Cholesterol Modulation of the Expression of Mitochondrial Aconitase in Human Prostatic Carcinoma Cells

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Abstract

Mitochondrial aconitase (mACON) is the key enzyme for the citrate oxidation in the mitochondrial Krebs cycle. Cholesterol treatment (10 μg/ml of cholesterol and 1 μg/ml of 25-hydroxycholesterol) for 24 h stimulates mACON enzymatic activity in human prostatic carcinoma cells (PC-3) and hepatoma cells (HepG2). Mevastatin, a cholesterol synthesis antagonist, blocked the effect of cholesterol treatment on mACON. The cholesterol treatment stimulated mACON enzymatic activity, which enhanced the citrate utility but decreased intracellular ATP levels in PC-3 cells. The immunoblotting and transient gene expression assays demonstrated that cholesterol treatment enhances the gene expression of mACON. Mutation of the putative sterol response element (SRE) from GACGCCCCACT to GACGCCCATAT abolished the stimulating effects of cholesterol on the promoter activity of mACON gene. The results suggest that cholesterol treatment induces the mACON gene expression through the SRE signal transduction pathway. Our study demonstrated the deregulation of cholesterol on the citrate metabolism.

Key Words: prostate, cholesterol, citrate, mevastatin, PC-3, HepG2, ATP, sterol response element

Introduction

Mitochondrial aconitase (mACON; aconitase hydratase, EC4.2.1.3) is the enzyme responsible for the interconversion of citrate and isocitrate in the citric acid cycle (9). Citrate oxidation *via* the Krebs cycle is the primary pathway for energy production from carbohydrate and fat metabolism; however, the citrate synthesized by prostate cells is, for the most part, accumulated and secreted rather than oxidized (5). In the bioenergetic theory of prostatic malignancy, Costello and Franklin (6) suggested that normal citrate-producing prostatic epithelial cells become citrate-oxidizing cells following a transformation in which the mACON is not limiting. Previous *in vitro* study demonstrated that stable transfected mACON antisense human prostate carcinoma cells, PC-3, have lower citrate utility, intracellular ATP levels and cell proliferation than the mock-transfected cells (14).

The citrate appears to function as a feed-forward allosteric activator of the acetyl-CoA carboxylase, the rate-controlling enzyme of fatty acid biosynthesis (18, 19). In some tumor cells with high glycolysis the citrate extrudes from the mitochondria to the cytosol in order to feed cholesterol synthesis. The cholesterol in turn increases in mitochondrial membranes influenced the proton permeability, which regulates the ability to make ATP (1). However, an early report indicated that increasing the cholesterol in mitochondrial membranes is not sufficient enough to induce "truncation" of the citric acid cycle in rat liver cells (8). Although most of the nonheptatic cells do not express the bile acid synthetic pathway, cholesterol homeostasis is still tightly regulated by negative feedback controlling synthesis and uptake (27).

The human prostatic adenocarcinoma cells, LNCaP,

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express all enzymes required for endogenous lipogenesis and most, if not all, enzymes are controlled by androgens (28, 29). On the other hand, the mevalonate and cholesterol pathway for sterol biosynthesis and cholesterol metabolism, which are induced by stress, have been demonstrated in the androgen receptor-negative human prostatic carcinoma cells, PC-3 (26). The diazepam-binding inhibitor/acyl-CoA-binding protein (DBI/ACBP), a polypeptide has been implicated in acyl-CoA binding and transport, steroid genesis, and peptide hormone release, also is identified in the human prostatic epithelium cells (30). Those lipogenetic enzymes and DBI/ACBP have been identified as sterol regulatory element binding protein (SREBP) responsive gene, and androgen stimulates the expression of SREBP transcription that induces transcriptional activity of these genes in the human prostatic carcinoma cells (31). Other study indicated that overexpression of the peripheral-type benzodiazepine receptor, a cholesterol transporter, as a prognostic indicator of the aggressive phenotype in the prostate cancer suggesting that cholesterol metabolism is important in the prostate pathology (23). Therefore, the objectives of the present study are to determine the regulation of mACON and citrate metabolism by cholesterol in the human prostatic carcinoma cells, PC-3 cells, and human hepatoma cells, HepG2.

