Prolactin Specifically Regulates Citrate Oxidation and m-Aconitase of Rat Prostate Epithelial Cells

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The prostate gland of many animals, including humans, produces and secretes extremely high levels of citrate. To achieve this function, prostate secretory epithelial cells possess unique metabolic properties that permit accumulation and ultimate secretion (net citrate production) of citrate. Mounting evidence continues to support the concept that prostate epithelial cells possess a limiting mitochondrial (m)-aconitase activity that minimizes citrate oxidation and results in the accumulation of citrate synthesized by the cells. Recent studies have revealed that prolactin (PRL) stimulates net citrate production of rat lateral prostate (RLP). The mechanism of this PRL effect has not been established. The current studies were concerned with the possibility that PRL might be involved in the regulation of citrate oxidation and m-aconitase of prostate cells. Studies were conducted with RLP, RVP (rat ventral prostate), RDP (rat dorsal prostate), and kidney cells. The results showed that PRL in vitro and in vivo decreased citrate utilization and the level of m-aconitase in RLP cells, and conversely increased citrate utilization and m-aconitase in RVP cells. Furthermore, PRL had no effect on either RDP or kidney cells. The effects of PRL on both citrate utilization and m-aconitase of RLP and RVP were abolished by cycloheximide and actinomycin. Mitochondrial studies revealed that PRL decreased citrate oxidation of RLP and increased citrate oxidation of RVP, but had no effect on isocitrate oxidation. In conclusion, these studies establish that PRL has a physiological role in the regulation of citrate oxidation in prostate, and that this action is associated with PRL regulation of the biosynthesis of m-aconitase. Furthermore, the effects of PRL are cell-specific and targeted at m-aconitase.

THE PROSTATE GLAND of humans and other animals has the unique function of accumulating and secreting extraordinarily high levels of citrate. This function results from the unique citrate-producing metabolic capability of prostate secretory epithelial cells. In contrast, essentially all other cells oxidize citrate as a major source of energy or use it as a source of acetate for lipogenesis. For details of the biochemical pathway of net citrate production by prostate cells, we refer to our recent review articles.[1,2] The most important metabolic characteristic of these cells is the limited ability to oxidize citrate due to a rate-limiting mitochondrial (m)-aconitase (EC 4.2.1.3) activity. Consequently, most of the citrate synthesized by these cells is accumulated and ultimately secreted as a major component of prostatic fluid.

The function of prostate citrate production is under endocrine hormone regulation, as reviewed previously.[2] Testosterone had long been considered the major hormonal regulator of prostate growth and function, including citrate production. However, it is becoming evident that prolactin (PRL) is also an important regulator of prostate citrate production.[2,3-6] Recent studies[7-9] have demonstrated that PRL stimulates mitochondrial aspartate aminotransferase (mAAT) and pyruvate dehydrogenase (PDH) activities, the two key regulatory enzymes involved in citrate synthesis by prostate epithelial cells.

Net citrate production is determined by the relative rates of citrate synthesis and citrate utilization. An increase in net citrate production can result from an increase in citrate synthesis and/or a decrease in citrate utilization. Since current evidence demonstrates that PRL increases citrate synthesis, it became essential to determine its effect on citrate utilization. Since citrate-producing prostate cells are characterized by a limited m-aconitase activity and limited citrate oxidation, the effect of PRL on these relationships had to be established. The present studies provide the first report of the role of PRL in the regulation of prostate citrate oxidation and the cell-specificity of PRL effects. The studies establish that PRL is an important physiological regulator of prostate citrate oxidation and that m-aconitase is the key enzyme involved in this regulation.

MATERIALS AND METHODS

These studies were conducted with male Wistar rats weighing 250 to 350 g. Handling and treatment of rats were in accordance with National Institutes of Health (NIH) guidelines. Preparation of the cells and mitochondria isolated from prostatic tissue has been described previously.[10-12] Purified rat PRL (25 IU/mg) was obtained from the NIH National Hormone and Pituitary Program.

In vivo experiments involving bromocryptine and PRL treatment of rats were conducted as follows. Bromocryptine treatment involved two successive subcutaneous injections (1 mg per rat) administered 24 hours apart. The animals were killed 16 hours after the second injection. Control animals were similarly treated with bromocryptine vehicle. This treatment induced a marked hypoprolactinemia (see the Results). In experiments involving PRL treatment of bromocryptine-treated rats, rat PRL (0.5 mg per animal) was injected subcutaneously at the time of the second bromocryptine injection and repeated 12 hours later. The rats were killed 4 hours after the second PRL injection. Control rats were injected with PRL vehicle. This protocol is based on our earlier studies demonstrating that these conditions reversed the hypoprolactinemic effects of bromocryptine treatment.[9] After completion of the treatment regimen, the rats were killed and the appropriate tissues were resected and placed in cold Hanks balanced salt solution or buffered sucrose solution (50 mmol/L HEPES in 250 mmol/L sucrose, pH 7.2). The resected tissues were immediately used for cell or mitochondrial preparations.

