Blackwell Publishing

Asian J Androl 2006; 8 (3): 307–315
DOI: 10.1111/j.1745-7262.2006.00139.x

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·*Original Article*·

Manganese antagonizes iron blocking mitochondrial aconitase expression in human prostate carcinoma cells

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Abstract

Aim: To investigate the possible role of manganese in the regulation of mitochondrial aconitase (mACON) activity human prostate carcinoma cell line PC-3 cells. **Methods:** The mACON enzymatic activities of human prostate carcinoma cell line PC-3 cells were determined using a reduced nicotinamide adenine dinucleotide-coupled assay. Immunoblot and transient gene expression assays were used to study gene expression of the mACON. The putative response element for gene expression was identified using reporter assays with site-directed mutagenesis and electrophoretic mobility-shift assays. **Results:** *In vitro* study revealed that manganese chloride ($MnCl_2$) treatment for 16 h inhibited the enzymatic activity of mACON, which induced the inhibition of citrate utility and cell proliferation of PC-3 cells. Although results from transient gene expression assays showed that $MnCl_2$ treatment upregulated gene translation by approximately 5-fold through the iron response element pathway, immunoblot and reporter assays showed that $MnCl_2$ treatments inhibited protein and gene expression of mACON. This effect was reversed by co-treatment with ferric ammonium citrate. Additional reporter assays with site-directed mutagenesis and electrophoretic mobility-shift assays suggested that a putative metal response element in the promoter of the *mACON* gene was involved in the regulation of $MnCl_2$ on the gene expression of mACON. **Conclusion:** These findings suggest that manganese acts as an antagonist of iron, disrupting the enzymatic activity and gene expression of mACON and citrate metabolism in the prostate. *(Asian J Androl 2006 May; 8: 307–315)*

Keywords: citrate; adenosine triphosphate; proliferation; PC-3; metal response element; prostate carcinoma cell line

1 Introduction

Aconitase (aconitase hydratase, EC4.2.1.3) is the enzyme responsible for the interconversion of citrate and isocitrate in the citric acid cycle [1]. Our previous *in vitro* study using the stable-transfected mitochondrial aconitase (mACON) antisense human prostate carcinoma cell cell line PC-3 illustrated the key role of mACON in citrate utility and bioenergy [2]. There are two different aconitases in mammalian cells, cytosolic and mitochondrial, which are encoded by two different genes [3]. Both aconitases contain a (4Fe-4S) cluster that is required for their enzymatic activities. The conserved iron responsive element (IRE) contains an approximately 30-nucleotide, RNA hairpin stem-loop located in the 5'-untranslated region of ferritins, 5-aminolevulinate synthase and mACON mRNAs,

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Received 2005-12-14 Accepted 2006-01-06

and in the 3'-untranslated region of transferrin receptor mRNA [4, 5]. Although the function of mACON is not directly involved in the regulation of the iron pool in cells, *in vitro* study demonstrates the deregulative effect of iron on the human *mACON* gene in prostate carcinoma cells [5].

Manganese is ubiquitous in mammalian systems and is essential for proper development and function; nonetheless, chronic overexposure to this metal elicits potent toxic effects [6]. Recently, epidemiological study revealed that iron status may affect manganese homeostasis in biological systems, although the mechanism remains to be elucidated [7]. Early *in vitro* and *in vivo* studies showed that manganese treatment significantly inhibits aconitase activity, which can lead to the disruption of mitochondrial energy production and cellular iron metabolism in the brain [8]. Although previous study in our laboratory has demonstrated that the regulative effect of iron on the human *mACON* gene is on the translational and transcriptional levels in PC-3 cells [5], there is no information yet to determine whether or not manganese disrupts the citrate metabolism of human prostate cells.

Animal studies have shown striking lesions in the kidneys and prostate gland after oral high-dose administration of manganese acetate for 63 days in Sprague–Dawley rats [9]. Although an epidemiological study from Japan suggested that manganese might be related to prostatic carcinogenesis [10], the adverse health effects of manganese on the human prostate are still unknown. The objectives of the present study were to evaluate the regulatory link between manganese and iron on gene expression of mACON and citrate utilization in human prostatic carcinoma cells. The putative mechanisms for the regulation of manganese on gene translation/transcription of mACON were investigated.

2 Materials and methods

2.1 Cell culture and chemicals

PC-3 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Fetal bovine serum (FBS) was purchased from HyClone (Logan, UT, USA) and all culture media used in the present study were from Invitrogen (Grand Island, NY, USA). Manganese chloride ($MnCl_2$), ferric ammonium citrate (FAC) and other general chemicals or drugs used in the present study were purchased from Sigma Chemical Company (St. Louis, MO, USA). The bicinchoninic acid (BCA) protein concentration assay kit was purchased from Pierce (Rockford, IL, USA). PC-3 cells were cultured in RPMI 1640 medium containing 10% FBS. The medium was changed twice a week.

