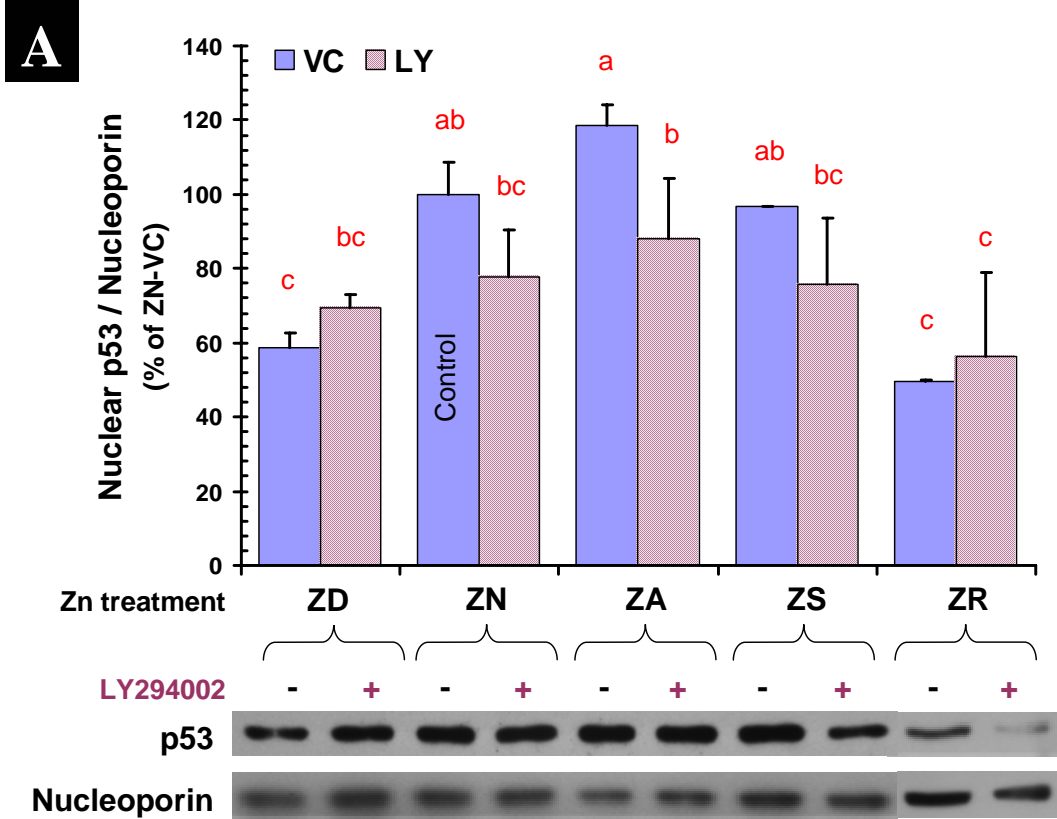


Figure 4.5. Effect of zinc on the (A) nuclear and (B) cytoplasmic levels of p53

protein. LNCaP cells were cultured in zinc-deficient (ZD; 0 $\mu\text{mol/L}$ zinc), zinc-normal (ZN; 4 $\mu\text{mol/L}$ zinc), zinc-adequate (ZA; 16 $\mu\text{mol/L}$ zinc), and zinc-supplemented (ZS; 32 $\mu\text{mol/L}$ zinc) media for 6 days. One subgroup of ZD cells was depleted with ZD medium for 5 days and then repleted with ZS medium for 1 day to provide the zinc-repleted (ZR) cells. Cells were treated with vehicle control (■, VC) or LY294002 (▨, LY) for 16 hours before harvesting. Nuclear and cytoplasmic extracts were subjected to SDS-PAGE and immunoblotting using anti-p53 (DO-1) antibody. To control equal loading, blots of nuclear and cytoplasmic extract were reprobed with anti-nucleoporin (N-19) or anti-GAPDH (FL-335) antibody, respectively. Protein bands were detected using enhanced chemiluminescence reagents and quantified using densitometry. Bar represents means \pm SEM from three separate experiments. Blot shown is a representative of three independent experiments. All three experiments showed similar results. Different letters indicate significant differences among groups ($p < 0.05$); treatments with the same letters indicate no significant difference.