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For studies involving in vitro effects of PRL, the following procedure was used. Normal rats served as donor animals. The appropriate tissues were resected from eight to 10 rats, and fresh cell preparations were obtained. Cells were suspended in buffered sucrose. Aliquots of cell suspension were incubated with PRL (0.5 μg/mL) or vehicle in reaction flasks. Incubation was conducted in reaction flasks placed in a metabolic shaker at 37°C for 3 hours. These conditions were based on our earlier study[9] that demonstrated an in vitro effect of PRL on PDH of rat prostate over the range of 0.1 to 1.0 μg/mL PRL. This approximates the physiological range of PRL.

Citrate utilization of cells was determined as follows. Aliquots (1.0 mL) of cell suspension obtained from in vivo or in vitro experiments were pipetted into reaction flasks. Citrate (0.8 mmol/L) was added to each flask. Zero-time flasks were obtained by immediate addition of trichloroacetic acid ([TCA] 7% final concentration). The remaining flasks were incubated for 60 minutes at 37°C in a Dubnoff metabolic shaker. TCA was added to the remaining flasks. The contents of each flask were centrifuged at 1,000 × *g* for 5 minutes. The protein-free extract was assayed for citrate by fluoroenzymatic assay.[13] The difference in citrate between zero-time and 60-minute incubation represents citrate utilization. The protein concentration of cell suspensions was also determined,[14] and citrate utilization is reported as citrate used per 60 minutes per milligram protein. All reaction flasks were prepared in triplicate.

Citrate and isocitrate oxidation was determined as follows. The mitochondria preparation from prostate tissue was obtained. The mitochondrial suspension was sonicated to eliminate permeability problems. Aliquots of mitochondrial suspension were incubated with either citrate or isocitrate (0.4 mmol/L) and NADP (0.8 mmol/L) in a final volume of 0.5 mL buffered sucrose medium. The reaction tubes were placed on a rotary shaker at 26°C for 30 to 60 minutes. Reactions were terminated by addition of TCA (7% final concentration). The TCA extract was assayed for citrate and isocitrate. Aliquots of mitochondrial preparation were also assayed for protein. Citrate and isocitrate oxidation is expressed as the decrease in substrate per 60 minutes per milligram protein. All reactions were prepared in triplicate. Citrate and isocitrate oxidation was linear over the course of the incubation period.

The level of m-aconitase enzyme was determined by Western blots using procedures previously described.[10] The m-aconitase antibody (kindly provided by Dr Paul Srere) was raised against purified porcine heart m-aconitase. Results of this study demonstrate that the antibody cross-reacts with rat tissue m-aconitase. Furthermore, the antibody is specific for m-aconitase in that cytosolic (c-)aconitase is not detected. This was confirmed by the absence of any detectable aconitase in cytosolic preparations, whereas whole homogenate and mitochondrial preparations elicited positive signals with the antibody. We used highly purified m-aconitase (kindly provided by Dr Mary Claire Kennedy) and partially purified m-aconitase (Sigma, St Louis, MO) as standards for identification of m-aconitase in the rat tissue. An 82-kd band, which is consistent with the molecular weight of m-aconitase, was identified as m-aconitase. The immunoblots were subjected to optical densitometry, and relative optical density (OD) units were used to estimate levels of m-aconitase enzyme, which are presented as OD units per milligram protein. The levels determined were within the linear range of OD units.

All experiments were repeated at least once to confirm the results. The data represent the typical results repeatedly obtained in these studies.

RESULTS

Earlier studies[3,4] had demonstrated that PRL in vivo increased citrate levels of rat lateral prostate (RLP) and monkey prostate. Therefore, we initially determined the effects of alterations in the PRL status of rats on citrate utilization by prostate epithelial cells. In the first studies, we used bromocryptine treatment of rats to induce a hypoprolactinemic condition. The regimen used typically results in a decrease in serum PRL from 25 to 46 ng/mL in normal rats to 2.1 to 4.2 ng/mL in bromocryptine-treated rats, versus a hypophysectomized control level of 1.6 ng/mL. The effects of bromocryptine treatment on citrate utilization of prostate cells are presented in Fig 1. The results demonstrate that bromocryptine treatment markedly decreased (−77%) citrate utilization of rat ventral prostate (RVP) cells, and conversely markedly increased (+575%) citrate utilization of RLP cells. In contrast, bromocryptine treatment had no effect on citrate utilization of rat dorsal prostate (RDP) cells.

In addition to citrate utilization, we also determined the effects of bromocryptine treatment on m-aconitase levels of prostate cells. Figure 1 shows that bromocryptine treatment decreased m-aconitase in RVP cells, increased m-aconitase in RLP cells, and had no effect on m-aconitase in RDP cells. Thus, the effects of bromocryptine treatment on m-aconitase levels correlated perfectly with its effects on citrate utilization.