Materials and Methods

Cell Culture and Chemicals

PC-3 and the HepG2 cell lines were obtained from the American Type Culture Collection (ATCC). The culture media were purchase from Life Technologies (Rockville, MD, USA). The fetal bovine serum (FBS) was obtained from HyClone (Logan, UT, USA). The cholesterol, 25-hydroxcholesterol, mevastatin, and lipoprotein-free fetal bovine serum (LPF-FBS) were purchased from Sigma (St. Louis, MO, USA). The BCA protein concentration assay kit was purchase from PIERCE (Rockford, IL, USA). The cells were maintained in RPMI 1640 containing 10% fetal bovine serum and the medium was changed twice a week. The cells which were precultured in RPMI 1640 medium with 5% LPF-FBS for 48 h before treatment were regarded as cholesterol starved.

Mitochondrial Aconitase Activity Assay

After cholesterol treatments (10 μg/ml cholesterol and 1 μg/ml 25-hydroxycholesterol) for 24 h, the mACON enzymatic activity of cells was determined after treating cells with digitonin by the NADH-coupled assay as described previously (14). The reaction was carried out in 100 mM Tris-HCl, 1 mM $MgCl_2$, 1 mM $NADP^+$, 1 U of ICDH (isocritate dehydrogenase), and 1 mM citrate. The mACON enzymatic activities of the cells were adjusted according to the concentration of protein in the mitochondrial extract, which was measured using a BCA protein assay kit.

Western Blotting of Human Mitochondrial Aconitase and β-Actin

Cells were lysed with lysing buffer [62.5 mM Tris (pH 6.8), 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 7 M urea, 5 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride] after cholesterol treatments as indicated. Equal amounts of proteins were separated by 7.5% SDS-polyacrylamide gel and analyzed with an ECL detection system as described by the manufacturer (Amersham BioSciences, New Territories, Hong Kong). The blot was probed with diluted 1:500 bovine mACON antiserum (kindly given by Dr. R.B. Franklin) and 1:1000 diluted β-actin antiserum (C11, Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Citrate and Intracellular ATP Assay

Cells (1×10^5 cells/well) were cultured in 6-well-plates until 80% confluent. After cholesterol treatment, the citrate concentrations in the media and the concentrations of intracellular ATP were determined using an ATP assay kit as described previously (16). The citrate concentrations in the medium and the intracellular ATP were adjusted according to the protein concentrations of the whole cell extract, as determined using the BCA protein assay kit.

Luciferase and β-Galactosidase Assay

The human mACON gene containing 18 exons (GenBank accession no. HSACO2G01-HSACO2G17) was isolated and sequenced in our laboratory. An *EcoRI* digested 9-kb DNA fragment was cloned and identified as the 5'-flanking region of the mACON gene. One DNA fragment (-158 to +38) was cloned into the luciferase reporter vector (pGL3; Promega Biosciences, San Luis Obispo, CA, USA) designed as pbGL188 by digesting this 9-kb DNA fragment with *Sac I* and *Xho I* as described previously (15). The reporter vector that contains the mutation of SRE (pbGLSREm) was constructed by using the QuikChange site-directed mutatgenesis kit (Stratagene, La Jolla, CA, USA) using the complement double-strand primers 5'GCCTTCACCGTGACGCCCATATCTTCCGGGCACGC-3' (underlined sequence represented the mutation site; only present the sense strand). The reporter vector that contains the mutation of the cAMP response element (pbGLCREm) was constructed as described previously

