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Zinc blocks gene expression of mitochondrial aconitase in human prostatic carcinoma cells

Ke-Hung Tsui[1,2], Phei-Lang Chang[1,2] and Horng-Heng Juang[2,3]*

[1]*Department of Urology, Chang Gung Memorial Hospital, Kwei-Shan, Taiwan, Republic of China*
[2]*Chang Gung Bioinformatics Center, Chang Gung Memorial Hospital, Kwei-Shan, Taiwan, Republic of China*
[3]*Department of Anatomy, Chang Gung University, Kwei-Shan, Taiwan, Republic of China*

Mitochondrial aconitase (mACON) contains a [4Fe-4S] cluster as the key enzyme for citrate oxidation in the human prostatic epithelial cell. Although there is accumulating evidence indicating that accumulation of high levels of zinc in prostate epithelial cells causes reduced efficiency of citrate oxidation, zinc regulation on the mACON is still not well understood. From *in vitro* studies, zinc chloride treatment has been developed using humic acid as the carrier (Zn-HA) in human prostatic carcinoma cells, PC-3. Zn-HA treatment (0.1–10 μM) restricts mACON enzymatic activity, which attenuates citrate utility and decreases intracellular ATP levels in PC-3 cells, whereas the effect is blocked by adding the zinc chelator, diethylenetriaminepentaacetic acid (DTPA). Immunoblot, ribonuclease-protection and transient gene-expression assays indicate that Zn-HA treatments inhibit mACON gene expression. Mutation of the putative metal response element (MRE) from CTCGCCTTCA to TGATCCTTCA abolishes Zn-HA inhibition of mACON promoter activity. Our results have demonstrated that zinc possesses a specific regulatory mechanism on the mACON gene, and a biologic function of the putative metal regulatory system in mACON gene transcription has been identified.
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Key words: prostate; zinc; metal response element; humic acid; aconitase

Zinc is a homeostatically regulated, essential mineral required for numerous metalloenzymes, which influence metabolic function for cell growth, replication, osteogenesis and immunity.[1] Although studies of the relationship between zinc and prostate carcinogenesis remain controversial, the evidence suggests that zinc may play a significant role in the prostate.[2–9] Total zinc levels in the prostate are 10 times higher than in other soft tissues, and this ability of zinc to accumulate in the prostate is lost during prostate carcinogenesis.[2,10] It has been demonstrated that zinc treatment restricts growth of prostate cancer cells *via* cell-cycle arrest, apoptosis and necrosis both *in vitro* and *in vivo*.[3,6,11] Some studies have reported especially high levels of zinc in the mitochondria of prostate epithelial cells, where zinc inhibits mitochondrial aconitase (mACON), resulting in decreased citrate oxidation.[4,10,12,13]

Aconitase (aconitase hydratase, EC4.2.1.3) is essential to carbohydrate and energy metabolism, and initiates the interconversion of citrate and isocitrate in the citric acid cycle.[14] Our previous *in vitro* study using the stable transfected mACON antisense human prostate carcinoma cell, PC-3, has illustrated the key role of mACON in citrate utility, and regulation of intracellular ATP levels in the prostate.[15] Zinc-based mACON regulation processes in the human prostate are still not fully understood. Previous study has determined conserved iron-responsive elements (IRE) in the 5′-untranslated region of the human mACON gene, showing that iron upregulates gene translation and transcription of mACON in human prostatic carcinoma cells.[16]

The objective of our study was to determine the regulation of zinc on the mACON expression and the association with intracellular ATP levels, citrate utility and cell proliferation in human prostate carcinoma cells. The mechanisms of this zinc regulation are also discussed.

Material and methods

Cell culture and chemicals

PC-3 cells were obtained from the American Type Culture Collection (Manassas, VA). The humic acid, zinc chloride ($ZnCl_2$), ferric ammonium citrate (FAC) and diethylenetriaminepenta-acetic acid (DTPA) were purchased from Sigma (St. Louis, MO). Humic acid, a deep brown organic acid that forms chelate compounds with heavy metals, was dissolved in deionizer water at 1.29 mg/ml and centrifuged at 10,000*g* for 10 min to remove insoluble particulate matter as described.[17] The zinc chloride stock solution was dissolved in 1 mM humic acid solution at a concentration of 100 mM (termed Zn-HA in this study). FBS was purchased from HyClone (Logan, UT). All culture media and reagents were purchased from Life Technologies (Rockville, MD). The cells were cultured in RPMI 1640 containing 10% FBS, and the medium was replaced twice a week.

Mitochondrial aconitase and lactate dehydrogenase enzymatic activity assays

After cell treatments with varying concentration of $ZnCl_2$ or Zn-HA, the enzymatic activities of mitochondrial aconitase and lactate dehydrogenase were assayed as described.[18] Cells were resuspended with 500 μl of 0.25 M sucrose-buffer plus 50 mM HEPES and 0.007% digitonin. The cytosol supernatant was centrifuged and prepared for the lactate dehydrogenase (LDH) enzymatic activity assay. Pelleted mitochondria were resuspended in 100 μl of HDGC (20 mM HEPES [pH 7.5], 1 mM DTT, 10% glycerol and 2 mM trisodium citrate, 0.5 mg/L leupeptin, 0.7 mg/L pepstatin and 0.2 mM PMSF). The membrane of the mitochondrial particle was broken apart in the presence of 1% Triton X-100, and the mitochondria extract tested for aconitase activity by NADH-coupled assay. The protein concentrations of the mitochondrial and cytosol fractions were measured using the BCA protein assay kit (Pierce, Rockford, IL). The enzymatic activity of cellular mACON and LDH were adjusted by the protein concentration.