Although bromocryptine treatment results in hypoprolactinemia, it also induces other systemic effects. Therefore, it was important to determine if PRL was associated with the in vivo effects of bromocryptine treatment. Consequently, we determined the effects of PRL administration to bromocryptine-treated rats. The results (Fig 2) demonstrate that PRL administration markedly increased (+96%) citrate utilization of RVP cells, and conversely markedly decreased (−76%) citrate utilization of RLP cells. PRL administration had no effect on citrate utilization of RDP cells. As a representation of "nonprostate" typical citrate-oxidizing cells, kidney cells were included in these studies. PRL treatment had no effect on citrate utilization of kidney cells. Furthermore, PRL increased the level of m-aconitase in RVP, decreased m-aconitase in RLP, and had no effect on m-aconitase in RDP or kidney cells. Therefore, the effects of PRL on m-aconitase levels were identical to its effects on the citrate utilization of RVP, RLP, RDP, and kidney cells. Furthermore, the results of PRL administration are consistent with the effects of bromocryptine treatment described earlier. Therefore, the combined studies represented in Figs 1 and 2 demonstrate that prolactin in vivo is an important regulator of citrate utilization and m-aconitase levels in RVP and RLP cells, but has no effect on RDP or kidney cells. It is also important to note in Fig 2 that the rate of citrate utilization and the level of m-aconitase in kidney cells are significantly greater than in prostate cells. This is consistent with our earlier reports that citrate oxidation and m-aconitase activity of the prostate are uniquely low when compared with other soft tissues.[1,15,16]

The in vivo studies described earlier demonstrate that

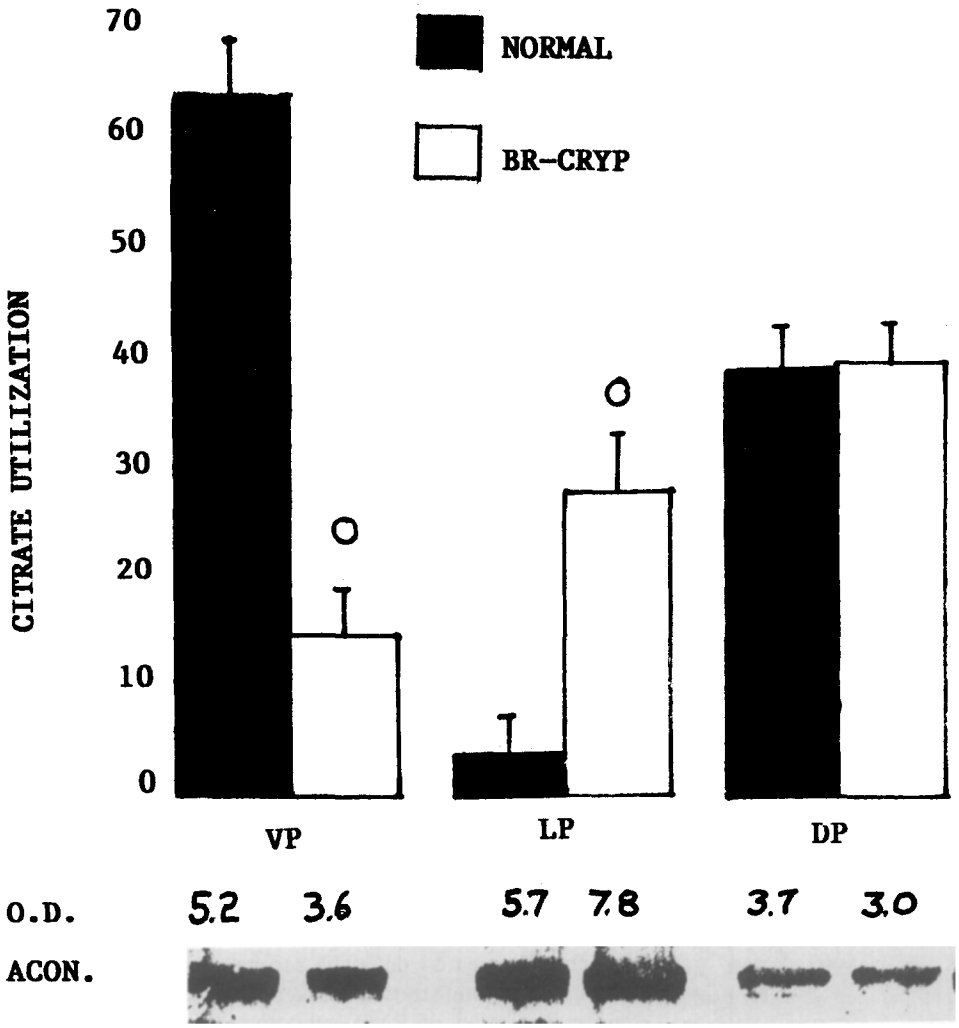

Fig 1. In vivo effects of bromocryptine (BR-CRYP) treatment on citrate utilization and m-aconitase of prostate epithelial cells. VP, ventral prostate; LP, lateral prostate; DP, dorsal prostate. Immunoblots of m-aconitase were developed from 100 μg cell protein. Citrate utilization is expressed as nmol citrate/mg protein/60 min. $^{o}P < .01$.

alterations in the PRL state of the animal have major and specific effects on citrate utilization and m-aconitase levels of prostate epithelial cells. However, such studies do not reveal if these effects are due to the direct action of PRL on prostate epithelial cells or to other systemic effects of PRL. To address this issue, the in vitro effects of PRL were investigated. For these studies, the prostate lobes and kidneys were obtained from normal donor rats, and isolated cells were prepared from each tissue. Aliquots of cells were exposed to either PRL (0.5 μg/mL) or vehicle for 3 hours. Citrate utilization and m-aconitase levels of PRL-treated and control cells were determined. The results (Fig 3) demonstrate that PRL markedly increased (+91%) citrate utilization of RVP cells, while markedly decreasing (−57%) citrate utilization of RLP cells. PRL treatment had no significant effect on citrate utilization of either RDP or kidney cells. Correspondingly, Fig 3 shows that PRL increased the level of m-aconitase in RVP cells, while markedly decreasing it in RLP cells. PRL had no effect on m-aconitase levels of RDP or kidney cells. These results provide compelling evidence that the in vivo effects of PRL are the result of direct and specific effects of PRL on RVP and RLP epithelial cells.