2.2 Mitochondrial aconitase and lactate dehydrogenase enzymatic activity assay

PC-3 cells and HepG2 cells were incubated with RPMI 1640 medium with 2% FBS and different concentrations of $MnCl_2$ (1 μmol/L, 10 μmol/L and 100 μmol/L) and FAC (10 μg/mL) for 16 h. The enzymatic activities of mACON and lactate dehydrogenase (LDH) were measured as previously described [5]. The mitochondria particles were prepared by digitonin digestion and the mACON enzymatic activities were determined using a reduced nicotinamide adenine dinucleotide-coupled assay ($n = 5$). The LDH activities in the cytoplasm fraction were assayed at 30ºC for the amount of pyruvate consumed. The enzymatic activities of mACON and LDH were adjusted according to the protein concentrations of mitochondrial and cytosolic extracts, respectively, which were determined using the BCA protein assay kit.

2.3 Western blot of human mitochondrial aconitase and β-actin

PC-3 cells were incubated in RPMI 1640 medium with 2% FBS and different concentrations of $MnCl_2$ (1 μmol/L, 10 μmol/L and 100 μmol/L) for 16 h. Cells were lysed with lysing buffer (62.5 mmol/L Tris-HCl [pH 6.8], 2% sodium dodecylsulfate [SDS], 10% glycerol, 5% β-mercaptoethanol and 7 mol/L urea) and the protein concentrations of aliquoted samples were measured using the BCA protein assay kit. Equal amounts of protein (60 μg) were loaded onto a 7.5% SDS-polyacrylamide gel, separated by electrophoresis, transferred to a nitrocellulose membrane, and antibody binding was assayed using an electrochemiluminescence detection system, as described by the manufacturer (Amersham Biosciences, New Territories, Hong Kong, China). The membrane was probed with a 1: 500 dilution of antibovine mACON antiserum (a gift from Dr R. B. Franklin) or a 1: 1000 diluted anti-β-actin antiserum (C11; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The intensity of different bands was analyzed using GeneTools of ChemiGenius (Syngene, Cambridge, UK).

2.4 Cell proliferation assays

Cell proliferation in the experiments comparing the

proliferation rates of cells in response to $MnCl_2$ was measured by the conversion of a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxylmethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay into a formazan product by viable cells; the absorbance was then measured at a wavelength of 490 nm using the cell Titer96$_{ueous}$ cell proliferation assay kit (Promega Bioscience, San Luis Obispo, CA, USA). In this assay, 5 000 cells were seeded into each well of a 96-well plate with RPMI 1640 medium and 10% FBS, and incubated for 48 h. The medium was changed to RPMI 1640 medium with 2% FBS and different concentrations of $MnCl_2$ for an additional 3 days. MTS dye solution was added and the plates were read 3 h later. The cell numbers in each well were determined using a microplate reader (Dynex Technologies, Chantilly, VA, USA).

2.5 Citrate and intracellular adenosine triphosphate (ATP) assay

PC-3 cells were incubated for 48 h in RPMI 1640 medium with 2% FBS and different concentrations of $MnCl_2$ (0 , 0.1 μ, 1 , 10 and 100 μmol/L). The citrate concentrations in the media and intracellular adenosine triphosphate (ATP) levels were measured according to previously described methods [2]. The citrate concentrations and the intracellular ATP levels were adjusted according to the protein concentrations of whole-cell extracts, which were measured using the BCA protein assay kit.

2.6 Site-direct mutagenesis and luciferase assays

The reporter vector, pGL188, containing the promoter (–158 to +38) of the human *mACON* gene was constructed as previously described [5]. The reporter vectors containing the mutated metal response element (MRE), antioxidant response element (ARE), sterol response element (SRE) and Sp1 binding site (Sp1) were constructed using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) as previously described [11]. The complementary double-stranded primers used for the *in vitro* site-directed mutagenesis were 5'-GGCGCCGTGTGGGAGAGCTCTTTAATGCGACCTCATC-3' (for pAREm), 5'-GCCTTCACCGTGACGCCCATATCTTCCGGGCA CGC-3' (for pSREm), 5'-CACTCTTCCGGGCACGAAACTGCCCAAAGGCTTTA-3' (for pSp1m) and 5'-CAGGGTTCTAGGGAGGCTGATCCTTCACCGTGACG-3' (for pMREm), the underline representing the mutation site. PC-3 cells (1×10^4/well) were cultured into the individual wells of a 24-well plate in RPMI 1640 medium with 10% FBS 1 day before transfection. Cells were transiently co-transfected with 1 μg/well of luciferase reporter vector and 0.5 μg/well of β-galactosidase (β-GAL) expression vector (pCMVSPORTβgal; Invitrogen) using TransFast transfection reagent (0.6 μg/well; Promega Bioscience) as previously described [5]. Cells were lysed with 200 μL of Luciferase Cell Culture Lysis Reagent (Promega Bioscience). For the luciferase assay, 20 μL of cell lysate was used. For the β-GAL enzyme assay, 100 μL of cell lysate was used, as detailed by the manufacturer (Promega Bioscience). The luciferase activity was determined as relative light units using the LumiCount luminometer (Packard BioScience, Meriden, CT, USA) and adjusted by the β-GAL activity.