Immunoblot assay of human mitochondrial aconitase and β-actin

After treatment using various dosages of Zn-HA with/without DTPA in RPMI 1640 medium with 5% FBS for 16 hr, cells were lysed with lysis buffer (62.5 mM Tris [pH 6.8], 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 7 M urea, 5 μg/ml leupeptin, and 1 mM phenylmethylsulphonyl fluoride). Equal amounts of protein were separated by 7.5% SDS-polyacrylamide gel and analyzed using the ECL detection system as detailed by the manufacturer (Amersham Bioscience, New Territories, Hong Kong). The blot was probed with diluted 1:500 bovine mACON antiserum (kindly

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***Correspondence to:** Department of Anatomy, Chang Gung University, 259 Wen-Hua 1st Road, Kwei-Shan, Tao-Yuan 333, Taiwan, Republic of China. Fax: +011-886-3-2118112.
E-mail: hhj143@mail.cgu.edu.tw
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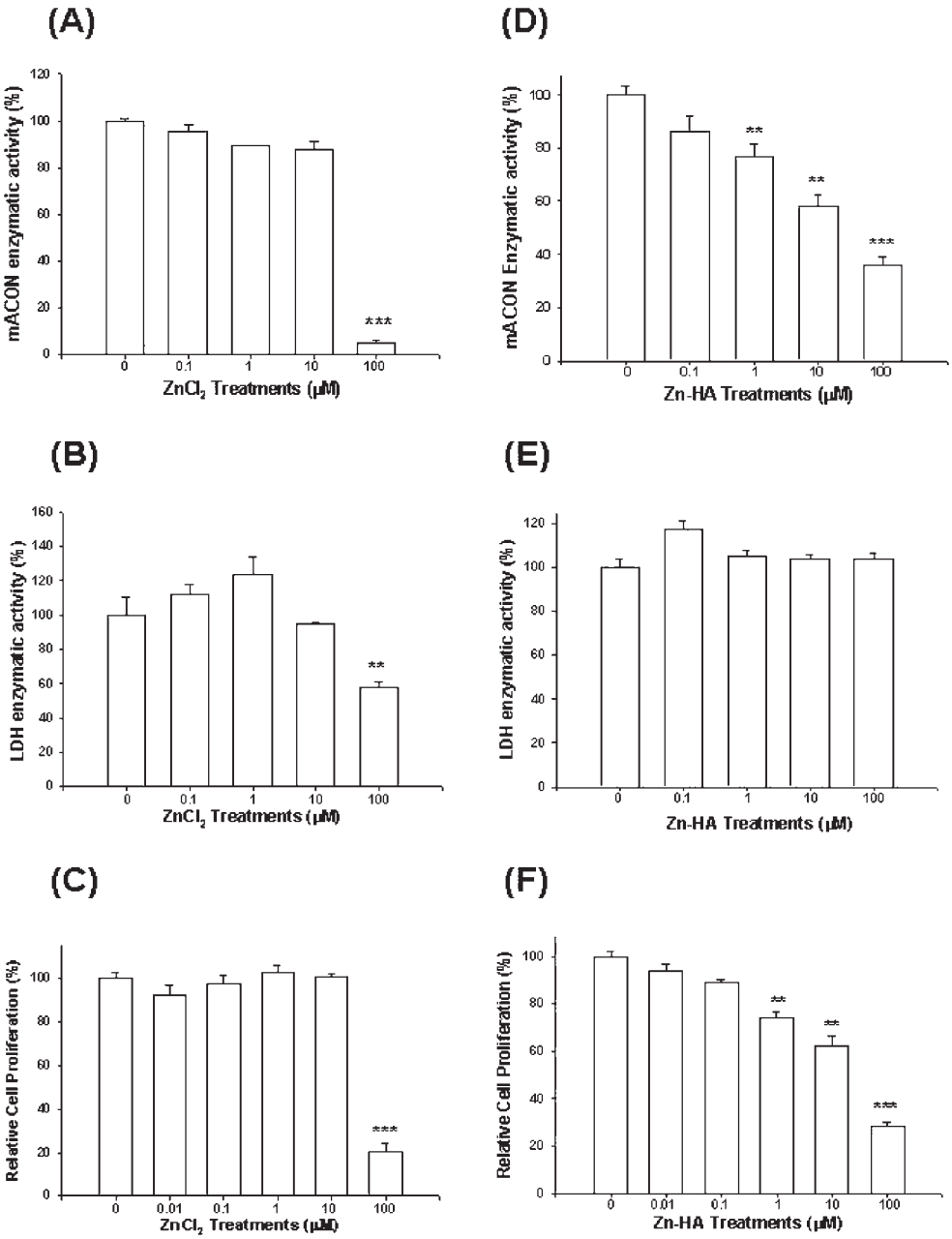