In the studies described earlier, we determined the change in the level of citrate after incubation of cells in the presence of added (exogenous) citrate. A decrease in the level of citrate has been described as "citrate utilization." However, such a change could be due to alterations in the level of endogenous citrate production. Therefore, we conducted a parallel study to that presented in Fig 3. In this study, exogenous citrate was excluded and the effect of PRL on endogenous citrate levels of the cell preparations was determined. In the absence of any exogenously added substrates, endogenous citrate levels of RVP, RDP, RLP, and kidney cell preparations were negligible and unchanged during the incubation period. PRL had no effect on endogenous citrate levels during the incubation period. Consequently, the decrease in citrate levels described in the previous experiments is appropriately described as "citrate utilization."

Recent studies[7-9] have demonstrated that PRL stimulates the biosynthesis of mAAT and PDH E1α in prostate epithelial cells. Consequently, it was important to determine if the action of PRL on citrate utilization and m-aconitase might also involve the biosynthesis of m-aconitase or other factors. To gain insight into this relation-

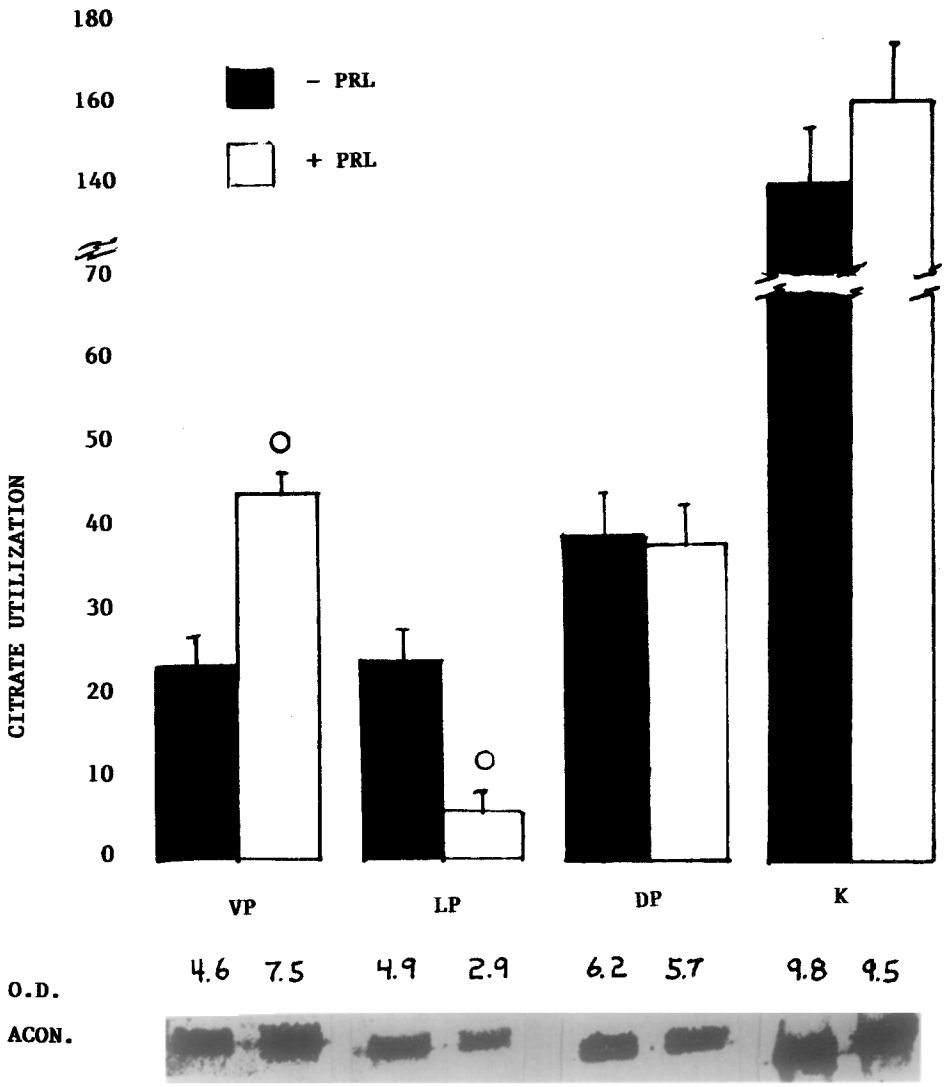

Fig 2. In vivo effects of PRL administration to bromocryptine-treated rats on citrate utilization and m-aconitase levels of isolated cells. See Fig 1.