2.7 Reporter assay for iron response element

The constructions of IRE reporter vectors were as previously described [6]. An 878 bp DNA fragment containing the cytomegalovirus (CMV) enhance/promoter and a transcriptional start site from pcDNA3 (Invitrogen; Carlsbad, CA, USA) was subcloned into the pGL3 basic reporter vector at the *Bgl II* and *Hind III* sites. This reporter vector was designated as pCMVGL3. A double-stranded DNA oligonucleotide annealed by two complementary oligonucleotides (5'-AGTTTAATGCGACCTCATCTTTGTCAGTGCACAAAATGGCGC-3' and 5'-CATGGCGCCATTTTGTGCACTGACAAAGATGAGGTCGCATTAA-3') was ligated into pCMVGL3 at the *Hind III* and *Noc I* sites, and this reporter vector was designated as pCMVIREGL3. The mutation of the iron response element (pCMVIRE12mGL3) was constructed using the QuikChange site-directed mutagenesis kit (Stratagene), with a pair of complementary primers (IREm12 5'-GCGACCTCATCTTTGTCAXXXXCAAAATGGCGCCCTAC-3' where X represents the deletion sites). Transient transfection and reporter assays were performed as described above.

2.8 Electrophoretic mobility-shift assay

An electrophoretic mobility-shift assay (EMSA) was performed as previously described [12]. The double-stranded DNA fragment containing the putative MRE response element (MRE; 5'-GTTTCATCCTGGGCATGTCTCCTCTGCCTTTG-3') was 5'-end labeled with γ-P^{32}ATP using T4 polynucleotide kinase. Nuclear extracts of PC-3 cells with or without pretreatment with $MnCl_2$

were gathered with the NE-PER nuclear and cytoplasmic extraction reagents as described by the manufacturer (Pierce). The 5'-end-labeled MRE (MRE probe; 5 nmol) was incubated with 2 μg of nuclear extract from PC-3 cells in 20 μL of binding buffer (25 mmol/L HEPES buffer, pH 7.9, 50 mmol/L KCl, 1 mmol/L $MgCl_2$, 0.5 mmol/L EDTA, 0.5 mmol/L dithiothreitol, 0.5 mmol/L phenylmethylsulfonyl fluoride and 4% glycerol) containing 0.1 μg of poly (dI-dC)-poly(dI-dC). The binding shift was challenged with 50-fold double-stranded MRE without the 5'-end having been labeled with γ-P^{32}ATP. Protein-DNA complex formation was analyzed on 4% polyacrylamide gels by autoradiography.

2.9 Statistical analysis

Results are expressed as mean ± SEM of at least three independent replications of each experiment. Statistical significance was determined using paired *t*-test analysis with the SigmaStat program for Windows (version 2.03; SPSS Inc., Chicago, IL, USA).

3 Results

In vitro study using human prostate carcinoma cells (PC-3 cells) revealed that $MnCl_2$ treatment (0–100 μmol/L) for 16 h significantly inhibited mACON enzymatic activity (Figure 1A). The results indicated 13% inhibition at 1 mmol/L and 46% inhibition at 100 μmol/L for PC-3 cells. $MnCl_2$ treatments did not affect cytosolic LDH activity of cells, indicating that even up to 100 μmol/L of $MnCl_2$ treatment did not induce necrosis of PC-3 cells (Figure 1B). In contrast, the inhibitory effect of $MnCl_2$ was itself attenuated by the co-treatment with 100 μg/mL of FAC (Figure 1C). Additionally, combinations of $MnCl_2$ and FAC did not affect the LDH enzymatic activity (Figure 1D). The decreased mACON enzymatic activity enhanced citrate secretion from PC-3 cells (Figure 2A). Attenuation of citrate utility in the Krebs' cycle caused a