FIGURE 1 – Modulation by zinc chloride and Zn-HA of mACON enzymatic activity and cell proliferation in PC-3 cells. Cells were treated with 3 ml of different concentrations of $ZnCl_2$ (*a–c*) or Zn-HA (*d–f*) in RPMI 1640 with 5% FBS for 16 hr. Cells were harvested and mitochondria particles prepared using digitonin digestion. The mACON enzymatic activities (*a,d*) were determined by NADP-coupled assay in the presence of 1% Triton X-100 to disassociate the mitochondrial membrane ($n = 5$). The LDH activities (*b,e*) in the cytoplasm fraction were assayed at 30°C for the amount of pyruvate consumed. Experimental data are presented as mean percentage ± SE ($n = 6$) of the enzymatic activity induced by $ZnCl_2$ treatment relative to the control-treated sample (**$p < 0.05$; ***$p < 0.01$). Cells (5,000 cells/well; $n = 8$) were incubated with l $ZnCl_2$ (C) or Zn-HA (F) (as indicated) in 100 μl RPMI 1640 medium with 5% FBS for 48 hr, and the cell number determined by MTS assay. Each treatment point represents mean-percentage stimulation of absorbance at 490 nm induced by $ZnCl_2$ treatment relative to the mock-treated analog (**$p < 0.05$; ***$p < 0.01$).

gifted by Dr. R.B. Franklin) and 1:1000 diluted β-actin antiserum (C11, Santa Cruz Biotechnology, Santa Cruz, CA).

Ribonuclease protection assay

Human mACON cDNA was linearized using *Nco*I digestion. The RNA probes (mACON and β-actin) were synthesized by ribonuclease protection assay kit (PRA II; Ambion, Austin, TX) with T3 RNA polymerase, as described previously.[15] RNA probe (1×10^5 cpm) was incubated with 30 μg of total RNA at 95°C for 5 min, and then incubated at 48°C overnight. Unprotected single-stranded RNA was removed with 5 U RNase A and 20 U RNase T1. The result of the ribonuclease protection was separated for analysis on 5% denaturing polyacrylamide gels.

Cell proliferation assays

Cell proliferation in response to $ZnCl_2$ or Zn-HA was measured with the MTS assay kit (Promega Biosciences, San Luis Obispo, CA). Cells (5,000 cells/well) were grown in 100 μl RPMI 1640 medium with 5% FBS and different dosages of $ZnCl_2$ or Zn-HA for 48 hr. Cells were washed twice with PBS, then incubated with freshly prepared, combined MTS/phenazine methosulfate (1:1 by volume) solution for 3 hr at 37°C in a humidified 5% CO_2 atmosphere. The absorbance of the formazan product was measured at 490 nm by ELISA microplate reader (Dynex Technologies, Chantilly, VA).

Citrate and intracellular ATP assay

PC-3 cells (2×10^5 /well) were incubated in the 6-well plate until 80% confluence was achieved. After Zn-HA treatment in RPMI 1640 medium with 5% FBS for 48 hr, the media citrate concentrations and intracellular ATP levels were measured as described.[15] The citrate concentrations in the supernatant were estimated by tracking NADH oxidation from the coupled reactions. The citrate concentrations in the media and the intracellular ATP levels were adjusted to the protein concentrations of the whole cell extract, using the BCA protein assay kit (Pierce).