ship, we investigated the effects of cycloheximide and actinomycin on citrate utilization and m-aconitase levels of RVP and RLP cells. For these studies, the in vitro system described earlier was used. The effects of PRL alone (0.5 μg/mL), PRL plus cycloheximide (2×10^{-6} mol/L), and PRL plus actinomycin (8×10^{-6} mol/L) were compared. The results (Fig 4) demonstrate that both cycloheximide and actinomycin abolished the stimulatory effect of PRL on citrate utilization and m-aconitase level of RVP cells. Cycloheximide and actinomycin also abolished the inhibitory effect of PRL on citrate utilization and m-aconitase level of RLP cells. It is also important to note that neither cycloheximide nor actinomycin had any effect on the endogenous (control) rate of citrate utilization. These results provide strong evidence that both the stimulatory effect of PRL on RVP cells and the inhibitory effect on RLP cells are dependent on de novo RNA and protein synthesis.

All the studies described above consistently demonstrated that PRL induced essentially identical effects on cellular citrate utilization and m-aconitase levels. This leads to the expectation that the effect of PRL is on the rate of citrate oxidation mediated by its effect on m-aconitase. The use of whole-cell preparations in the previous experiments did not provide direct evidence to link the effects on citrate utilization to citrate oxidation and m-aconitase. To address this issue, we determined the effects of PRL on citrate oxidation of mitochondrial preparations. For these studies, we used the in vivo effects of PRL administration on bromocryptine-treated rats as described earlier (Fig 2). After treatment, RLP and RVP were resected, homogenized, and processed to obtain the mitochondrial fraction. Mitochondrial preparations were incubated with either citrate or isocitrate to determine citrate and isocitrate utilization. NADP was added as the required cofactor for isocitrate oxidation (IDH activity). The results show (Fig 5) that PRL treatment significantly increased (+82%) citrate oxidation of RVP mitochondria, while decreasing (−60%) citrate oxidation of RLP mitochondria. PRL treatment had no effect on citrate oxidation of RDP or kidney mitochondria. In contrast, PRL had no effect on isocitrate oxidation of either RVP, RLP, or kidney mitochondria (results not shown). Consequently, the effects of PRL on citrate oxida-

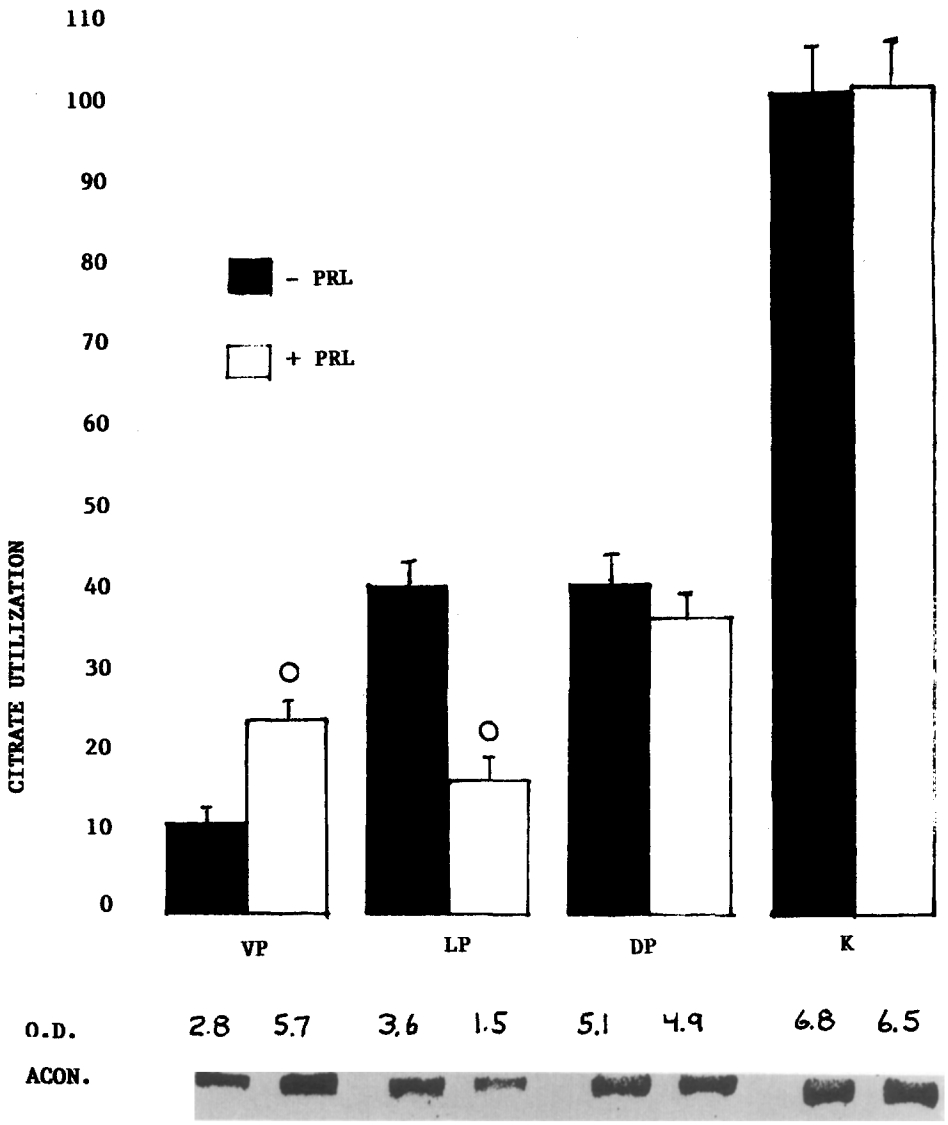

Fig 3. In vitro effects of PRL on citrate utilization and m-aconitase levels of isolated cells. See Fig 1.