(15). Cells were plated onto 24-well plates at 1×10^4 cells/well 1 day prior to transfection. Cells were transiently transfected using TransFast transfection reagent (0.6 μg/well; Promega Biosciences) with 0.5 μg/well of the non-sterol-regulated cytomegalovirus promoter β-galactosidase expression vector (pCMVSPORTβgal; Life Technologies) and 1 μg/well of pbGL188 reporter vector or mutant forms of reporter vectors in the serum reduction medium (OPTI-medium; Life Technologies) for 4 h. The cells continued to incubate in RPMI 1640 with 5% FBS until treated with cholesterol in RPMI-1640 medium with 5% LPF-FBS for 24 h. For the cholesterol starvation experiment, the cells were cultured in RPMI-1640 medium with 5% LPF-FBS for an additional 24 h, and with or without cholesterol treatment in the same medium for the next 24 h thereafter. The reaction was stopped by adding 200 μl of Luciferase Cell Culture Lysis reagent (Promega Biosciences); the cell extracts were then assayed for the activities of luciferase and β-galactosidase as described previously (17).

Electrophoretic Mobility Shift Assays

The nuclear extract of PC-3 cells was extracted with the NE-PER nuclear and cytoplasmic extraction reagents as described by the manufacturer (PIERCE). The double-strand of the putative SRE (5' GCCTTCACCGTGACGCCCCACTCT TCCGGC-ACGC-3') is the 5'-end labeled with γ-P^{32}ATP by T_4 polynucleotide kinase. The 5'-end-labeled SRE was incubated with nuclear extract from PC-3 cells and the binding-shift was challenged with wild-type double stranded SRE or the mutated form of double strand SRE (5'-GCCTTCACCGTGACGCCCATATCT-TCCGGGCAC GC-3') without 5'-end labeled with γ-P^{32}ATP. The protocol for the gel-shift assay was that described before (32).

Statistical Analysis

Results are expressed as means ± SEs of at least three independent replication of each experiment. Statistical significance was determined using Student's paired-*t* test analysis with the SigmaStat program for Windows version 2.03 (SPSS Inc, Chicago, IL, USA).

Results

The PC-3 cells and the HepG2 cells were cultured in the RPMI 1640 medium with 10% FBS until 80% confluent. The culture media were changed to PRMI 1640 medium containing 5% LPF-FBS with or without cholesterol treatment (10 μg/ml cholesterol and 1 μg/ml 25-hydroxycholesterol) for an additional 24 h. Results indicated that cholesterol treatment significantly stimulated the mACON enzymatic activity in both PC-3 (Fig. 1A) and HepG2 cells (Fig. 1B). However, the increase of the mACON enzymatic activity in the PC-3 cells was more significant than in the HepG2 cells after cholesterol treatment. The stimulating effect of cholesterol on the mACON enzymatic activity was blocked by treating cell with mevastatin, a cholesterol synthesis antagonist (Fig. 1C & 1D).

Interestingly, the culture conditions affected the activities of mACON in different cell types. When cells were cholesterol starved (precultured in PRMI 1640 medium with 5% LPF-FBS for 48 h) before the cholesterol treatment, we found that cholesterol treatment inhibited the mACON enzymatic activity of the PC-3 cells but not the HepG2 cells (Fig. 1A & 1B). It seemed that cholesterol had a divergent effect on the mACON enzymatic activity of the prostate cells. Moreover, the endogenous mACON enzymatic activity in the PC-3 cells after cholesterol starvation for 48 h (0.148 ± 0.003 U/μg mitochondria) significantly decreased by about 60% compared to the PC-3 cells that were cultured in the normal medium (0.727 ± 0.029 U/μg mitochondria). The endogenous mACON enzymatic activity in the HepG2 cells after cholesterol starvation also decreased by 30%. The results suggest that HepG2 cells are more restricted to the starvation medium than PC-3 cells.