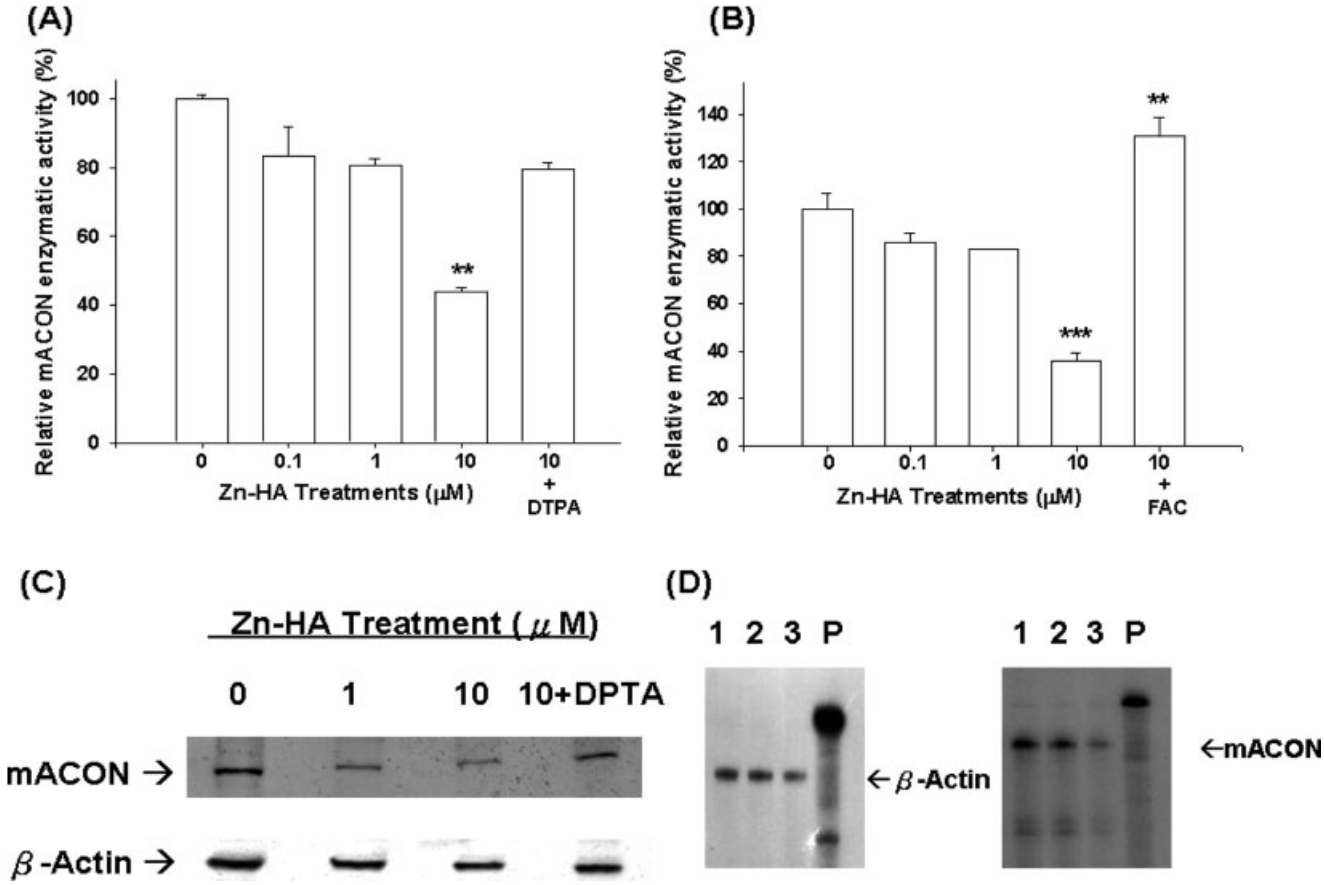

FIGURE 2 – Modulation by Zn-HA, zinc chelator or ferric ammonium citrate of mACON enzymatic activity and gene expression in PC-3 cells. Cells were treated with 3 ml of different concentrations of Zn-HA (Zn) as indicated and DTPA (*a*) or FAC (*b*) in RPMI 1640 with 5% FBS for 16 hr. The mACON enzymatic activities in the mitochondrial fraction were assayed as described in Material and Methods. Experimental data are presented as mean percentage ± SE ($n = 6$) of enzymatic activity induced by treatments relative to the control sample (**$p < 0.05$; ***$p < 0.01$). Cells were treated with different concentrations of Zn-HA (Zn) with or without DPTA (as indicated) for 24 hr. Cells were harvested and lysed to extract protein for the immunoblot assay (*c*) and to extract mRNA for the ribonuclease protection assay (*d*) (1, control; 2, 1 μM Zn-HA; 3, 10 μM Zn-HA; P, free probe).

Luciferase and β-galactosidase assay

The reporter vector, pGL188, containing the human mACON promoter was constructed as described.[18] The pGLCMVIRE reporter vector containing bulge/loops of iron response element controlled by the cytomegalovirus (CMV) enhancer/promoter were constructed as described previously.[16] Several putative response elements, including metal response element (MRE), antioxidant response element (ARE), sterol response element (SRE), and Sp1 binding sites, were found on the promoter of the mACON gene using simple DNA sequence analysis with GCG sequence analysis software (Accelrys Inc., San Diego, CA). The reporter vectors containing mutated metal response element (pMREm), sterol response element (pSREm), Sp1 binding site (pSp1m) and antioxidant response element (pAREm) were constructed using the QuikChange Site-directed Mutagenesis kit (Stratagene, La Jolla, CA). The complement double-strand primers used for the *in vitro* site-directed mutagenesis were 5′-GGCGCCGTGTGGGAGAGCTCTTTAATGCGACCTCAT C-3′ (for pAREm), 5′-GCCTTCACCGTGACGCCCATATCTTCCGGGCACGC-3′ (for pSREm), 5′-CACTCTTCCGGGCACGAAACTGCCCAAAGGCTTTA-3′ (for pSp1m), and 5′-CAGGGTTCTAGGGAGGCTGATCCTTCACCGTGACG-3′ (for pMREm; mutation site is underlined). One day before the transfection, cells were plated onto 24-well plates at 1×10^4 cells/well and transiently transfected using 1 μg/well luciferase reporter vector and 0.5 μg/well β-galactosidase expression vector, as described previously.[18] The transfected cells were washed twice in RPMI 1640 medium and immediately treated with different concentrations of Zn-HA, as indicated in RPMI 1640 medium with 5% FBS for an additional 16 hr. Cells were lysed with 200 μl of Luciferase Cell Culture Lysis Reagent (Promega Bioscience). Twenty microliters of cell lysate was used for luciferase assay and 100 μl of cell lysate for β-galactosidase (β-GAL) enzyme assay, as detailed by the manufacturer (Promega Bioscience). The luciferase activity was determined as relative light units (RLU) using the LumiCount luminometer (Packard BioScience, Meriden, CT) and adjusted by the β-GAL activity.

Electrophoretic mobility-shift assay

The electrophoretic mobility shift assay was carried out as described previously.[19] The double-stranded DNA fragment containing the putative metal response element (5′-GTTTCA-TCCTGGGCATGTCTCCTCTGCCTTTG-3′) was 5′-end labeled with γ-P^{32}ATP using T4 polynucleotide kinase. Nuclear extracts of the PC-3 cells were gathered using NE-PER nuclear and cytoplasmic extraction reagents as described by the manufacturer (PIERCE). The 5′-end-labeled MRE (MRE probe; 5 nM) was incubated with 2 μg of nuclear extract (NE) from PC-3 cells in 20 μL of binding buffer (25 mM HEPES buffer [pH 7.9], 50 mM KCL, 0.05 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulphonyl fluoride and 10% glycerol) containing 0.5 μg poly (dI-dC)-poly(dI-dC). The binding-shift was challenged with 50-fold or 100-fold double-stranded MRE or its double-stranded

mutational form (MREm; 5′-GTTTCATCCTGGGAGGATCTG-CCTTTG-3′) without labeling of the 5′-end having with γ-P^{32}ATP. Protein-DNA complex formation was analyzed on 4% polyacrylamide gels by autoradiography.