Figure 4.6

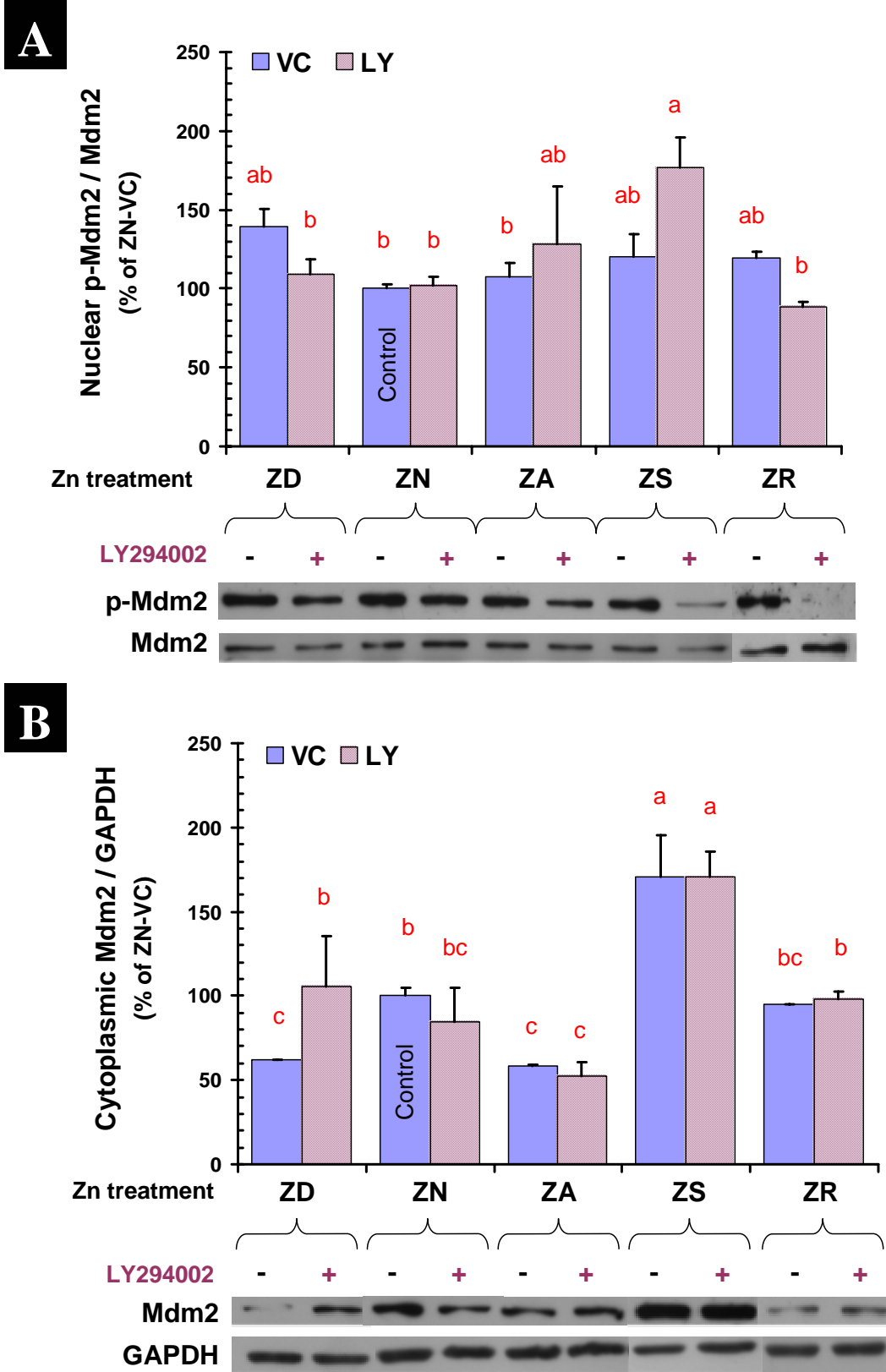


Figure 4.6. Effect of zinc on the (A) nuclear and (B) cytoplasmic levels of Mdm2

protein. LNCaP cells were cultured in zinc-deficient (ZD; 0 $\mu\text{mol/L}$ zinc), zinc-normal (ZN; 4 $\mu\text{mol/L}$ zinc), zinc-adequate (ZA; 16 $\mu\text{mol/L}$ zinc), and zinc-supplemented (ZS; 32 $\mu\text{mol/L}$ zinc) media for 6 days. One subgroup of ZD cells was depleted with ZD medium for 5 days and then repleted with ZS medium for 1 day to provide the zinc-repleted (ZR) cells. Cells were treated with vehicle control (■, VC) or LY294002 (▨, LY) for 16 hours before harvesting. Nuclear extracts were subjected to SDS-PAGE and immunoblotting using anti-phospho-Mdm2 (Ser166) antibody and reprobed with anti-Mdm2 (C-18) antibody for controlling equal loading. Similarly, cytoplasmic protein extracts were probed with anti-Mdm2 (C-18) antibody and reprobed with anti-GAPDH (FL-335) antibody. Protein bands were detected using enhanced chemiluminescence reagents and quantified using densitometry. Data represent an average from two different donors of LNCaP. Bar represents means \pm SEM from two separate experiments. Blot shown is a representative of two independent experiments. Both experiments showed similar results. Different letters indicate significant differences among groups ($p < 0.05$); treatments with the same letters indicate no significant difference.

DISCUSSION

Previous studies have shown that higher than 50 $\mu\text{mol/L}$ of zinc could induce Akt phosphorylation in fibroblasts and adipocytes (Tang and Shay 2001), lung epithelial cells (Kim et al. 2000, Wu et al. 2003, 2005, Bao and Knoell 2006), and breast cancer epithelial cells (Ostrakhovitch and Cherian 2004), while zinc deficiency lead to hypophosphorylation of Akt in fibroblasts (Clegg et al. 2005). However, this zinc effect on Akt phosphorylation has never been examined in prostate cells. Approximately 10% of Americans do not consume the estimated average requirement for zinc and could be at risk for zinc deficiency (Wakimoto and Block 2001). In contrast, epidemiological evidence showed that men on high doses of zinc supplements (>100 mg/d) had increased risk for prostate cancer (Leitzmann et al. 2003). In view of the prevalence of dietary zinc deficiency and supplementation and the clinical significance of low zinc status in prostate cancer, we designed the current study to examine the influence of *physiologic* level of zinc on cell cycle progression via PI3K/Akt signaling pathway in human malignant prostate LNCaP cells.

Laboratory studies have found an inhibitory effect of zinc on prostate cancer cell growth via cell cycle arrest at 15 $\mu\text{mol/L}$ in LNCaP and PC-3 cells (Liang et al. 1999), necrosis at 500 $\mu\text{mol/L}$ in LNCaP and at 200 $\mu\text{mol/L}$ in PC-3 cells (Iguchi et al. 1998), or NF- κ B inactivation at 7.5 $\mu\text{mol/L}$ in DU-145 and PC-3 cells (Uzzo et al. 2002, 2006). However, it is important to note that zinc concentration higher than 32 $\mu\text{mol/L}$ is *non-physiological* in which cells would never be exposed *in situ*. The normal circulating levels of zinc as in plasma is ~ 15 $\mu\text{mol/L}$, of which about 66% is mobile, transportable zinc available for uptake by cells (Costello and Franklin 1998). Since 16 $\mu\text{mol/L}$ is the

normal zinc levels found in the human plasma and 32 $\mu\text{mol/L}$ of zinc is the highest attainable level by oral supplementation in humans, we used them as zinc-adequate and zinc-supplemented groups, respectively, in our study. Therefore, the zinc concentrations chosen in our study are *physiologically* relevant.

LNCaP cells were selected as our model system because: a) they represent early progression of prostate cancer tissue which are still androgen-responsive; b) they contain wild-type p53 unlike other prostate cancer cell lines; c) their response to nutrients in Akt phosphorylation has been well-documented (Wu et al. 2006, Hu et al. 2005, Ni et al. 2005, O'Kelly et al. 2006); and d) they have a higher basal endogenous zinc level and accumulate higher intracellular zinc levels than PC-3 cells do (Costello et al. 1999). After treating LNCaP cells with various concentrations of zinc for 5 days, their zinc status was receptive to zinc concentration in the medium (**Figure 4.1B**). But the zinc status reached a plateau at higher than 4 $\mu\text{mol/L}$ zinc. The zinc uptake of LNCaP cells is obviously less responsive than normal prostate cells because a progressive increase in zinc uptake by normal prostate cells is evident even at the highest concentration tested (32 $\mu\text{mol/L}$) (Han et al. 2007). These findings are similar to previous observations that prostate cancer cells, unlike normal prostate cells, lack the ability to accumulate zinc (Hasumi et al. 2003). Down-regulation of zinc uptake transporter ZIP during cancer initiation and/or progression was proposed as the responsible mechanism (Franklin et al. 2003). On the other hand, zinc-deficient treatment was able to deplete zinc status of LNCaP cells to 25% of ZN controls (**Figure 4.1B**). And our zinc repletion approach was sufficient to normalize zinc deficiency in LNCaP cells.