It is suggested that limiting of mACON activity prevents citrate from entering the Krebs cycle. Our results indicated that cholesterol stimulates mACON enzymatic activity, which significantly blocked 30% of citrate secretion from the PC-3 cells (Fig. 2A). The mean citrate concentration in the medium was 4.12 ± 0.16 nM/mg of cells for PC-3 cells and 0.74 ± 0.04 nM/mg of cells for HepG2 cells. However, cholesterol treatment only showed a little effect on the citrate secretion from the HepG2. Our results, unexpectedly, showed that cholesterol treatments do not enhance, but inhibit, the intracellular ATP biosynthesis of both PC-3 cells and HepG2 cells (Fig. 2B & 2D). The mean intracellular ATP level was 264.75 ± 19.59 pmol/μg of cells for PC-3 cells and 140.52 ± 4.97 pmol/μg of cells for HepG2 cells. It seemed that cholesterol treatments may also interrupt other bioenergetic pathways in prostatic carcinoma cells.

The immunoblot assay demonstrated that cholesterol's divergent effect on mACON expression of the PC-3 cells depended on the pretreatment conditions (Fig. 3B). A putative SRE (CGTGACGCCCCACTC) was found in the sequence of the DNA fragment (-158 to +37) containing the mACON promoter, according to simple sequence analysis (Fig. 3A). This putative SRE contained the consensus sequences of MLTF-HMGCoAred (CGTGAC), CNBP-SRE (STGSSGYG), and (Sp1)-U2snR.2 (ACGCCC) (13, 21, 22). The

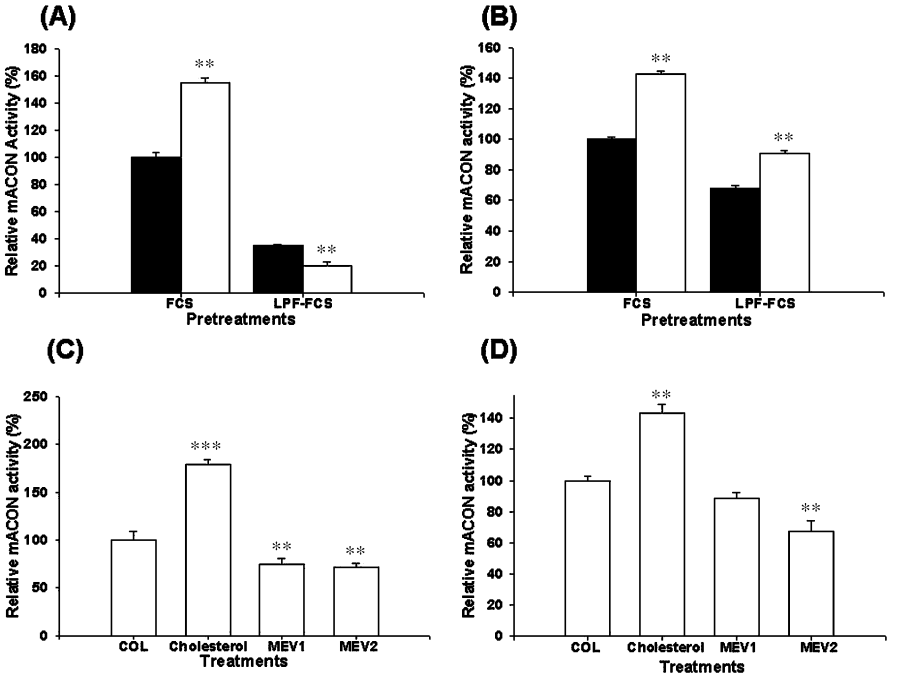

Fig. 1 Divergent modulation of cholesterol treatment on the enzymatic activity of the mitochondrial aconitase of PC-3 cells and HepG2 cells. PC-3 cells (A) and HepG2 cells (B) were treated with or without cholesterol starvation for 48 h. Cells were mock-treated with 0.1% ethanol (control group; black box) or were treated with 10 g/ml of cholesterol and 1 μg/mL of 25-hydroxycholesterol (cholesterol treatment group) for an additional 24 h. PC-3 cells (C) and HepG2 cells (D) were treated with 0.1% ethanol (control group; n = 5), cholesterol treatment, 5 μM mevastatin (MEV1), or 50 μM mevastatin (MEV2) for 24 h without cholesterol starvation pretreatment. Mitochondria particles were prepared and the enzymatic activity of mACON was determined as described under "Materials and Methods." Data from experiments are presented as mean percentage ± SE (n = 5) of the enzymatic activity induced by cholesterol treatments relative to sample with control treatment (**represents $P < 0.05$).