Statistical analysis

Results are expressed as the mean ± SE of at least 3 independent replications of each experiment. Statistical significance was determined by pair *t*-test analysis using SigmaStat software for Windows (version 2.03; SPSS Inc., Chicago, IL).

Results

In vitro study using the human prostate carcinoma cell, PC-3, showed that $ZnCl_2$ treatment (0.1–10 μM) for 16 hr did not influence mACON enzymatic activity until the $ZnCl_2$ concentration reached 100 μM (Fig. 1*a*). This $ZnCl_2$ blocking effect on mACON enzymatic activity may be due to the cytotoxicity of the high concentration of zinc chloride, because the concentration of intracellular lactate dehydrogenase was markedly decreased (Fig. 1*b*). However, when PC-3 cells are $ZnCl_2$ dosed for 16 hr with humic acid as the carrier (Zn-HA treatment), the results of enzymatic assay show that Zn-HA treatment significantly inhibits mACON enzymatic activity but not LDH enzymatic activity (Fig. 1*d*,*e*). MTS assay showed that Zn-HA treatments for 48 hr inhibit cellular proliferation in PC-3 cells, by contrast, whereas $ZnCl_2$ without the humic acid carrier does not affect proliferation of PC-3 cells except at cytotoxic dosages (Fig. 1*c*,*f*).

This mACON enzymatic activity was reduced to 50% by Zn-HA treatment at 10 μM. Contrastingly, the obstructive effect of Zn-HA was itself blocked by cells co-treated with the zinc chelator, DTPA, or transferrin-independent iron donor, FAC (Fig. 2*a*,*b*). Immunoblot and ribonuclease protection assays confirmed that Zn-HA treatments inhibit not only enzymatic activity but also the mACON gene expression in PC-3 cells (Fig. 2*c*,*d*).

The citrate concentrations in the culture media were multiplied 1.6-fold after 10 μM Zn-HA treatment (Fig. 3*a*). Attenuating citrate utility in the Krebs cycle caused a 40% decrease in intracellular ATP level in the Zn-HA treated cells compared to the mock-treated groups (Fig. 3*b*). These results suggest that Zn-HA treatments inhibit mACON enzymatic activity in PC-3 cells, producing attenuated citrate utility and bioenergy.

A CMV enhancer/promoter with the sequence of an identical sequence to the iron response element (IRE) of human mACON cDNA, driving luciferase reporter vector (pCMVIREGL3) was designed to observe the modulation of Zn-HA treatment on the gene translation of mACON. Results from the reporter assays indicate that Zn-HA treatment does not affect gene translation though the IRE pathway (Fig. 4*a*). Measurement of transient gene expression with 5′-deletion assay shows that Zn-HA treatment affects mACON gene expression and that this depends on the response element on the DNA fragment, which is located in the 5′-flanking region (−158 to +38) of the mACON gene (Fig. 4*b*). The inhibiting effect of Zn-HA treatment on the promoter activity of the human mACON gene in PC-3 cells was restricted by co-treatment with DTPA and FAC (Fig. 4*c*). Further, this mACON gene promoter activity was stimulated 3.5-fold by FAC. Our findings suggest that Zn-HA treatment acts as an iron antagonist, disrupting enzymatic activity and mACON gene expression in the prostate. Mutation of MRE from CTCGCCTTCA to TGATCCTTCA using site-directed mutagenesis abolished the repressive effects of Zn-HA treatment (Fig 4*d*). The sequence and putative response elements of pGL188 are presented in Figure 5*a*. The EMSA results also show an unknown transcription factor bound on this specific MRE sequence after Zn-HA treatment (Fig. 5*b*).

Discussion

The normal human prostate accumulates the highest zinc levels of any soft tissue. Zinc content is increased in benign prostate hyperplasia but decreased in prostate cancer by comparison with normal tissue.[2,20] The uniquely high accumulation of mitochondrial zinc seems to be due to the zinc transporters and high cytosolic levels of zinc-transportable ligands, such as citrate and aspartate, in prostate cells.[21–24]

A growing body of evidences suggests that the loss of this unique capacity to retain high levels of zinc is an important factor in the development and progression of malignant prostate cells.[3,7,13] Further, it has been demonstrated in a number of studies that zinc supplementation may attenuate prostate cancer tumorigenicity, however, the results of other epidemiological and laboratory investigations of the relationship between prostate-cancer incidence and zinc are conflicting.[3–9] Moreover, *in vivo* and *in vitro* studies from different laboratories also indicate, conversely, that either high dosage levels of zinc supplementation or zinc deficiency, respectively, may cause cell apoptosis in prostate or non-prostate cells.[25–29]