The focus of previous studies in low zinc condition was rather acute, which lasted from 30 minutes to 72 hours (Liang et al. 1999, Iguchi et al. 1998, Uzzo et al. 2002, 2006). Even though these studies did include a group of cells treated with 0-1.5 $\mu\text{mol/L}$ zinc, none have shown a successful depletion of cellular zinc status. In the study of Liang et al. (1999), the zinc status of cells treated with 1.5 $\mu\text{mol/L}$ zinc was even higher than that of cells growing at normal condition. This suggested that their 48 hours of depletion process versus 5 days in our study might not be long enough to induce zinc deficiency. Furthermore, a substantial amount of zinc coming from fetal bovine serum (FBS) was not being controlled for (Iguchi et al 1998, Uzzo et al. 2002, 2006). Our chelating strategy was able to deplete the zinc from FBS containing medium to $<0.1 \mu\text{mol/L}$, without eliminating other growth components in FBS. Moreover, Liang et al. (1999) switched the culture condition from medium containing 10% FBS to serum-free medium 24 hours prior to various zinc treatments. According to standard LNCaP cell culture protocol, the number of cells would slowly decline at a FBS concentration of 0.01% (Horoszewicz et al. 1983). Consequently, whether the serum deprivation approach adapted by Liang et al. (1999) would induce the growth inhibitory effect of zinc warrants further investigation.

Studies have established an association of low intracellular zinc concentrations with the induction of apoptosis. Both p53 and NF- κ B are known pathways that negatively and positively, respectively, affecting cell proliferation in various cell types. Observations from our laboratory have shown that zinc deficiency amplifies nuclear p53 accumulation in human hepatoblastoma HepG2 cells (Alshatwi et al. 2006, Reaves et al. 2000), as well as in normal human bronchial epithelial cells (Fanzo et al. 2001) and human aortic endothelial cells (Fanzo et al. 2002) in the absence of apoptosis. Others

have found that zinc deficiency inhibits NF- κ B promoter activities in human LNCaP, PC-3 and DU-145 prostate cancer cells (Uzzo et al. 2002, Iguchi et al. 1998), human lung fibroblasts (Ho et al. 2003), rat glioma cells (Ho and Ames 2002), mouse 3T3 fibroblasts (Clegg et al. 2005, Chou et al. 2004), and human neuroblastoma IMR-32 cells (Mackenzie et al. 2002) in the presence of apoptosis. Despite these contradictory findings on zinc deficiency-induced apoptosis, our zinc-deficient LNCaP cells did not exhibit accumulation at sub-G0 phase of the cell cycle (**Figure 4.2A**). Thus, LNCaP cells did not undergo apoptosis in zinc deficiency. As compared to zinc-normal cells, higher percentage of zinc-deficient, zinc-adequate, zinc-supplemented, and zinc-repleted cells was retained at G0/G1 phase, with proportionally less at S phase (**Figure 4.2B**). This suggested that more non-zinc-normal cells were undergoing proliferation, which might be a result of Akt hyper-phosphorylation. Surprisingly, we found that Akt phosphorylation was 75%, 81%, 81%, and 115% higher in zinc-deficient, zinc-adequate, zinc-supplemented, and zinc-repleted cells, respectively, than zinc-normal controls (**Figure 4.3**). Cell cycle data validated our assumption that once Akt is phosphorylated, it actively promotes cell survival and inhibits apoptosis. Akt has been shown to exert its anti-apoptotic function by 1) preventing the release of cytochrome c from mitochondria; 2) preventing BAD from interacting with Bcl-2 protein; 3) preventing the conversion of procaspase-9 to its active, pro-apoptotic caspase; and 4) stimulating NF- κ B-mediated transcription of genes that can suppress apoptosis (Kennedy et al. 1999, Khwaja 1999, Cardone et al. 1998, Burow et al. 2000).

More recently, another anti-apoptotic role of Akt has been described in p21 phosphorylation. p21 is a cyclin-dependent kinases (CDK) inhibitor that can arrest the

cell cycle in response to DNA damage, generally in a p53-dependent manner. The anti-proliferative effects of p21 are assisted by its ability to bind to proliferating cell nuclear antigen (PCNA) and block DNA synthesis required for S phase of the cell cycle.

However, in opposition to its anti-proliferative functions, p21 can be pro-proliferative when localized in the cytoplasm. When p21 is phosphorylated by Akt at Thr145, p-p21 is retained in the cytosol and loses the ability to interact with PCNA. This is because the phosphorylation of p21 at Thr145 results in two modifications of p21. Firstly, the Thr145 site lies adjacent to the nuclear localization signal (NLS) domain, which prevents p21 from interacting with importins and hence blocking nuclear translocation (Harreman et al. 2004). Secondly, Thr145 residue lies within the consensus binding sequence for PCNA. Thus, Thr145 phosphorylation of p21 by Akt can disrupt the hydrogen bonding between p21 and PCNA, thereby destabilizing the complex (Warbrick 2000). Further consequence of cytoplasmically retained p21 is to facilitate the assembly and activity of cyclin D-cdk4 complex, which promotes cell cycle progression through G1 phase into S phase (Boonstra 2003). We observed a high Akt phosphorylation, low nuclear p21, and a high ratio of G1/S in zinc-deficient LNCaP cells (**Figures 4.3, 4.4A, and 4.2A**). These results led us to postulate that p21 might be phosphorylated by Akt in zinc deficiency, so less cytoplasmic p21 was translocated into the nucleus. This, in turn, maintained the cell cycle progression from G1 to S phase. Future study should be designed to determine whether zinc deficiency-inhibited nuclear p21 accumulation has any effect on the expression of cyclins and the activity of cyclinD/CDK4 complex in LNCaP cells.

When comparing the magnitude of Akt hyper-phosphorylation in zinc-deficient malignant prostate LNCaP cells (**Figure 4.3**) with normal prostate cells (Han et al. 2007),

it was 75% vs. 42% greater than their respective zinc-normal controls, respectively. This might be due to the fact that normal prostate retain wild-type PTEN that can negatively regulate the phosphorylation of Akt to keep it at basal level. However, PTEN gene in malignant prostate cells, like LNCaP in this case, is deleted and mutated, thus incapable of inhibiting Akt phosphorylation. Therefore, the present data indicated that malignant prostate cells were susceptible to Akt phosphorylation in either zinc-deficient, -adequate, -supplemented, or -repleted state probably due to a non-functional PTEN. The use of a specific inhibitor of PI3K/Akt pathway, LY294002, normalized the Akt hyper-phosphorylation in zinc-deficient, -adequate, -supplemented, and -repleted cells to that of zinc-normal cells (**Figure 4.3**). These data confirmed that the observed Akt hyper-phosphorylation was indeed induced via PI3K signaling.