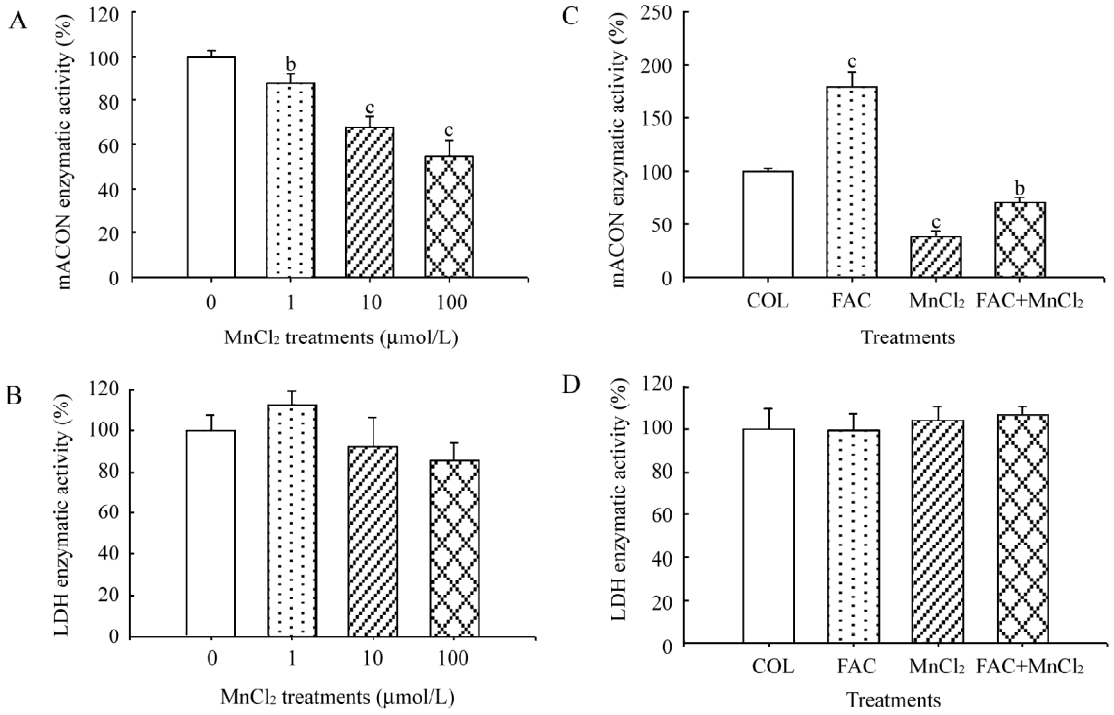

Figure 1. Modulation of manganese chloride ($MnCl_2$) and ferric ammonium citrate (FAC) on mitochondrial aconitase (mACON) enzymatic activity in PC-3 cells. Cells were treated with 3 mL of different concentrations of $MnCl_2$ and FAC, as indicated, for 16 h. (A, C): mACON enzymatic activities were determined using a reduced nicotinamide adenine dinucleotide-coupled assay. (B, D): Lactate dehydrogenase (LDH) activities in the cytoplasm fraction were assayed at 30ºC for the amount of pyruvate consumed. Experimental data are presented as the percentage (mean ± SEM, $n = 6$) of the enzymatic activity induced by $MnCl_2$ or FAC treatments, relative to control-treated (COL) samples ($^bP < 0.05$; $^cP < 0.01$).