tion occur at the m-aconitase step. It was then important to determine if concurrent changes in the level of m-aconitase in mitochondrial preparations accompanied the effects of PRL on citrate oxidation of RVP and RLP cells. The results (Fig 5) demonstrate that PRL treatment increased m-aconitase level and citrate oxidation of RVP cells, while decreasing m-aconitase level and citrate oxidation of RLP cells. Thus, results obtained with the mitochondrial preparations are identical to the cell studies described earlier. It is important to note that the effects of PRL on m-aconitase levels of RVP and RLP mitochondrial preparations were the same as for RVP and RLP cell preparations. This confirms that the effects of PRL are on m-aconitase. Therefore, collectively, these studies demonstrate that PRL increases citrate oxidation of RVP cells and decreases citrate oxidation of RLP cells, and that these effects are mediated through PRL regulation of m-aconitase. The results also demonstrate that the effects of PRL are not generalized effects on mitochondrial enzymes, but are specifically targeted at m-aconitase.

DISCUSSION

These studies comprise the first report that clearly establishes an important role of PRL in the regulation of citrate oxidation of prostate epithelial cells. In previous studies, we reported that PRL regulates mAAT and PDH E1α, the key regulatory enzymes involved in citrate synthesis of prostate cells. These effects are specific for prostate epithelial cells in that liver or kidney cells are unaffected by PRL. The fact that the effects of PRL on m-aconitase, mAAT, and PDH E1α occur in vitro and in vivo provides evidence that the regulation of prostate citrate production is a physiological role of PRL in males, as we recently proposed.[2,5]

The diversity of the effects of PRL on citrate oxidation and m-aconitase of different prostate epithelial cells is an important relationship. The current studies clearly demonstrated that citrate oxidation and m-aconitase levels were stimulated in RVP, inhibited in RLP, and unaffected in RDP by PRL. These three prostatic lobes are derived from