Indifferent from previous findings on zinc deficiency-induced p53 nuclear accumulation in HepG2 cells (Alshatwi et al. 2006), normal bronchial epithelial cells (Fanzo et al. 2001), and normal primary aortic endothelial cells (Fanzo et al. 2002), we did not observe that in LNCaP cells. Unexpectedly, nuclear p53 level was 41% and 51% lower in zinc-deficient and -repleted LNCaP cells, respectively (**Figure 4.5A**). However, markedly suppressed level of nuclear p21 in zinc-deficient LNCaP cells (**Figure 4.4A**) is consistent with our previous observation in HepG2 cells (Alshatwi et al. 2006). We hypothesized that reduced nuclear p21 level by zinc deficiency in LNCaP cells might be partly due to suppressed p53 transactivational activity. This assumption may be confirmed by conducting a luciferase promoter assay.

In an attempt to unravel the rationale behind zinc deficiency-inhibited nuclear p53 level, we examined the Akt-Mdm2-p53 signaling axis by measuring the extent of Mdm2

phosphorylation in the nucleus. According to current literature, phosphorylated Akt can phosphorylate Mdm2 and promote its translocation from the cytoplasm to the nucleus (Mayo and Donner 2001). Consequently, the phosphorylated Mdm2 (p-Mdm2) can form a complex with p53 and p300 to poly-ubiquitinate p53 (Mayo and Donner 2001). Poly-ubiquitinated form of p53 is then shuffled to the proteasome for degradation (Grossman et al. 2003). As Akt hyper-phosphorylation was apparent in zinc deficiency (**Figure 4.3**), we expected more p-Mdm2 to be detected from zinc-deficient than zinc-normal cells. However, nuclear p-Mdm2 level was indifferent between these groups (**Figure 4.6A**). As a result, Akt did not exert any effect on inhibiting nuclear p53 protein level through Mdm2 in zinc deficiency. Thus, Akt might signal through other downstream effectors, i.e. mTOR and p70 S6 kinase, in zinc-deficient LNCaP cells.

The effect of zinc supplementation on p21 expression and cell cycle progression was previously examined in malignant prostate cells by Liang et al. (1999). After 24 hours of incubation with 15 $\mu\text{mol/L}$ zinc, cell cycle arrest at G2/M phase was accompanied by increased mRNA levels of p21 in LNCaP (p53^{+/+}) and PC3 (p53^{-/-}) cells, which indicated that the impaired cell cycle progression might be independent of p53. Even though the protein level of p21 was not reported in their study, we did not observe increased p21 protein level in either nuclear or cytoplasmic fraction nor G2/M arrest after treating LNCaP cells with zinc-adequate (16 $\mu\text{mol/L}$) and zinc-supplemented (32 $\mu\text{mol/L}$) media for 6 days (**Figure 4.4**). Their study was done in serum-starved cells prior to and during zinc treatment. This discrepancy in culture condition and zinc treatment might have caused the G2/M arrest in their study.

Cell cycle arrest at G1 phase induced by PI3K inhibitor LY294002 was previously reported in LNCaP cells (Yang et al. 2005). Treatment with LY294002 induced a markedly higher percentage of LNCaP cells being retained at G0/G1 phase (**Figure 4.2B**). A large blockage of G1 to S progression was evident as G1/S ratio was 8-, 4-, 5-, 6-, and 7-fold higher in LY294002-treated zinc-deficient, zinc-normal, zinc-adequate, zinc-supplemented, and zinc-repleted cells, respectively, than their respective untreated counterparts (**Figure 4.2A**). This observation underlines the possibility that zinc deficiency may predispose the LNCaP cells to become more sensitive to LY294002.

Together, the results of this study suggested that because LNCaP cells have an inactive PTEN, Akt phosphorylation occurs in either low or high zinc status. In zinc deficiency, suppressed nuclear p53 accumulation and unaffected nuclear Mdm2 phosphorylation indicated a non Akt-Mdm2-p53 signaling axis in LNCaP cells. Yet, suppressed nuclear and cytoplasmic p21 levels in zinc-deficient LNCaP cells might be a result of p21 phosphorylation by Akt, which inhibited p21 nuclear entry and manifested cytoplasmic p21 degradation. As a result, more zinc-deficient cells survived and progressed through G0/G1 phase of the cell cycle. In summary, these results suggested that zinc deficiency-induced cell cycle progression might be maintained through Akt-p21 signaling axis in LNCaP cells.

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CHAPTER 5.

GENERAL DISCUSSION

This dissertation was designed to examine the influence of physiologic levels of zinc on cell cycle progression via the PI3K/Akt signaling pathway in human normal prostate epithelial cells (PrEC) and human malignant prostate LNCaP cells. These cells were selected because of their different responsiveness to zinc uptake and ability to express wild-type phosphatase and tensin homologue (PTEN) gene. Findings from this dissertation suggested that the zinc deficiency resulted in PTEN phosphorylation, which was unable to suppress Akt phosphorylation in PrEC. This zinc deficiency-induced Akt phosphorylation led to Mdm2 phosphorylation and less nuclear accumulation of p53, thus exerting less of the tumor-suppressive effect of p53 and promoting cell survival. For that reason, the progression of cell cycle was signaling through an Akt-Mdm2-p53 axis in PrEC. On the other hand, LNCaP cells have an inactive PTEN, therefore Akt phosphorylation occurs in either low or high zinc status. Suppressed nuclear p53 accumulation in zinc-deficient LNCaP cells might indicate an Akt-independent mechanism. Yet, suppressed nuclear p21 levels in zinc-deficient LNCaP cells might be a result of p21 phosphorylation by Akt, which inhibited p21 nuclear translocation and promoted cell cycle progression. In summary, these results suggested that zinc deficiency-induced cell cycle progression might be maintained through Akt-Mdm2-p53 signaling axis in PrEC while Akt-p21 signaling axis in LNCaP cells. Although only

subtle differences were observed among the treatment groups, the future studies listed below are proposed in order to strengthen the findings of the current study.