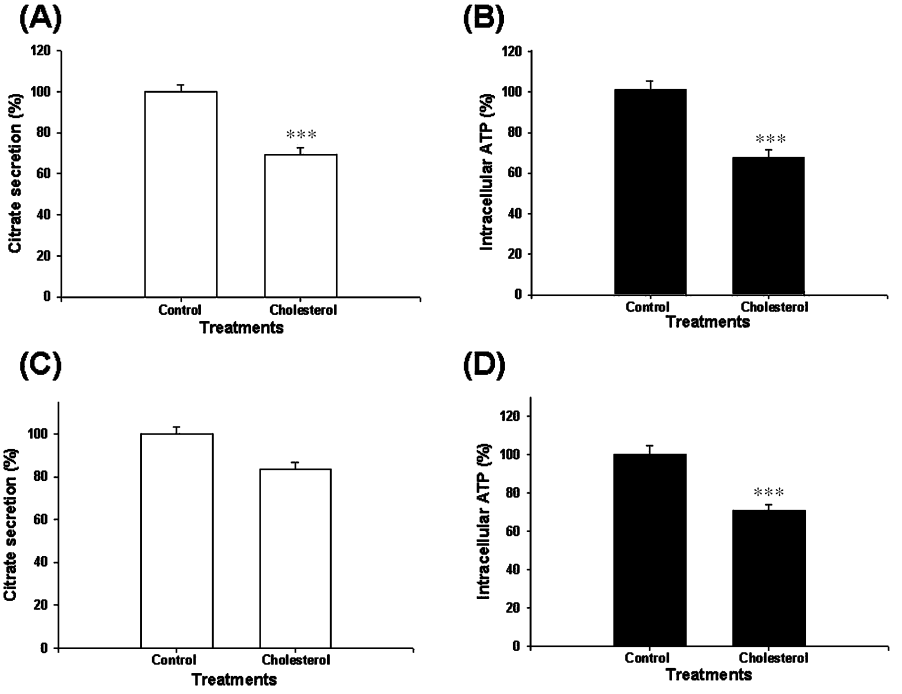

Fig 2 Cholesterol treatment modulation of citrate secretion and intracellular ATP levels in PC-3 and HepG2 cells. PC-3 Cells (A, C) and HepG2 cells (B, D) were cultured in RPMI1640 medium with 10% FBS until 70%-80% confluent. After treating with 0.1% ethanol (control group) or 10 μg/mL of cholesterol and 1 μg/mL of 25-hydroxycholesterol (cholesterol group) in RPMI1640 medium with 5% CF-FBS for 48 h, cells were harvested for determination of intracellular ATP levels and the media were collected for citrate assay as described under "Materials and Methods". Data from experiments are presented as mean percentage ± SE (n = 6) of the citrate concentration and ATP levels induced by cholesterol treatments relative to samples with control treatment (***represents $P < 0.01$).