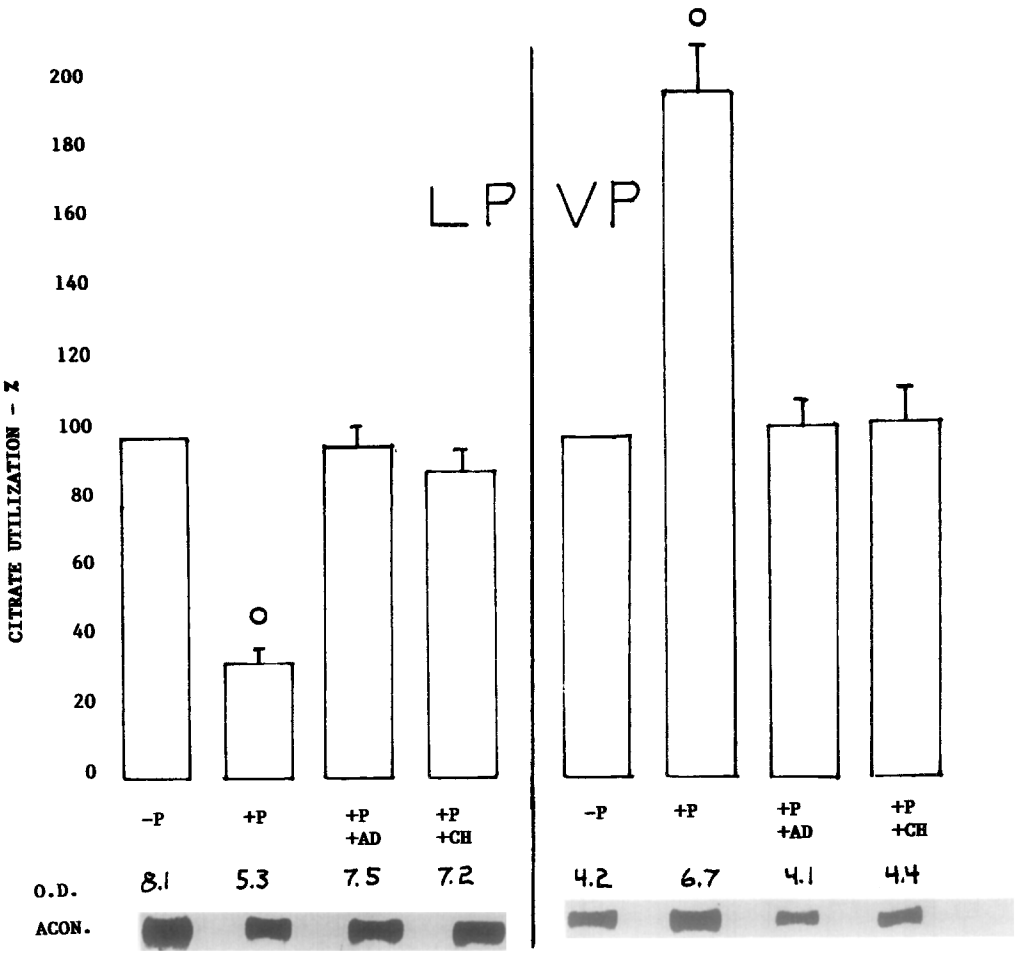

Fig 4. In vitro effects of PRL P), cycloheximide (CH), and acti- ıomycin (AD) on citrate utiliza- ion and m-aconitase levels of solated RVP and RLP epithelial :ells. Citrate utilization is re- ›orted as % of control (-P). See ig 1.

different embryonic origins. Therefore, the secretory epithelial cells of each lobe are embryologically differentiated. With regard to function, RVP and RLP epithelial cells are citrate-producing cells, whereas RDP cells are not. Consequently, the responses to PRL coincide with the three embryologic cell lines. Based on these results, three types of prostate epithelial cells can be identified: (1) citrate-producing cells in which PRL stimulates citrate oxidation and m-aconitase biosynthesis, as represented by RVP cells, (2) citrate-producing cells in which PRL inhibits citrate oxidation and m-aconitase biosynthesis, as represented by RLP cells, and (3) non–citrate-producing cells that are unresponsive to PRL, as represented by RDP cells. The current studies demonstrate that the capability of citrate oxidation of prostate epithelial cells is variable and depends on the origin of the cell and the influence of hormonal factors. These relationships might prove to be important considerations in the pathogenesis of malignant prostate cells.

The specificity of the responses of RVP, RLP, and RDP observed in this study has been similarly observed in our previous studies. PRL increases the level of PDH E1α in RLP cells, but not in RVP or RDP cells.[9] PRL increases mAAT of RLP cells but not of RVP cells. Prins[17] reported that PRL specifically increased the concentration of androgen receptor in RLP, but not in RVP or RDP. The mechanism by which prostate cell specificity in response to PRL is achieved is now an important issue that needs to be resolved. This cell specificity is not due to the presence or absence of PRL receptors. For example, hepatocytes contain PRL receptors and are responsive (proliferation and oncogene expression) to PRL,[18-23] yet PRL has no effect on mAAT, PDH E1α, or m-aconitase of liver cells, whereas it does regulate these enzymes in prostate cells.

Even more perplexing than the issue of cell specificity are the opposite effects of PRL on RVP and RLP cells. Whereas PRL increased citrate oxidation and m-aconitase of RVP cells, it inhibited citrate oxidation and m-aconitase of RLP cells. PRL reportedly stimulates net citrate production of RLP.[3,6] Our recent studies have demonstrated that PRL stimulates the biosynthesis of mAAT and PDH E1α of RLP cells.[7-9] These are the two key regulatory enzymes responsible for the production of oxaloacetate and acetyl coenzyme A necessary for citrate synthesis by prostate epithelial cells. The current studies now demonstrate that PRL inhibits citrate oxidation, which, along with its stimulatory effect on citrate synthesis, results in increased net citrate production of RLP. In contrast to its stimulatory effect on the biosynthesis of mAAT and PDH E1α in RLP cells, PRL exerts an inhibitory effect on m-aconitase. The results with cycloheximide and actinomycin provide strong evidence that this effect involves RNA and protein synthe-