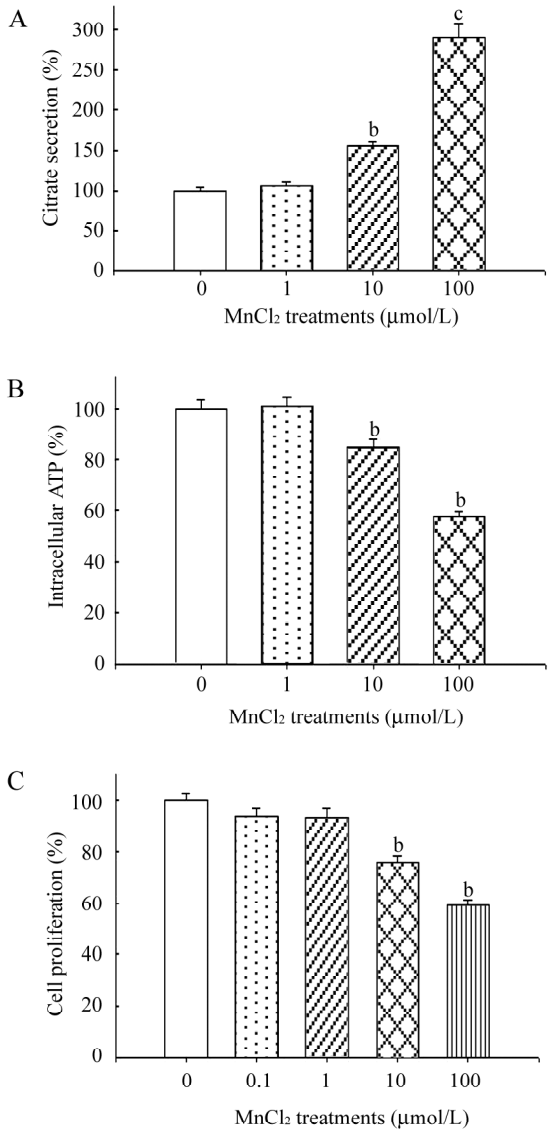

Figure 2. Effects of manganese chloride ($MnCl_2$) on intracellular adenosine triphosphate (ATP) levels, citrate utility and cell proliferation in PC-3 cells. Cells were treated with different concentrations of $MnCl_2$, as indicated, for 48 h. (A): Media were collected for the citrate assay. (B): Cells were harvested for determination of intracellular adenosine triphosphate (ATP) levels. Data are presented as the percentage (mean ± SEM, $n = 6$) of the citrate concentration and ATP levels resulting from $MnCl_2$ treatments, relative to the control samples with no treatment ($^bP < 0.05$; $^cP < 0.01$). (C): Cells (5 000 cells/well; $n = 8$) were incubated with $MnCl_2$ and the viable cell numbers were determined using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxylmethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay. Each treatment point represents the percentage (mean ± SEM) of stimulations of absorbance at 490 nm induced by $MnCl_2$ treatments relative to control samples with no treatment ($^bP < 0.05$; $^cP < 0.01$).

40% decrease of intracellular ATP levels in $MnCl_2$-treated cells in comparison to the control samples with non-treated groups (Figure 2B). The MTS assay showed that $MnCl_2$ treatments for 48 h inhibited cellular proliferation of PC-3 cells (Figure 2C).

A CMV enhancer/promoter containing the sequence for an IRE of human mACON cDNA-driving luciferase reporter vector (pCMVIREGL3) was constructed to observe the modulation of $MnCl_2$ on the gene translation of mACON. The results from the reporter assays indicated that $MnCl_2$ treatment (100 μmol/L) upregulated 5-fold of the luciferase activity of pCMVIREGL3, but had no effect on pCMVGL3 reporter vectors (Figure 3A). Continued study revealed that $MnCl_2$, similarly to FAC, upregulated the luciferase activity of the pCMVIREGL3 reporter vector through the IRE pathway, because this effect was blocked by deleting four nucleotides in the IRE sequence (Figure 3B). However, the immunoblot assay indicated that $MnCl_2$ treatment inhibited *mACON* gene expression in PC-3 cells (Figure 3C). The unexpected contrary results indicated that $MnCl_2$ induced gene translation, but decreased mACON protein abundance. Therefore, we continue to study the regulatory effect of $MnCl_2$ on the gene transcription of mACON. The transient gene expression with the reporter vector containing the DNA fragment, which is located at the 5'-flanking region (–158 to + 38) of the *mACON* gene, showed that $MnCl_2$ treatment affected *mACON* gene expression in a dosage-dependent manner (Figure 3D). The promoter activity of the *mACON* gene decreased by 45% when PC-3 cells were treated with 100 μmol/L $MnCl_2$. Although the inhibitory effect of $MnCl_2$ on the promoter activity of the human *mACON* gene in PC-3 cells was attenuated when cells were co-treated with FAC, the combination of FAC and manganese treatments did not restore 100% activity (Figure 4A). In combination with similar results shown in Figure 1C, this suggests that the regulation of mACON by $MnCl_2$ and FAC may not be through the same signal pathway. Several putative response elements, including the MRE, ARE, SRE and Sp1 binding sites, were found on the promoter of the *mACON* gene using simple DNA sequence analysis with the Genetics Computer Group (GCG) program (Accelrys, Inc., San Diego, CA, USA). Mutation of *MRE* from CTCGCCTTC to CTGATCCTTC using site-directed mutagenesis abolished the repressive effects of $MnCl_2$ treatment (Figure 4B). However, mutation of *ARE* upregulated the promoter activity of mACON after $MnCl_2$