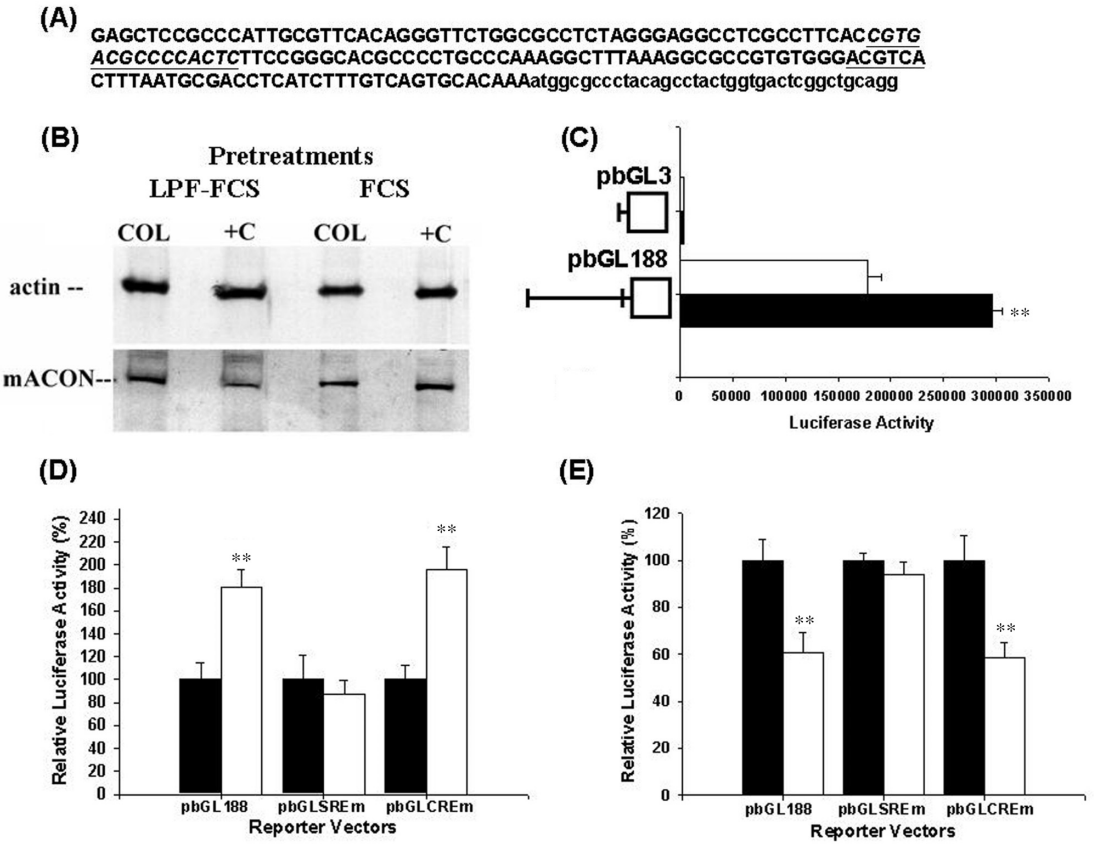

Fig. 3 Cholesterol treatment modulation of mACON gene transcription is dependent on the sterol response element. (A) Promotor sequence of the mACON gene that was cloned in the pbGL188 reporter vector (italic underline represents the putative sterol response element, plain underline represents the cAMP response element and the small capitals represent the first exon of the human mACON gene). (B) PC-3 cells were treated with 0.1% ethanol (COL) and cholesterol treatment (+C) for 24 h with (LPF-FBS) or without (FBS) the pretreatment of cholesterol starvation for 48 h. The mitochondrial aconitase (mACON) and the β-actin (actin) of PC-3 cells after cholesterol treatment were determined using immunoblot assay. (C) Transient gene expression indicated that cholesterol treatment increases the mACON promoter activity of PC-3 cells. The transfected-cells were mock-treated with 0.1% ethanol (black box; n = 6), and cholesterol treatment (white box) with (E) or without (D) the pretreatment of cholesterol starvation for 24 h. Data are from experiments done in quadruplicate and are presented as the mean percentage of stimulation (± SE) of luciferase activity induced by cholesterol treatments relative to control treatment, with **indicating $P < 0.05$.