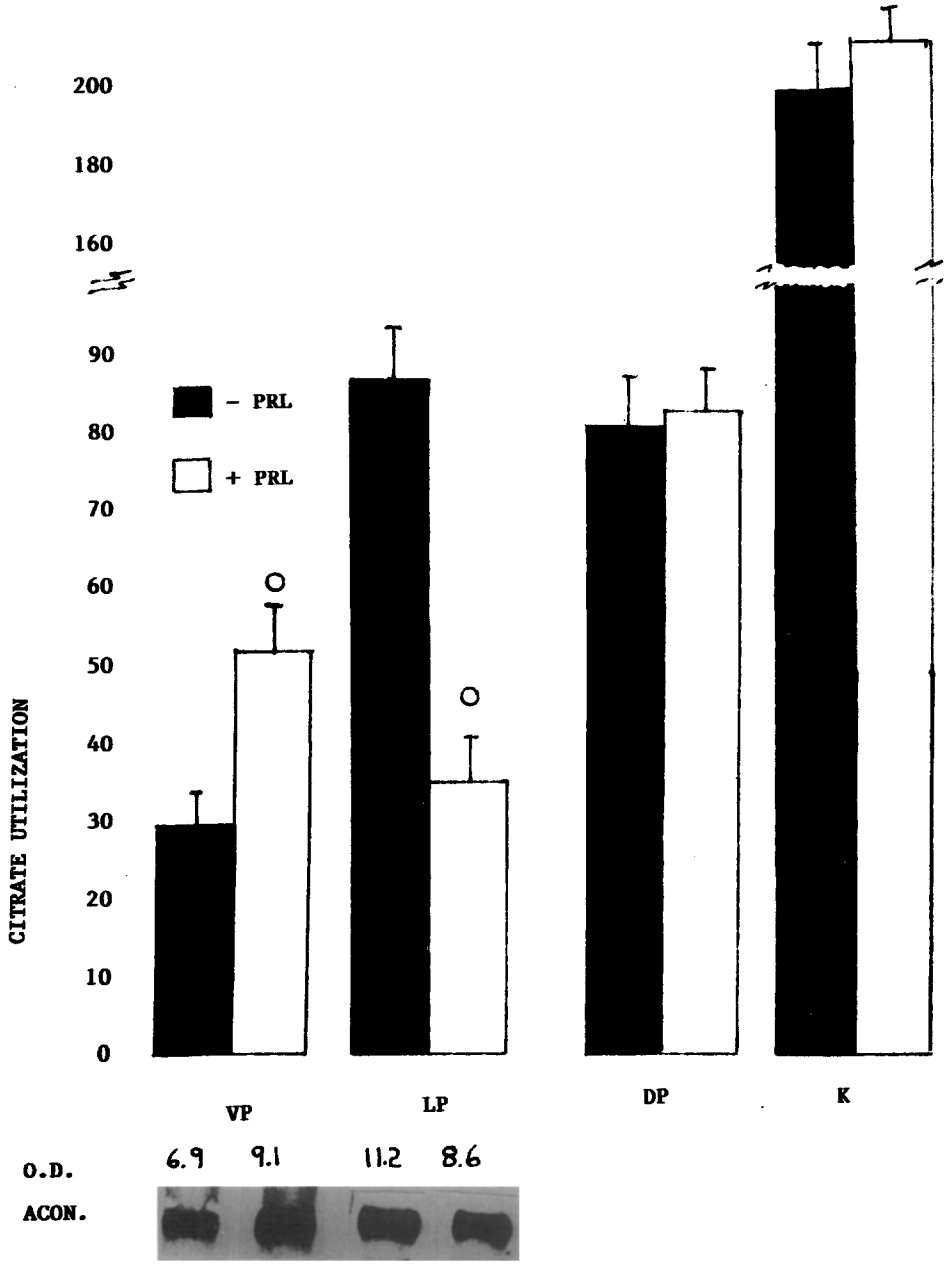

Fig 5. In vivo effects of PRL administration to bromocryptine-treated rats on citrate and isocitrate oxidation and on m-aconitase levels of RVP and RLP mitochondria. See Fig 1.

sis. This would suggest that PRL might stimulate the biosynthesis of a "m-aconitase inhibitory factor" that inhibits the biosynthesis of m-aconitase in RLP cells. This possibility requires further study.

In contrast to its inhibitory effect on RLP cells, PRL markedly stimulated citrate utilization and m-aconitase level of RVP cells. It is important to note that, in contrast to RLP, PRL has no stimulatory effect on net citrate production of RVP.[3,6] PRL stimulation of citrate oxidation would be consistent with its inability to increase citrate levels of RVP. The mechanism by which PRL increases the level of m-aconitase in RVP cells needs to be addressed. The inhibition of this effect by cycloheximide and actinomycin provides evidence that PRL stimulates the biosynthesis of m-aconitase, possibly at the gene level. Our recent studies have demonstrated that PRL does stimulate expression of the mAAT gene in RLP cells.[8] It also regulates several growth-promoting genes in various cells.[19,21] Consequently, it is plausible to suggest that the m-aconitase gene in RVP cells is a PRL-responsive gene.

The metabolic implications of the current observations are extremely important. This is the first report that PRL regulates citrate metabolism in any cells. m-Aconitase represents the entry step of citrate into the Krebs cycle. By alterations of citrate oxidation at this step, PRL must have profound effects on the intermediary metabolism of RVP and RLP cells, including energy production, cellular respiration, and reactions coupled to the Krebs cycle. It is also

important to note that in typical cellular metabolism m-aconitase is not considered a regulatory enzyme. m-Aconitase is generally described as catalyzing an equilibrium reaction, that is not a rate-limiting step in intermediary metabolism. Because of this, essentially all cells typically maintain a citrate to isocitrate ratio of approximately 9:1 or 10:1, which reflects the equilibrium of m-aconitase. However, citrate-producing prostatic tissue exhibits a citrate to isocitrate ratio of approximately 30:1 to 40:1.[1,22,23] We have proposed that prostate cells contain a uniquely limiting m-aconitase reaction that limits citrate oxidation and permits the characteristic high levels of citrate production and secretion. This concept is also supported by the observation[24] that fluoroacetate treatment of rats had little effect on citrate oxidation and citrate levels of the prostate, while markedly inhibiting citrate oxidation and increasing citrate levels of virtually all other tissues. The present studies additionally confirm the unique relationship that m-aconitase is a regulated and regulatory enzyme in prostate epithelial cells and PRL is an important regulator of m-aconitase. This unique m-aconitase relationship is consistent with the specialized function of the prostate, ie, production and secretion of extremely high levels of citrate. A rate-limiting, regulatory m-aconitase reaction would represent the most efficient metabolic alteration to optimize the function of net citrate production. The mechanisms involved in the regulatory aspects of m-aconitase and the impact on intermediary metabolism of prostate epithelium are issues that need to be elucidated.

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