Effect of zinc on cellular zinc status

After treating cells with various concentrations of zinc for 6 days, the zinc uptake of PrEC is more responsive than LNCaP cells, even at the highest concentration studied (32 $\mu\text{mol/L}$) (**Figures 3.1B and 4.1B**). The zinc status of LNCaP cells tended to reach a plateau beyond the zinc concentration that is normally found in standard culture media (4 $\mu\text{mol/L}$). These data are coherent to previous observations that prostate cancer cells, unlike normal prostate cells, lack the ability to accumulate zinc (Hasumi et al. 2003). Down-regulation of zinc uptake transporter ZIP during cancer initiation and/or progression was proposed as the responsible mechanism (Franklin et al. 2003). In contrast, zinc-deficient treatment was able to deplete zinc status of PrEC and LNCaP cells to 59% and 75%, respectively, of their respective ZN controls. Thus, zinc depletion of PrEC is also more readily achieved than LNCaP cells. Overall, the responsiveness of PrEC, to zinc concentration of the media, in zinc uptake is greater than LNCaP cells.

Previous studies have shown that *supra-physiologic* levels of zinc at 50 $\mu\text{mol/L}$ or higher could induce Akt phosphorylation (Tang and Shay 2001, Kim et al. 2000, Wu et al. 2003, 2005, Bao and Knoell 2006), while zinc deficiency lead to hypo-phosphorylation of Akt (Clegg et al. 2005). To our knowledge, this dissertation is the first to use prostate cells to examine the physiologic effect of zinc on Akt phosphorylation. We used <1 $\mu\text{mol/L}$ zinc to represent zinc deficiency. Zinc concentration of 4 $\mu\text{mol/L}$ is usually found in standard culture media, thus was used to represent normal level and

internal control. Since 16 $\mu\text{mol/L}$ is the normal zinc levels found in the human plasma and 32 $\mu\text{mol/L}$ of zinc is the highest attainable level by oral supplementation in humans, we used them as zinc-adequate and zinc-supplemented groups, respectively, in our study. A randomized, double-blind, and placebo controlled primary prevention trial in reducing the frequency of cancers and heart diseases revealed that a daily supplementation of 20 mg zinc for 2 years would result 16.2 \pm 3.9 $\mu\text{mol/L}$ zinc in the plasma (Malvy et al. 2001). Thus, the zinc concentration used in this dissertation would approximately translate into <1.25, 5, 20, and 40 mg/d for zinc-deficient, -normal, -adequate, and –supplemented treatments, respectively.

The current Recommended Dietary Allowance (RDA) for zinc for adult male is 11 mg/d (Institute of Medicine 2001). And the established tolerable upper levels (UL), which is the highest intake associated with no adverse health effects, for zinc is 40 mg/d (Institute of Medicine 2001). To put these values into context, our chosen ranges of dose met the current recommendation but without reaching the limit for toxicity. In order to understand the dose-dependent relationship between dietary zinc intake and prostatic zinc level, future study should focus on the use of 3-day food records and primary sample taken from subjects randomly assigned to various levels of zinc supplement.

Effect of zinc on p53 and p21 nuclear accumulation and cell cycle progression

Studies have established an association of low intracellular zinc concentrations with the induction of apoptosis. Both p53 and NF- κ B are known pathways that positively and negatively, respectively, affecting apoptosis in various cell types. Others have found that zinc deficiency inhibits NF- κ B promoter activities in human LNCaP, PC-3 and DU-145

prostate cancer cells (Uzzo et al. 2002, Iguchi et al. 1998), human lung fibroblasts (Ho et al. 2003), rat glioma cells (Ho and Ames 2002), mouse 3T3 fibroblasts (Clegg et al. 2005, Chou et al. 2004), and human neuroblastoma IMR-32 cells (Mackenzie et al. 2002) in the presence of apoptosis. However, observations from our laboratory have shown that zinc deficiency amplifies nuclear p53 accumulation in human hepatoblastoma HepG2 cells (Alshatwi et al. 2006, Reaves et al. 2000), as well as in normal human bronchial epithelial cells (Fanzo et al. 2001) and human aortic endothelial cells (Fanzo et al. 2002) in the absence of apoptosis. Despite these contradictory findings on zinc deficiency-induced apoptosis, neither of our zinc-deficient PrEC or LNCaP cells underwent apoptosis. Both zinc-deficient cells did not exhibit accumulation at sub-G0 phase of the cell cycle (**Figures 3.2A and 4.2A**). As suggested by our findings of zinc deficiency-induced phosphorylation of Akt in both PrEC and LNCaP cells, the absence of apoptosis might be a result of phosphorylated Akt (p-Akt), which is known for its pro-survival activity.

In contrast to our laboratory's previous findings on zinc deficiency-induced p53 nuclear accumulation in HepG2 cells (Alshatwi et al. 2006, Reaves et al. 2000), normal human bronchial epithelial cells (Fanzo et al. 2001), and normal human aortic endothelial cells (Fanzo et al. 2002), we did not observe that in both prostate cells. Surprisingly, nuclear p53 level was 32% and 41% lower in zinc-deficient PrEC and LNCaP cells, respectively (**Figures 3.5A and 4.5A**). In response to various types of cellular stress, p53 is extensively post-translationally modified. Namely, the phosphorylation and ubiquitination of p53 have been implicated in the regulation of p53 protein levels as well as its DNA binding and transcriptional activities. p21, a cyclin-dependent kinases (CDK)

inhibitor, can be transcriptionally activated via either a p53-dependent or -independent mechanism. Suppressed nuclear p21 protein level, mRNA abundance, and promoter activity were previously reported in zinc-deficient HepG2 cells (Wong et al. 2007). Despite p53 nuclear accumulation was observed in the same treatment condition and cell type (Alshatwi et al. 2006), p21 expression might be suppressed via p53-independent mechanism (Wong et al. 2007). We hypothesized that the reduced nuclear p21 level by zinc deficiency in LNCaP cells (**Figure 4.4A**) might be due to suppressed p53 transactivational activity. To test this assumption, future experimental design involving the usage of p53-expressing (LNCaP, p53^{+/+}) and p53-null (PC-3, p53^{-/-}) cells will permit the determination of whether nuclear p21 is suppressed via p53-dependent or -independent mechanism in zinc deficiency.

Moreover, we hypothesized that higher nuclear p21 protein level observed in zinc-supplemented PrEC and lower in zinc-deficient LNCaP cells might be directly influenced by an enhanced or depressed transcriptional activity of p21 gene, respectively. Under the conjecture that nuclear p21 protein levels measured in these prostate cells are simply affected by the transcriptional process, our assumption may be confirmed by conducting a luciferase promoter assay. To be specific, this assay will involve the transient transfection of a p21-promoter-luciferase gene into PrEC and LNCaP cells. This approach will provide data to support the hypothesis that the translational and transcriptional processes are depressed by a reduction in p21 gene promoter activity in zinc-deficient LNCaP cells. Furthermore, p21 promoter activity should be measured in zinc-supplemented PrEC, in which higher level of nuclear p21 was found, to provide

additional lines of evidence for up-regulated transcription and possibly translational processes by zinc supplementation.