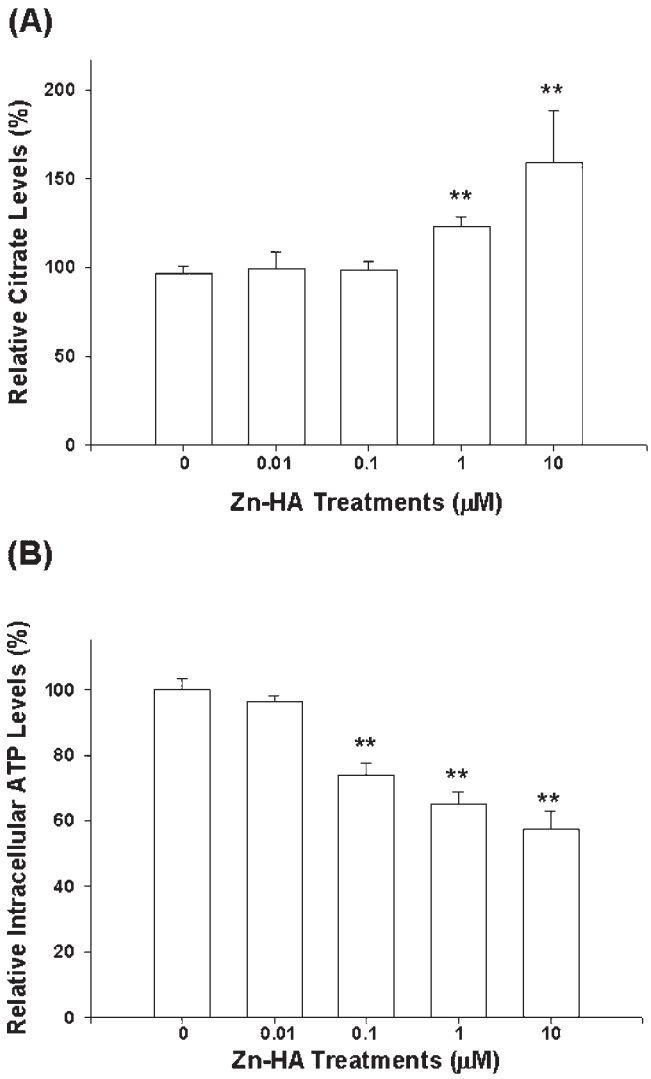

FIGURE 3 – Regulation by Zn-HA of intracellular ATP levels and citrate utility in PC-3 cells. Cells (2×10^6 cells/flask) were treated with 3 ml of different concentrations of Zn-HA (as indicated) in RPMI 1640 with 5% FBS for 16 hr ($n = 6$). The media were collected for citrate assay (*a*) and cells were harvested for determination of intracellular ATP levels (*b*) as described in Material and Methods. Experimental data are presented as mean percentage ± SE ($n = 6$) of citrate concentration and ATP levels resulting from Zn-HA treatment relative to control sample (**$p < 0.05$).

