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Et al.

Branched Fatty Acids in Dairy and Beef Products Markedly Enhance α-Methylacyl-CoA Racemase Expression in Prostate Cancer Cells *in Vitro*[1]

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Abstract

An enzyme previously identified as α-methylacyl-CoA racemase (AMACR) is overexpressed in high-grade prostatic intraepithelial neoplasia and in a majority (60–100%) of prostate cancers (CaPs) as compared with normal and benign hyperplastic lesions of the prostate, where it is minimally expressed. This enzyme is required for the β-oxidation of branched-chain fatty acids, which include phytanic acid and its α-oxidation product, pristanic acid. Interestingly, there is an established correlation between CaP risk and the consumption of dairy and beef products, which also contain marked quantities of these two phytols. In this context, it has also been reported that sex steroids influence lipogenesis through the induction of fatty acid synthase in CaP-derived cell lines and CaP tissues. These findings indicate a potential role for AMACR and the possible influence of sex steroids in both the early development and subsequent progression of CaP. Despite the recent interest in AMACR as a histological marker for CaP, little is known about the regulation of this enzyme and its role in CaP development. To identify potential AMACR-regulating factors, we treated LNCaP cells (an androgen-responsive CaP-derived cell line) and NPrEC cells (a normal prostate basal epithelial cell line) with increasing concentrations of pristanic acid, phytanic acid, 5α-dihydrotestosterone, and 17β-estradiol. Neither the biologically potent androgen 5α-dihydrotestosterone nor 17β-estradiol had any apparent effect on AMACR expression at the protein or transcriptional levels in either cell line. Conversely, pristanic acid and, to a much lesser extent, phytanic acid markedly increased AMACR protein levels selectively in the LNCaP cell line, but not the NPrEC cell line. However, no change was measured at the transcriptional level in either cell line. AMACR is therefore significantly increased at the protein level in CaP cells, through what appears to be the stabilizing effect of the same fatty acids that are present at appreciable concentrations in beef and dairy products, which have been associated with CaP risk. Our findings therefore provide a link between the consumption of dietary fatty acids and the enhanced expression of AMACR, an enzyme that may play an important role in genesis and progression of CaP.

Introduction

CaP[3] is the most commonly diagnosed cancer in American men (1). In an attempt to identify genes overexpressed in CaP, thus gaining insight into the etiology of this form of cancer, several investigators have recently used high-throughput cDNA screening techniques (2–7). Xu *et al.* (7) used a cDNA library subtraction method in conjunction with high-throughput microarray screening, resulting in the identification of three genes that were differentially expressed in normal human prostate as compared with CaP. One of the genes described by Xu *et al.* (7) as *P504S* and previously identified as AMACR was found to be overexpressed in CaP tissues (7–9). The significance of those findings was subsequently expanded on by Jiang *et al.* (10, 11), who were the first to report that AMACR was significantly up-regulated in 94–100% of CaP and prostatic intraepithelial neoplasia lesions tested at the RNA and protein levels. This was in sharp contrast to the near complete lack of expression in both benign hyperplastic and normal prostate glands (11). In two recent reports, Rubin *et al.* (6) and Luo *et al.* (12) confirmed and extended the findings of Jiang *et al*. (10, 11). In addition, Luo *et al.* (12) found strong AMACR expression in 62% of the metastatic CaP lesions tested and reported that both hormone-refractory and non-hormone-refractory metastatic lesions were immunohistochemically positive for AMACR. It is important to stress that all investigators have demonstrated low to undetectable expression of AMACR in normal human prostatic epithelium. Interestingly, it was recently reported that AMACR is also strongly expressed in several other human carcinomas and their precursor lesions, implying a basic mechanistic role for this enzyme throughout the early stages of cancer formation (13). However, most current interest regarding AMACR has

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[3] The abbreviations used are: CaP, prostate cancer; AMACR, α-methylacyl-CoA-racemase; AR, androgen receptor; CS, calf serum; DHT, 5α-dihydrotestosterone; ER, estrogen receptor; E_2, 17β-estradiol; FBS, fetal bovine serum; IHC, immunohistochemical; LDH, lactate dehydrogenase; mAb, monoclonal antibody; PSA, prostate-specific antigen; PFBz, pentafluorobenzyl; GC-MS, gas chromatography-mass spectrometry; CAS, Casodex.

involved its expression in the prostate, where it has been touted as a new molecular marker for prostate cancer (10, 12, 14).