Another possibility that might have an effect on nuclear p21 protein levels is its subcellular localization and stability. The subcellular localization of p21 has been proposed to be critical for the regulation of p21's anti-proliferative and pro-proliferative functions (Vivanco and Sawyers 2002). In the nucleus, p21 can interact with proliferating cell nuclear antigen (PCNA) and block DNA synthesis required for S phase of the cell cycle. As opposed to its anti-proliferative functions, p21 can be pro-proliferative when localized in the cytoplasm. The Thr145 residue in the carboxyl terminus of p21 can be phosphorylated by Akt *in vitro* and *in vivo*. The phosphorylation of p21 on Thr145 prevents p21's nuclear entry and inhibits the interaction of p21 with PCNA (Li et al. 2002). This phosphorylated p21 (p-p21) is therefore cytoplasmically retained. Thus, the growth-inhibiting activity of p21 is inhibited through facilitating the assembly and activity of cyclin D-CDK 4 complex, limiting the binding of the CDK2 and CDK4 to p21, and attenuating the CDK2 inhibitory activity of p21 (Boonstra 2003). Consequently, cell cycle progression is mainly dependent on the relative levels of cytoplasmic and nuclear p21 protein.

In LNCaP cells, nuclear and cytoplasmic levels of p21 were 52% and 45% lower in zinc-deficient than zinc-normal group, respectively (**Figures 4.4A and 4.4B**). These results led us to postulate that p21 might be phosphorylated by p-Akt at Thr145 in zinc deficiency, so this phosphorylation of Thr145 prevents the translocation of p21 into the nucleus. When these p21 data are coupled with findings from flow cytometric analysis,

the zinc-deficient cells appeared to be able to progress from G1 to S phase of the cell cycle (**Figures 4.2A and 4.2B**).

This dissertation did not examine the extent of Thr145 phosphorylation of p21 is because whether this phosphorylation can alter the stability of p21 remains equivocal (Child and Mann 2006). Previous study has shown that p-Akt staining is correlated with cytoplasmic localization of p21 in breast tumors (Zhou et al. 2001). Perhaps an *in situ* immunofluorescent staining study would help determining the cytoplasmic and nuclear localization of p21. If transfecting zinc-deficient LNCaP cells with dominant-negative Akt mutant or with small interference RNA (siRNA, as a knock-down strategy) can restore the nuclear p21 localization and growth-inhibiting activity of p21, we then can conclude that p21 localization is dependent via Akt in zinc deficiency. Moreover, future studies should be designed to determine whether zinc deficiency-inhibited nuclear p21 accumulation has any effect on the expression of cyclins and the activity of cyclinD/CDK4 complex in LNCaP cells.

Recent studies have indicated that the nuclear import/export process of p21 is modulated oppositely by different isoforms of Akt: Akt1 and Akt2 (Héron-Milhavet et al. 2006). In cells Akt1 was silenced by siRNA, p21 localized in the nucleus and S-phase entry was inhibited. However, in cells over-expressing Akt2, increased nuclear localization of p21 with inhibited M-G1 progression was shown. In brief, p21 phosphorylation by Akt1 is required for cell proliferation, while Akt2 binding to p21 promotes cell cycle exit. To add another layer of complexity – the T145 phosphorylation of p21 by Akt1 prevents Akt2 binding to p21, while the binding of Akt2 prevents T145 phosphorylation of p21 by Akt1. On the other hand, p-Akt1 has been frequently detected

in prostate tumors (Ayala et al. 2004). Although we did not measure the specific Akt isoform in this dissertation, we hypothesized that our zinc deficiency-induced Akt phosphorylation and -reduced nuclear p21 protein level was a result of Akt1 phosphorylation.

Zinc-deficient treatment had no effect on nuclear p21 protein accumulation in PrEC (**Figure 3.7A**). Similarly, our laboratory previously found that p21 mRNA level remained unaffected by zinc deficiency in normal primary aortic endothelial cells (Fanzo et al. 2002). This might be due to the zinc status of zinc deficient-PrEC being not sufficiently depleted in these originally zinc-accumulating cells, to exert a suppressive effect on nuclear p21 protein level. As a result, zinc-deficient PrEC were able to progress through all three phases of the cell cycle. Treating PrEC with pharmacological inhibitor of PI3K/Akt pathway, LY294002, was able to increase nuclear p21 levels, but not in LNCaP cells (**Figures 3.7A and 4.4A**). Moreover, a strong blockage of G1 to S progression (as expressed by G1/S ratio) was only seen in LY294002 treated-zinc-deficient LNCaP cells (**Figure 4.2A**). These findings suggested the likelihood that LNCaP, as a malignant cell type, has become more susceptible towards LY294002 than normal cells.

Effect of zinc on Akt phosphorylation

We found that Akt phosphorylation was enhanced by zinc deficiency in both PrEC and LNCaP cells than their respective zinc-normal cells (**Figures 3.3A and 4.3**). When comparing the magnitude of Akt hyper-phosphorylation in zinc-deficient PrEC with LNCaP cells, it was 42% vs. 75% greater than their respective zinc-normal group. This

might be due to the fact that normal prostate cells, as PrEC in this case, retain wild-type PTEN that can negatively regulate the phosphorylation of Akt. However, the PTEN gene in malignant prostate cells like LNCaP is deleted and mutated, thus incapable of inhibiting Akt phosphorylation. Therefore, the present data indicated that normal prostate cells are only susceptible to Akt phosphorylation in zinc-deficient state. Probably due to non-functional PTEN, malignant prostate cells were susceptible to Akt phosphorylation in either zinc-deficient, -adequate, or -supplemented state. Apart from the usage of LY294002 in this dissertation, inhibition of PI3K/Akt signaling may also be achieved in future studies by ectopic expression of PTEN in LNCaP cells.