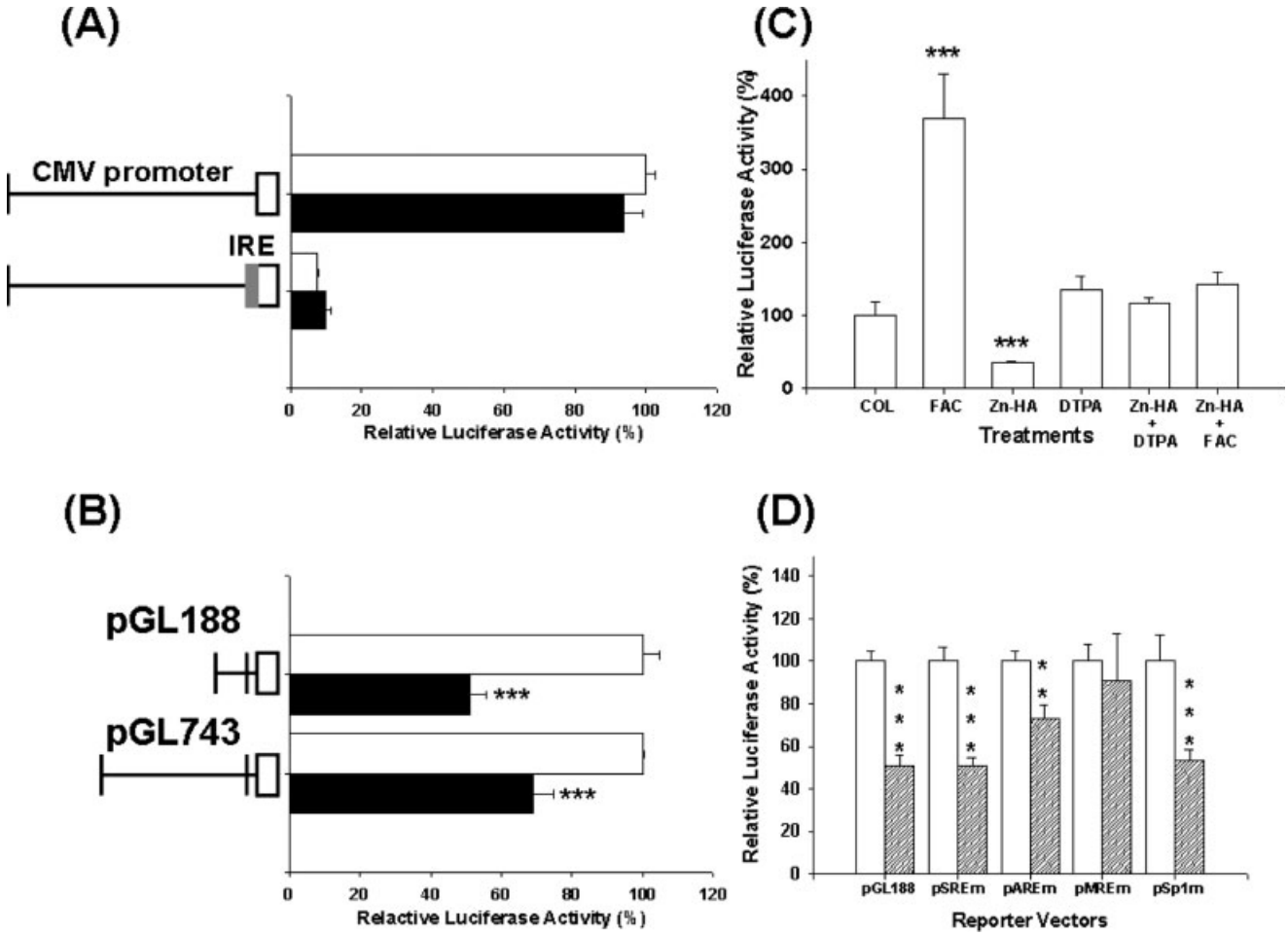

FIGURE 4 – Effect of Zn-HA on expression of human mACON gene in PC-3 cells. (*a*) Zn-HA regulation of mACON gene expression is independent of loop/bulge of iron response element on mACON gene (white and black boxes represent mock and 10 μM Zn-HA treatments, respectively). (*b*) Deletion assay shows that Zn-HA regulation of mACON gene promoter activity depends on the mACON 188 DNA fragment (white and black boxes represent mock and 10 μM Zn-HA treatments, respectively). (*c*) pGL188 reporter vector-transfected PC-3 cells were treated with ferric ammonium citrate (FAC), 10 μM Zn-HA (Zn-HA) or diethylenetriaminepenta-acetic acid (DTPA) for 16 hr. (*d*) PC-3 cells were transfected with different mutant forms of reporter vector (white and black boxes represent mock and 10 μM Zn-HA treatments, respectively). Experimental data were obtained in quadruplicate and are presented as mean percentage (±SE) luciferase activity produced by $ZnCl_2$ treatment relative to control-treated samples (**$p < 0.05$; ***$p < 0.01$).

Our present *in vitro* study confirms that high-dosage $ZnCl_2$ treatment causes cytotoxic effects on prostatic carcinoma cells characterized by loss of intracellular LDH. Reports from other laboratories have shown zinc-induced apoptosis in PC-3 cells through activation of caspase-9 and caspase-3, which cleaves the nuclear poly (ADP)-ribose polymerase, or decreasing mitochondrial trans-membrane potential and Bcl-2 protein levels.[4,25] However, immunoblot assay indicated that 10 μM Zn-HA treatment for 16 hr did not significantly downregulate the protein levels of caspase-3 in PC-3 cells in this study (data not shown). In yet another study, the zinc ionophore, pyrithione, was used to facilitate zinc transport across the cell membrane, demonstrating that zinc, at a physiological dosage, inhibits activation of NFκB transcription factor, which sensitizes cells to tumor necrosis factor (TNFα) and promotes paclitaxel-mediated cell death in PC-3 cells.[26]

Humic acid, a brown organic substance found to influence metal transport and uptake through soil layers in plants and vertebrates, was used as the carrier for zinc chloride in our present study. Humic acids occur naturally in soils and surface water. Increasing humic acid content in water promotes the formation of chelate compounds with heavy metals including zinc, which leads to decreased toxicity.[30] In our present study, even at higher dosages, we found that Zn-HA treatment has only modest cytotoxic effect on PC-3 cells. Although the precise functions of humic acid in zinc transportation and detoxification remain unknown, other studies have successfully used humic acid as a carrier in *in vitro* assay systems, demonstrating the regulation on gene expression by arsenic, cadmium, chromium or lead through differing pathway.[17]

A series of studies have shown that the main effect of zinc in the prostate is as a mACON inhibitor in citrate metabolism.[2,10,12,13,21–23] Our results indicate that Zn-HA inhibition of cellular proliferation in PC-3 cells is not due to the cytotoxic effects of $ZnCl_2$ but rather to restricted mACON gene expression, which attenuates citrate utility and intracellular ATP biosynthesis. These results are in agreement with those of analogous *in vivo* and *in vitro* studies conducted at other laboratories.[3,6,31,32]

Whether zinc directly influences mACON or simply acts as an Fe antagonist for mACON gene translation or transcription in the human prostatic epithelial cells remains unknown. Our study clearly demonstrated by using the reporter assay with a CMV enhancer/promoter and the IRE sequence of the human