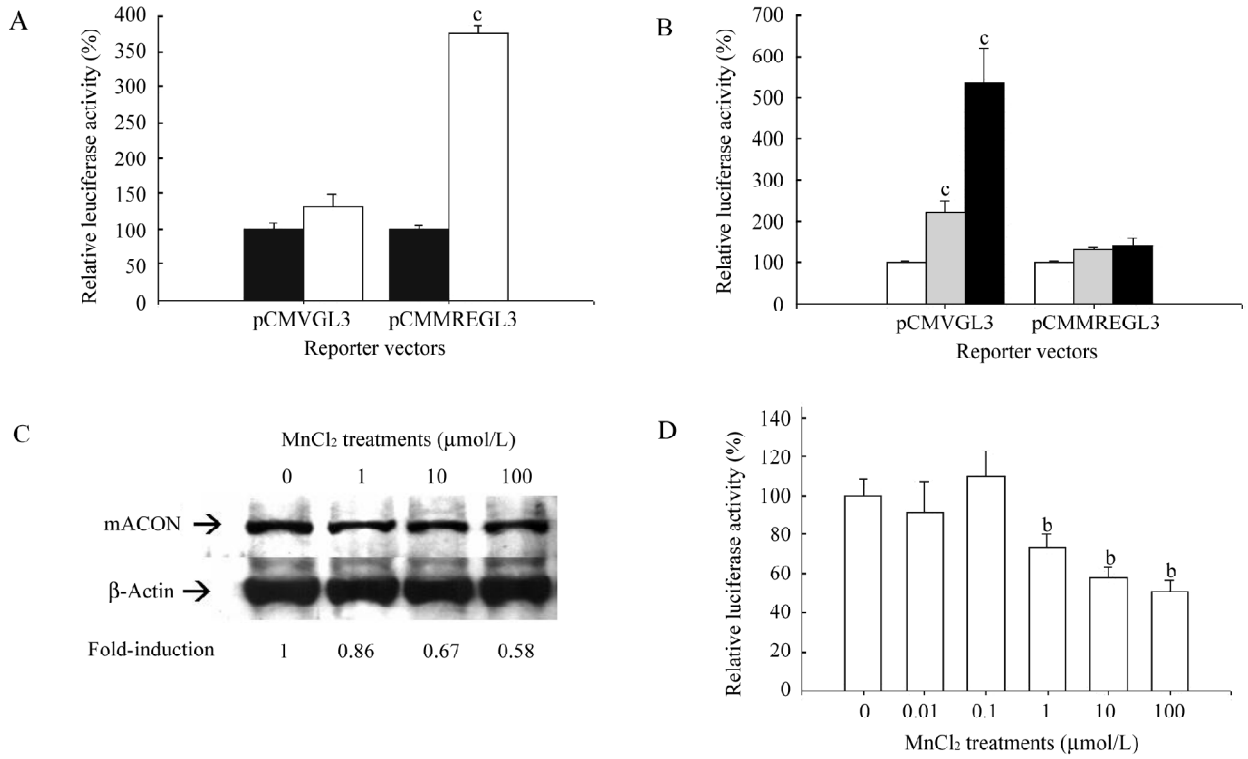

Figure 3. Modulation of manganese chloride ($MnCl_2$) on mitochondrial aconitase (*mACON*) gene expression in PC-3 cells. (A): $MnCl_2$ upregulates gene translation of mACON (black boxes represent control samples with no treatment and white boxes represent 100 μmol/L $MnCl_2$ treatment). (B): Regulation of $MnCl_2$ and ferric ammonium citrate on *mACON* gene expression is dependent on the loop/bulge of the iron response element on the *mACON* gene (white boxes represent control sample with no treatment; gray boxes represent 10 μg/mL ferric ammonium citrate (FAC) treatment; black boxes represent 100 μmol/L $MnCl_2$ treatments. (C): Cells were treated with different concentrations of $MnCl_2$, harvested and lysed to extract protein for use in the immunoblot assay with antibovine mACON antiserum (mACON) or antihuman β-actin antiserum (β-actin). (D): $MnCl_2$ downregulates the promoter activity of mACON. pGL188 reporter vector transfected PC-3 cells were treated with different concentrations of $MnCl_2$, as indicated. Experimental data are presented as the percentage (mean ± SEM) of luciferase activity resulting from $MnCl_2$ treatment relative to control samples with no treatment ($^bP < 0.05$; $^cP < 0.01$).

treatment. Results from EMSA also revealed an unknown transcription factor bound to this specific MRE sequence, which was inhibited by $MnCl_2$ treatment (Figure 4C).