Effect of zinc on Akt-Mdm2-p53 signaling axis

In accordance with zinc deficiency-induced Akt phosphorylation, Mdm2 phosphorylation was higher and p53 accumulation was lower in the zinc-deficient PrEC's nucleus than all other treatment groups (**Figures 3.3A, 3.4A, and 3.5B**). Treatment with inhibitor LY294002 was able to reverse zinc deficiency-induced Mdm2 phosphorylation. This agreed with the notion that p-Akt could subsequently phosphorylate Mdm2 and promote its nuclear translocation (Mayo and Donner 2001). Consequently, nuclear Mdm2 could complex with p53 and p300 to form poly-ubiquitinated p53, which was then shuffled to the proteasome for degradation (Mayo and Donner 2001, Grossman et al. 2003). Thus, cytoplasmic level of p53 was also lower in zinc-deficient PrEC than all other treatment groups. Despite our previous data showing that nuclear p300 level was markedly reduced by zinc deficiency in HepG2 cells (Alshatwi et al. 2006); it was unchanged in zinc-deficient PrEC (**Figure 3.6**). This suggested that the p300 was available in the nucleus to

form a complex with p53 and Mdm2 for manifesting p53 ubiquitination. As a result, Akt may exert an indirect effect on inhibiting apoptotic function of p53 tumor suppressor protein through Mdm2 in zinc deficiency. In contrast, the effects of Akt hyperphosphorylation on Mdm2 and p53 were not seen in LNCaP cells (**Figures 4.5 and 4.6A**). Thus, future studies should examine whether Akt might signal through other downstream effectors, i.e. mTOR and p70 S6 kinase, in LNCaP cells.

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APPENDIX

The main purpose of our experiments was to examine the zinc treatment effects at five different levels (ZD, ZN, ZA, ZS, and ZR). In addition, we explored the possibility of using an inhibitor LY294002 (LY) to normalize the increase or the decrease associated with specific zinc treatment to that of ZN-VC group. 'ZN' represents zinc-normal medium that contains zinc level comparable to standard culture protocol. 'VC' represents vehicle control treatment that contains 0.05% dimethyl sulfoxide (DMSO). Because LY was dissolved in 0.05% DMSO, our VC group was designed to be corresponded to LY group's control. For this reason, ZN-VC group was chosen to serve as our internal control group. Furthermore, we normalized each piece of data to their respective ZN-VC group. This helped us to show how different was the zinc-treated group (ZD, ZA, ZS, and ZR) compared to cells treated with normal condition (ZN).

Throughout this dissertation, the means of each measurement were compared using one-way analysis of variance (ANOVA). This statistical method assisted us to calculate two different variances and compare them. The first variance is a measure of the dispersion among the group means. The other variance is a measure of the dispersion within the groups themselves. We did a further set of *post-hoc* least significant difference (LSD) test called contrasts or multiple comparisons to determine which means were significantly different and in what directions the differences lies.

Alternatively, there are two ways to look at our data: first way – five sets of group representing the different zinc levels; and second way – two sets of group representing the presence or absence of inhibitor treatment. This type of study design is also called a

2×5 factorial design. Since our factor levels were chosen for a specific reason, we subjected our data to a fixed-effects model ANOVA. Because we had two independent factors (zinc and inhibitor), there were 10 possible factor combinations: ZD with VC, ZD with LY, ZN with VC, ZN with LY, ZA with VC, ZA with LY, ZS with VC, ZS with LY, ZR with VC, and ZR with LY. Since we studied all possible combinations of factor levels, we performed a factorial analysis of variance with a completed crossed design. The number of replicates representing each combination of factors was the same, so our design was balanced or orthogonal. Each replicate was randomly assigned to one of the treatment groups.

A two-way ANOVA is a rigorous way to compare zinc treatments lumping across inhibitor treatments, and then compare inhibitor treatments lumping across zinc treatments. This test looks for inhomogeneity of group means by comparing within-group variance to among-group variance. If among-group variance exceeds within-group by a certain amount, then we have evidence that groups come from populations with different parametric means. This test can also point out the interaction effects. In general, an interaction exists whenever the effect of one factor has on the mean depends on the level of another factor.

For p-Akt measurement in PrEC, we found zinc, inhibitor, and interaction of zinc×inhibitor effects are significant ($p<0.05$) as shown in **Table 1**. Thus, zinc and inhibitor each has an effect on p-Akt level. But this relationship must be interpreted in terms of the interaction of zinc joint with inhibitor. With or without inhibitor treatment makes a large difference on p-Akt level only in ZD and ZR groups, but not in ZN, ZA, and ZS group (**Figure 1**). Then, we performed *a posteriori* comparisons with the LSD

test for the detection of a difference among zinc treatments (**Table 2**). Again, findings from the LSD test indicated that p-Akt levels of ZD and ZR groups are significantly different from that of ZN, ZA, and ZS groups.

Table 1. ANOVA table for p-Akt

Tests of Between-Subjects Effects

Dependent Variable: PAKT

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	26157.051 ^a	9	2906.339	7.084	.000
Intercept	216505.269	1	216505.269	527.753	.000
ZINCTRT	7251.614	4	1812.903	4.419	.010
INHIBITO	13527.332	1	13527.332	32.974	.000
ZINCTRT * INHIBITO	5378.105	4	1344.526	3.277	.032
Error	8204.797	20	410.240		
Total	250867.117	30			
Corrected Total	34361.848	29			

a. R Squared = .761 (Adjusted R Squared = .654)