sequence is homologous to the consensus sequence of the sterol regulatory element 1 (5'-ATCACCCCAC-3') (10). The luciferase activity assay indicated that the reporter vectors (pbGL188) contained the cholesterol response element within the mACON promoter (Fig. 3C). Results from transient gene expression assays also indicated the cholesterol divergent effect on mACON promoter activity of PC-3 cells. The cholesterol treatment significantly increased the mACON promoter activity in PC-3 cells (Fig. 3D); however, the cholesterol treatment significantly inhibited the mACON promoter activity when the transfected cells were cholesterol starved for 48 h before cholesterol treatment (Fig. 3E). The result is similar to the finding from the assay of mACON enzymatic activity and immunoblot assay after the cholesterol treatment. When the SRE mutated from GACGCCCACT to GACGCCCATAT, the transient gene expression assay indicated that the cholesterol treatment loss the effect on the promoter activity of the mACON gene (Fig. 3C & 3D). However, mutation of the cAMP response element (CRE) to AGAGCT did not modify the effects of cholesterol on mACON promoter activity.

The electrophoretic mobility shift assays indicated that the nuclear extract from PC-3 cells bound to the P^{32}-labeled double-stranded oligonucleotide containing the putative wild-type SRE of the mACON promoter. However, this observed gel shift disappeared when the reaction mixture was challenged with unlabeled double-stranded oligonucleotide containing the SRE, but not for the oligonucleotide featuring a mutation at the SRE (Fig. 4). Our results suggest that the cholesterol treatment deregulates the expression of mACON gene through the SRE pathway in human prostatic carcinoma cells.

Discussion

Previous studies suggest that citrate oxidation by mACON is a rate limiting step in prostatic citrate metabolism by demonstrating the high citrate: isocitrate ratio of about 30:1 in the rat ventral prostate (5). *In vitro* studies using human carcinoma cells suggest that the regulation and the metabolic pathway of citrate synthesis and oxidation found in the rat ventral prostate could be extrapolated to human prostate carcinoma (14-17). Costello and Franklin proposed that the biochemical change in the citrate production likely preceded the histopathological identification of malignant cells, and the decrease in citrate production occurred early in malignancy (7).

It has been suggested that the increase in cholesterol is directly responsible for the deviations of mitochondrial functions. In the Coleman's (3) truncated Krebs cycle hypothesis, it is suggested that elevated cholesterol in the mitochondrial inner membrane induces a kinetic alteration of the tricarboxylate carrier that restricts intramitochondrial metabolism of citrate through aconitase in favor of its export to the cytoplasm where the ATP citrate lyase generates the acetyl-CoA necessary for *de novo* cholesterol synthesis. In glycolytic liver tumor cells, the citrate is preferentially extruded from the mitochondria to the cytosol where its two carbon units (acetyl-CoA) feed an already deregulated cholesterol synthesis (24, 25). However, other reports indicate that the excessive membrane cholesterol is not responsible for the citrate metabolic and bioenergetic changes of the hepatoma mitochondria (8). Correlation studies between rates of *de novo* cholesterol synthesis and cellular citrate concentration led to controversial results because these studies had serious limitations in that citrate concentrations were determined for the whole tissue rather than for the extramitochondrial compartment where cholesterol synthesis occurs. The hepatoma cells are not citrate-producing cells but citrate utilizing cells, which may account for the result that cholesterol treatment had only a small effect on citrate secretion from the HepG2 cells. The reason why cholesterol treatments did not downregulate mACON enzymatic activity after cholesterol-starvation in HepG2 cells in this study may have been due to that effect. HepG2 cells have greater cholesterol buffering capacity than the PC-3 cells (20); moreover, HepG2 cells (2.078 ± 0.027 U/μg mitochondria) have higher endogenous mACON enzymatic activities than PC-3 cells (0.727 ± 0.029 U/mg mitochondria).