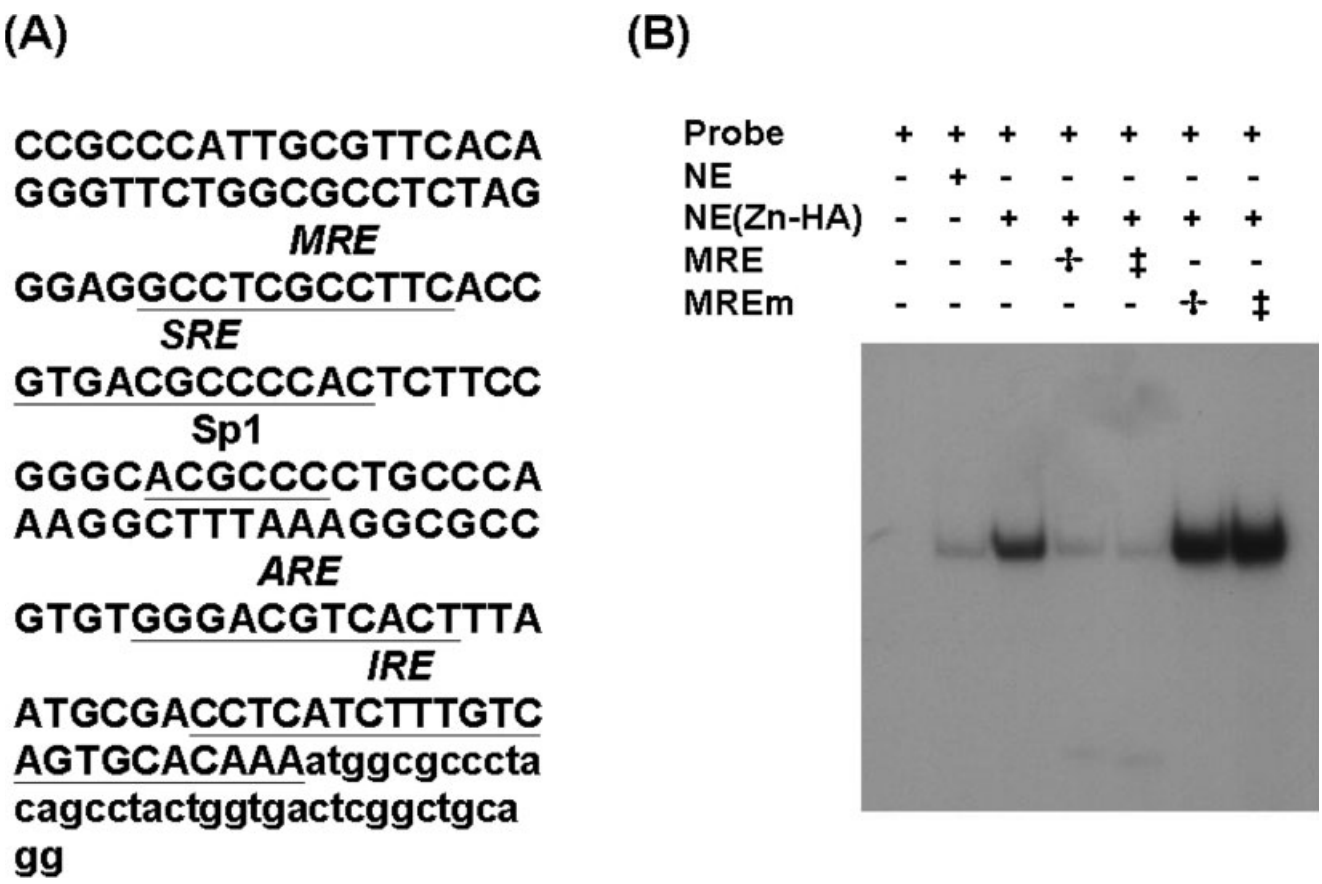

Figure 5 – Binding of nuclear protein of PC-3 cells to the putative metal response element of the promoter of human mitochondrial aconitase gene. (*a*) Sequence of DNA fragment cloned in pGL188 vector. The putative response elements (*MRE*, metal response element; *SRE*, sterol response element; Sp1, Sp1 binding site; *ARE*, antioxidant response element; *IRE*, iron response element) are underlined, the coding region of the first exon is in lower case. (*b*) The electrophoretic mobility shift assay was applied using the 32-bp MRE oligonucleotide probe end-labeled with ^{32}P (MRE probe) and nuclear extract from the PC-3 cells with (Zn-HA) or without (NE) Zn-HA treatment. The gel shift disappeared when the reaction mixture was challenged with 50-fold (†) or 100-fold (‡) unlabeled double-stranded oligonucleotide containing the putative metal response element (MRE), but not the oligonucleotide featuring a mutation (MREm).

mACON-driving luciferase reporter vector that zinc does not affect mACON gene translation through the IRE pathway. In previous research we have demonstrated by using the same reporter vector that FAC upregulated mACON gene translation through the IRE pathway.[16]

A number of studies have confirmed that zinc treatment induces MTF-1 expression, which deregulates the metallothionein gene through the MRE pathway.[33–37] Further, recent reports indicate that zinc upregulates gene expression of metallothionein and zinc transporters in human prostatic carcinoma cells, as well as the promoter activity of the probasin gene in the transgenic mouse prostate.[32,33,38] Immunoblot and ribonuclease protection assay results in our present study show that Zn-HA treatment inhibits mACON protein abundance and downregulates mACON gene expression. Several putative regulatory elements, including Sp1, MRE, SRE and ARE, that are important for mACON gene transcription have been demonstrated in our previous studies.[16,39] The putative MRE sequence on the mACON promoter is homologous with MRE (HTHXXGCTC; H = A, C, or T; X = any residue) on the metallothionein genes and the Cu/Zn-superoxide dismutase gene in *Saccharomyces cerevisiae*.[33,40] Transient gene expression assay in the present study showed Zn-HA blocked regulation of mACON promoter activity with mutation of the putative MRE from CTCGCCTTCA to TGATCCTTCA. The results from EMSA also indicate that blockage of mACON gene transcription by unknown factors after Zn-HA treatment binding to the MRE sequence.

Previous study indicates that FAC upregulation of mACON gene transcription may involve a putative antioxidant response element signal pathway, instead of the metal responsive element involved in zinc regulation of our study.[16] These studies seem to suggest a regulatory link between energy utilization and metal metabolism in human prostatic carcinoma cells, and that this may be an important determinant of prostate cancer onset. Our results demonstrate that Zn-HA treatment affects human mACON gene expression through the MRE signal pathway. We also argued that that zinc does not compete with iron in mACON expression at the translational level. Our study provides additional evidence in support of the notion that zinc homeostasis is linked to energy utilization through the modulation of gene expression of mACON in human prostate carcinoma cells, and also indicates that zinc and iron may reflect antagonistic regulatory mechanisms on the mACON gene at the transcriptional level.

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