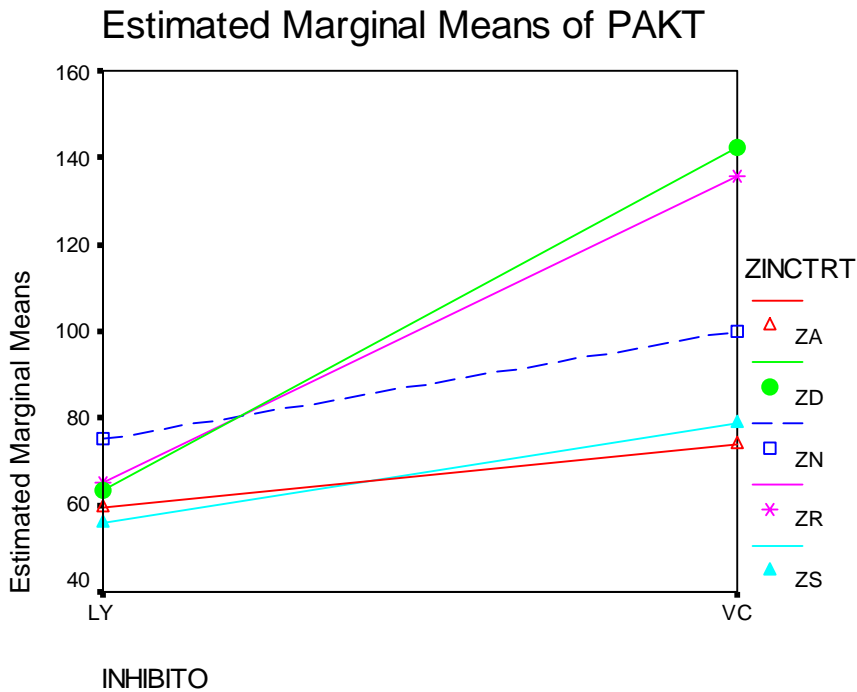


Figure 1. Interaction plot for p-Akt, Inhibitor x Zinc

Table 2. Multiple comparison table for p-Akt

Multiple Comparisons

Dependent Variable: PAKT
LSD

(I) TRT	(J) TRT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0	4	42.4567*	16.5376	.018	7.9598	76.9536
	10	79.0467*	16.5376	.000	44.5498	113.5436
	14	67.3267*	16.5376	.001	32.8298	101.8236
	16	68.5067*	16.5376	.001	34.0098	103.0036
	32	63.4967*	16.5376	.001	28.9998	97.9936
	116	83.2433*	16.5376	.000	48.7464	117.7402
	132	86.5700*	16.5376	.000	52.0731	121.0669
	320	6.9067	16.5376	.681	-27.5902	41.4036
1320	77.5267*	16.5376	.000	43.0298	112.0236	
4	0	-42.4567*	16.5376	.018	-76.9536	-7.9598
	10	36.5900*	16.5376	.039	2.0931	71.0869
	14	24.8700	16.5376	.148	-9.6269	59.3669
	16	26.0500	16.5376	.131	-8.4469	60.5469
	32	21.0400	16.5376	.218	-13.4569	55.5369
	116	40.7867*	16.5376	.023	6.2898	75.2836
	132	44.1133*	16.5376	.015	9.6164	78.6102
	320	-35.5500*	16.5376	.044	-70.0469	-1.0531
1320	35.0700*	16.5376	.047	5.731	69.5669	
10	0	-79.0467*	16.5376	.000	-113.5436	-44.5498
	4	-36.5900*	16.5376	.039	-71.0869	-2.0931
	14	-11.7200	16.5376	.487	-46.2169	22.7769
	16	-10.5400	16.5376	.531	-45.0369	23.9569
	32	-15.5500	16.5376	.358	-50.0469	18.9469
	116	4.1967	16.5376	.802	-30.3002	38.6936
	132	7.5233	16.5376	.654	-26.9736	42.0202
	320	-72.1400*	16.5376	.000	-106.6369	-37.6431
1320	-1.5200	16.5376	.928	-36.0169	32.9769	
14	0	-67.3267*	16.5376	.001	-101.8236	-32.8298
	4	-24.8700	16.5376	.148	-59.3669	9.6269
	10	11.7200	16.5376	.487	-22.7769	46.2169
	16	1.1800	16.5376	.944	-33.3169	35.6769
	32	-3.8300	16.5376	.819	-38.3269	30.6669
	116	15.9167	16.5376	.347	-18.5802	50.4136
	132	19.2433	16.5376	.258	-15.2536	53.7402
	320	-60.4200*	16.5376	.002	-94.9169	-25.9231
1320	10.2000	16.5376	.544	-24.2969	44.6969	
16	0	-68.5067*	16.5376	.001	-103.0036	-34.0098
	4	-26.0500	16.5376	.131	-60.5469	8.4469
	10	10.5400	16.5376	.531	-23.9569	45.0369
	14	-1.1800	16.5376	.944	-35.6769	33.3169
	32	-5.0100	16.5376	.765	-39.5069	29.4869
	116	14.7367	16.5376	.383	-19.7602	49.2336
	132	18.0633	16.5376	.288	-16.4336	52.5602
	320	-61.6000*	16.5376	.001	-96.0969	-27.1031
1320	9.0200	16.5376	.591	-25.4769	43.5169	
32	0	-63.4967*	16.5376	.001	-97.9936	-28.9998
	4	-21.0400	16.5376	.218	-55.5369	13.4569
	10	15.5500	16.5376	.358	-18.9469	50.0469
	14	3.8300	16.5376	.819	-30.6669	38.3269
	16	5.0100	16.5376	.765	-29.4869	39.5069
	116	19.7467	16.5376	.246	-14.7502	54.2436
	132	23.0733	16.5376	.178	-11.4236	57.5702
	320	-56.5900*	16.5376	.003	-91.0869	-22.0931
1320	14.0300	16.5376	.406	-20.4669	48.5269	
116	0	-83.2433*	16.5376	.000	-117.7402	-48.7464
	4	-40.7867*	16.5376	.023	-75.2836	-6.2898
	10	-4.1967	16.5376	.802	-38.6936	30.3002
	14	-15.9167	16.5376	.347	-50.4136	18.5802
	16	-14.7367	16.5376	.383	-49.2336	19.7602
	32	-19.7467	16.5376	.246	-54.2436	14.7502
	132	3.3267	16.5376	.843	-31.1702	37.8236
	320	-76.3367*	16.5376	.000	-110.8336	-41.8398
1320	-5.7167	16.5376	.733	-40.2136	28.7802	
132	0	-86.5700*	16.5376	.000	-121.0669	-52.0731
	4	-44.1133*	16.5376	.015	-78.6102	-9.6164
	10	-7.5233	16.5376	.654	-42.0202	26.9736
	14	-19.2433	16.5376	.258	-53.7402	15.2536
	16	-18.0633	16.5376	.288	-52.5602	16.4336
	32	-23.0733	16.5376	.178	-57.5702	11.4236
	116	-3.3267	16.5376	.843	-37.8236	31.1702
	320	-79.6633*	16.5376	.000	-114.1602	-45.1664
1320	-9.0433	16.5376	.591	-43.5402	25.4536	
320	0	-6.9067	16.5376	.681	-41.4036	27.5902
	4	35.5500*	16.5376	.044	1.0531	70.0469
	10	72.1400*	16.5376	.000	37.6431	106.6369
	14	60.4200*	16.5376	.002	25.9231	94.9169
	16	61.6000*	16.5376	.001	27.1031	96.0969
	32	56.5900*	16.5376	.003	22.0931	91.0869
	116	76.3367*	16.5376	.000	41.8398	110.8336
	132	79.6633*	16.5376	.000	45.1664	114.1602
1320	70.6200*	16.5376	.000	36.1231	105.1169	
1320	0	-77.5267*	16.5376	.000	-112.0236	-43.0298
	4	-35.0700*	16.5376	.047	-69.5669	-5.731
	10	1.5200	16.5376	.928	-32.9769	36.0169
	14	-10.2000	16.5376	.544	-44.6969	24.2969
	16	-9.0200	16.5376	.591	-43.5169	25.4769
	32	-14.0300	16.5376	.406	-48.5269	20.4669
	116	5.7167	16.5376	.733	-28.7802	40.2136
	132	9.0433	16.5376	.591	-25.4536	43.5402
320	-70.6200*	16.5376	.000	-105.1169	-36.1231	

*. The mean difference is significant at the .05 level.

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