Early studies showed that prostatic carcinoma cells significantly accumulated triglycerides and cholesterol ester after androgen treatments or heat shock (26, 28). Cholesterol homeostasis is tightly regulated in prostatic epithelial cells and may have significant implications in carcinogenesis. Several SREBP genes including acetyl-CoA carbocylase-α, Farnesyl diphosphate synthase, and HMG-CoA synthase may be critical downstream genes mediating a subset of androgen effects in the human prostate (12, 29). Our results demonstrate that cholesterol modulated mACON enzymatic activity in the hepatic and nonhepatic cells, and suggested that mACON may be involved in cholesterol homeostasis in the human prostate.

Although cholesterol treatments significantly decreased the citrate secretion from the PC-3 and HepG2 cells, our results indicated that the cholesterol treatments decreased the intracellular ATP levels of both cell types. The amount of ATP represents the actual energetic needs of the cell and may be an indication of the cell biochemical state. Another study suggested that latent ATPase activity increases 2 to 3-fold as a function of the extent of cholesterol enrichment in normal and tumor cells (4). Whether

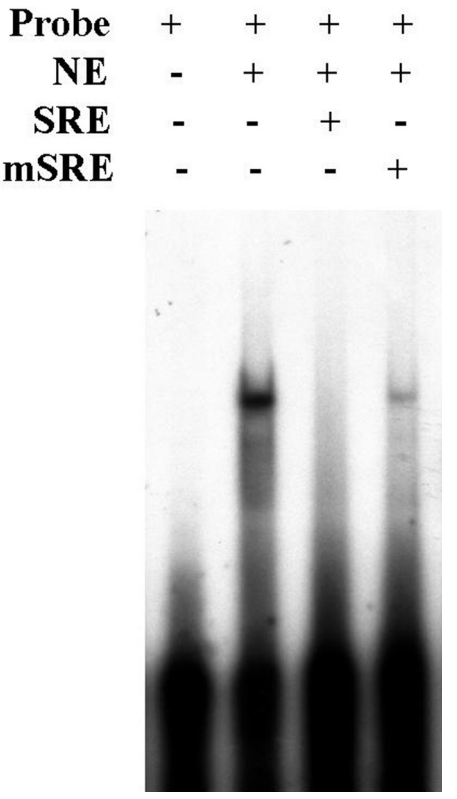

Fig. 4 PC-3 cell nuclear protein binding to the putative sterol response element of the human mitochondrial aconitase promoter. Electrophoretic mobility shift assay was performed as described under "Materials and Methods" using the 34-bp wild-type SRE oligonucleotide probe end-labled with ^{32}P (probe) and nuclear extract from PC-3 cells (NE). The gel shift disappeared when the reaction mixture was challenged with a 50-fold concentration of unlabeled double-stranded oligonucleotide containing the SRE but not the oligonucleotide featuring a mutation at the SRE (SREm).

cholesterol treatment in this study may directly control ATP biosynthesis or ATP utility pathways needs to be further investigated.

The sterol regulatory element-binding proteins (SREBP) are proposed to play a central role in coordinating control of lipogenic enzymes of the two major lipogenic pathways, the fatty acid synthesis and the cholesterol synthesis pathways (2, 33). Three SREBPs (SREBP-1a, -1c, and -2) stimulate the transcription of genes involved in the synthesis and the receptor-mediated uptake of cholesterol and fatty acids; however, SREBP-2 produces a higher ratio of cholesterol synthesis over fatty acid synthesis than does SREBP-1 (11). Although the mechanisms for cholesterol modulated mACON gene expression remains unknown, our result from transient gene expression with mutatgenesis indicated that cholesterol treatment regulates mACON gene expression dependent on the SRE, but not on the CRE, of the mACON promoter. Previous study indicated that the nitrosoinum ion was a signaling molecule for human mACON expression through the cAMP signal transduction pathway, which is specific in the prostate (15).

In summary, our study showed that cholesterol increased mACON gene expression of human prostatic carcinoma and heptoma cells and characterized a sterol response element in the promoter of the human mACON gene. The results indicated that human mACON is a sterol-regulation gene and demonstrated that cholesterol deregulates citrate acid metabolism.

Acknowledgments

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