4 Discussion

The incorporation of the antiknock additive methylcyclopentadienyl manganese tricarbonyl into gasoline worldwide has led to increased chronic environmental exposure to manganese [13]. Excessive manganese accumulation can result in adverse neurological, reproductive and respiratory effects in both laboratory animals and humans. Manganese is believed to exert toxicity by the formation of reactive oxygen species, disruption of cellular iron homeostasis, inhibition of complexes within the mitochondrial electron transport chain, generation of damaging radical quinones, or inhibition of total cellular aconitase activity [14]. The mechanisms of manganese toxicity in neurology have been well studied, and one of the major issues is the adverse effect of manganese on aconitase, which interferes with cellular iron homeostasis in the brain [8].

Manganese is found in all mammalian tissues with concentrations ranging from 0.1 μg/g to 4.9 μg/g of wet-tissue weight and from 4 μg/L to 14 μg/L in whole blood of normal adult humans; in contrast, manganese concentrations range between 3 μmol/L and 20 μmol/L in most tissues of adult rats [6, 14]. Manganese and iron have similar physical and chemical properties, and replacement of iron by manganese in the iron–sulfur cen-

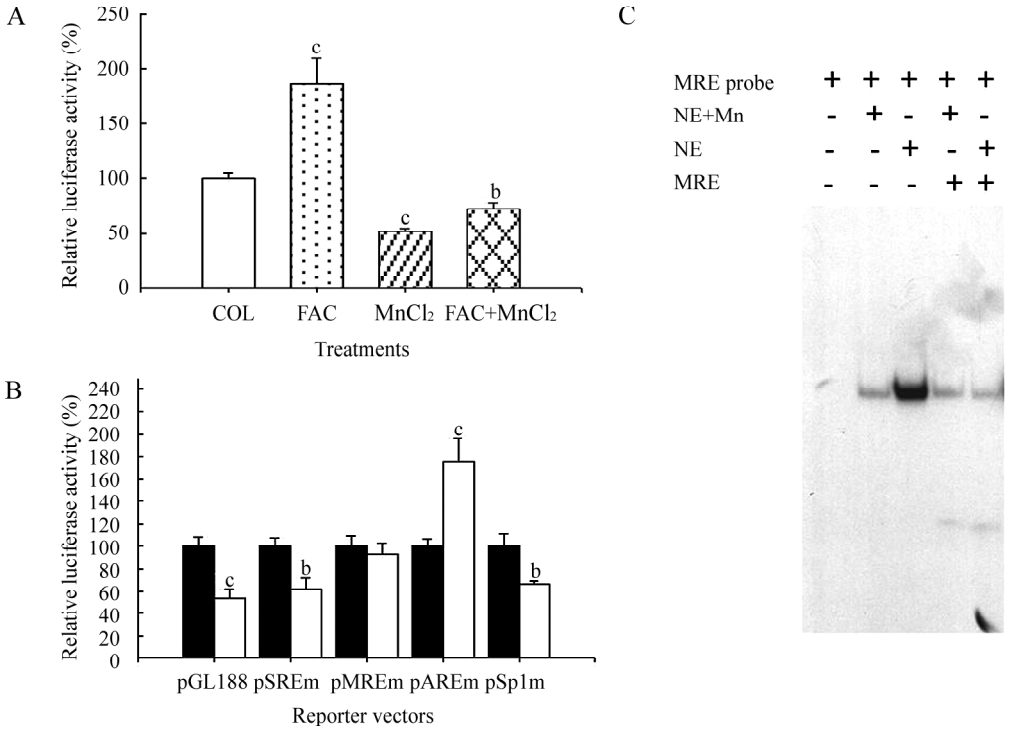

Figure 4. The putative metal response element (MRE) involves the modulation of manganese chloride ($MnCl_2$) on mitochondrial aconitase (*mACON*) gene expression in PC-3 cells. (A): pGL188 reporter vector-transfected PC-3 cells were treated with 100 μmol/L $MnCl_2$ (Mn), 10 μg/mL ferric ammonium citrate (FAC) or without treatment (COL). (B): PC-3 cells were transfected with different mutant-form reporter vectors (white boxes represent control samples with no treatment and black boxes represent 100 μmol/L $MnCl_2$ treatment). Experimental data are presented as the percentage (mean ± SEM) of luciferase activity resulting from $MnCl_2$ treatments relative to control samples with no treatment ($^bP < 0.05$; $^cP < 0.01$). (C): The electrophoretic mobility-shift assay used the 32 bp MRE oligonucleotide probe end-labeled with γ-^{32}P ATP (MRE probe) and nuclear extract (NE) from the PC-3 cells with (NE + Mn) or without (NE) $MnCl_2$ treatment. The gel shift disappeared when the reaction mixture was challenged with unlabeled double-stranded oligonucleotide (MRE) containing the putative MRE.

ter of aconitase results in inhibition of aconitase activity, as has been reported in published studies for more than two decades [1]. Results from the present study show that a non-toxic dose of manganese chloride significantly inhibits the enzymatic activity of mACON in human prostatic carcinoma cells. The inhibitory effect is reversible when the cells are co-treated with manganese and iron. This is the first report indicating that manganese inhibits mACON enzymatic activity in prostate cells *in vitro*.

Limiting mACON activity prevents citrate from entering the Krebs' cycle, which induces the accumulation and secretion of citrate from the prostate epithelial cells. This biochemical change in citrate production likely precedes early histopathological evidence of prostate malignancy. A bioenergetic hypothesis suggests that normal citrate-producing prostate epithelial cells become citrate-oxidizing malignant cells following transformation, which results in a net increase of 22 ATP/mol glucose and provides the energy for the activities associated with the process of malignancy [15]. In agreement with other studies, the results obtained here indicate that $MnCl_2$ inhibits the biosynthesis and enzymatic activity of mACON, the attenuation of citrate utility, cellular bioenergetic synthesis and the proliferation of PC-3 cells [2, 5, 11, 12]. The present study, along with a previous study from another laboratory, indicates that high concentrations (625 μmol/L–2.5 μmol/L) of manganese treatments could lead to the disruption of mitochondrial energy production [8].

Previous study from our laboratory indicated that FAC upregulation of *mACON* gene transcription may involve a putative antioxidant response element signal path-

way [5], instead of the metal response element involved in zinc and manganese regulation, as in the present study [12]. Together, these studies seem to suggest a regulatory link between energy utilization and metal metabolism in human prostatic carcinoma cells; this may be an important determinant of prostate cancer onset.

An increase in available cellular iron concentrations inhibits the binding of iron response element binding protein (IREP) to IRE, resulting in enhanced ferritin, mACON and 5-aminolevulinate synthase (eALAS) translation, and the repression of transferrin receptors [5, 16]. In the present study, we used the reporter vector with a CMV enhancer/promoter and the sequence of the human mACON IRE driving the luciferase to demonstrate that $MnCl_2$, similarly to FAC, upregulates the gene translation of mACON through the IRE pathway; moreover, $MnCl_2$ had greater efficacy (5-fold *vs.* 2-fold) than FAC did on the IRE signal pathway. The present study indirectly supports the hypothesis that manganese acts similarly to iron on cellular IREP–IRE binding activity, which alters cellular iron homeostasis [5, 8]. This result is also in agreement with other studies that have demonstrated that 200 μmol/L of manganese treatment inhibits the IREP–IRE binding activity in PC12 cells and human PLC hepatoma cells [17].

Although the results from the transient gene expression assay indicate that $MnCl_2$, similarly to FAC, enhances the translation of mACON, the immunoblot assay reveals that $MnCl_2$ treatment inhibits mACON protein abundance. The regulation of mACON promoter activity by $MnCl_2$ is blocked when the putative MRE mutates from CTCGCCTTCA to TGATCCTTCA. Results from EMSA also indicate that $MnCl_2$ inhibits unknown factors from binding to the MRE sequence, which blocks gene transcription of the mACON. The putative MRE sequence on the mACON promoter is homologous to the MRE (HTHXXGCTC; H = A, C or T; X = any residue) on the metallothionein genes and the Cu, Zn-superoxide dismutase gene in *Saccharomyces cerevisiae* [18]. The putative ARE sequence of the mACON promoter is homologous to TGACNNNGC, which has been demonstrated as the antioxidant binding site for NF-E2 relative factor 2 and Maf proteins in metal-inducible genes [19]. Although our previous study indicated that FAC upregulation of *mACON* gene transcription may also occur through the ARE signal pathway, results from the present study show that the ARE sequence in the mACON promoter is a negative regulation element in manganese treatment. The mechanism of neurotoxicity of manganese is partially a result of induction of oxidative stress, as shown in rat brains [20]. The contrary mechanism of FAC and $MnCl_2$ regulation on *mACON* gene transcription through the ARE-like recognition sequence needs further investigation.

The present study demonstrates that $MnCl_2$ competes with iron, which leads to inhibition of mACON enzymatic activity and interrupts citrate utility in human prostatic carcinoma cells *in vitro*. Although $MnCl_2$ can act as iron does to upregulate *mACON* gene translation through the bulge/loop of the IRE, $MnCl_2$ blocks the gene transcription of mACON through the MRE pathway.

Acknowledgment

The authors thank Dr R. B. Franklin for his generosity in providing the mACON antibody. This study was supported by research grants CMRP G1006 and G34026 from Chang Gung Memorial Hospital and by grants NSC 94-2320-B-182-017 and NSC 94-2314-B-182A-155 from the National Science Council (Taiwan, China).

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