Under normal physiological conditions, AMACR is expressed at appreciable levels and is transported to both the peroxisomal and mitochondrial compartments in a variety of tissues, including liver, kidney, skeletal muscle, gall bladder, and brain (8, 15–17). AMACR is responsible for the interconversion of R-configured β-methyl groups found within various small molecules that contain branched chains, such as phytols and bile acids, to the S form; a prerequisite for metabolism via the β-oxidation pathway (18, 19). As a result, AMACR is a required component of the oxidative metabolism and biosynthetic pathways of branched-chain fatty acids and bile acids, respectively. Pristanic acid, the α-oxidation product of phytanic acid, is one of the more common substrates acted upon by this enzyme (20–22). Interestingly, phytanic acid is a major component of dairy and beef products, resulting in low micromolar concentrations in the sera of individuals who consume a traditional Western diet (22–24). This branched-chain fatty acid is hydrolyzed from chlorophyll by the metabolizing enzymes in bacterium found in the rumen of animals such as cows and goats (25–28). As a result, total phytanic acid can reach very high concentrations in the sera and tissues of these animals (22, 23, 25–29). A correlation has been reported between a diet rich in dairy and beef products and a risk for CaP (8, 30, 31). Taken together, these findings indicate a potential role for AMACR in both the early development and subsequent progression of CaP.

The negligible expression of AMACR in normal prostatic epithelium, along with its overexpression in CaP, prompted us to investigate factors that may regulate this enzyme. For reasons mentioned above, along with a recent report that phytanic acid is capable of inducing a component of the α-oxidation pathway, our investigation focused on the influence of pristanic and phytanic acids on AMACR expression (32). In addition, we were interested in determining whether the biologically potent androgen DHT or E_2 has any regulating effect on the expression of this enzyme. The inclusion of DHT in these studies was prompted by findings that androgens influence lipogenesis and enhance the accumulation of neutral lipids through the induction of fatty acid synthase in AR-positive CaP cell lines (33–36). E_2 was also studied, due to the reported expression of ER-β in both prostatic basal epithelium and CaP, along with the existence of elements that interact with the ER, such as activator protein 1, in the promoter region of the rodent AMACR gene (37–39).

To investigate AMACR regulation *in vitro*, we chose cell lines that are highly representative of their cellular tissue counterparts. The LNCaP cell line is derived from lymph node metastasis and expresses AMACR in a manner comparable with that seen in primary and metastatic CaP tissues *in vivo* (10). As with a majority of primary CaP tissues, this cell line expresses functional AR and ER-β (37, 38). In addition, we developed an immortalized NPrEC cell line phenotypically identical to the basal cells that line the human prostatic acinus (40–42). Basal cells are considered to be the major proliferative epithelial component of the prostatic acinus and the purported precursor of secretory cells. This cell line was developed for these studies to assess AMACR expression in a cell type believed to play a role in the early stages of CaP development. Thus, the effects of dietary phytols and sex steroids on the regulation of AMACR were compared in cell lines that mirror the *in vivo* prostate cell types believed to be targets for carcinogens and key participants in neoplastic progression.

Materials and Methods

Cell Culture. The LNCaP, PC3, DU145, and MCF-7 cell lines were obtained from American Type Culture Collection (Manassas, VA) and were of low passage number at the time of each experiment. The PC3-AR cell line was stably cotransfected with the full-length wild-type AR and neomycin-resistant (NEO) gene as reported previously (43). DU145, PC3, and MCF-7 cell lines were all grown in DMEM:Ham's F-12 (Invitrogen, Carlsbad, CA). LNCaP cells were grown in RPMI 1640 (Invitrogen). The normal prostate epithelial NPrEC cells were obtained from Clonetics-BioWhittaker, Inc. (Walkersville, MD), and immortalized by the addition of a retroviral vector expressing human papilloma virus E6/E7 open reading frames as described previously (44). This NPrEC cell line was grown on the recommended PrEGM media supplied by Clonetics-BioWhittaker, Inc. The phenotype of these cells was then determined by comparing the pattern of protein markers with cellular counterparts in tissues (see "Immunocytochemistry").

All cells were seated on cell culture-treated T-75 vented or 24-well Falcon plates (BD Biosciences, Bedford, MA) and cultured at 37°C under 95% humidity and 5% CO_2 in 10 or 1.0 ml (respectively) of the media recommended by American Type Culture Collection containing 5% FBS (Invitrogen) for 24 h and changed to 5% charcoal-stripped serum (Sigma, St. Louis, MO) or serum-free medium supplemented with insulin transferrin selenium (ITS) (BD Biosciences) for 24 h before treatment. For all experiments, compounds were brought up in DMSO and diluted 1000× in media to the appropriate concentrations. Cells were treated with DHT, E_2, and phytanic acid (Sigma); pristanic acid (Lordan Lipids, Malmo, Sweden); and bicalutamide (CAS) extracted in DMSO from 50-mg tablets (Astra Zeneca, Wilmington, DE).

Immunoblotting. Western blot analysis was performed on total cell lysates that were prepared and diluted in M-Per reagent (Pierce, Rockford, IL) to a concentration of 2 μg/μl. Protein quantification was determined with BCA reagent (Pierce) and compared with a standard curve of bovine albumin on a μQuant UV-visible plate reader at 590 nm (BIO-TEK Instruments, Winooski, VT) with KC-Junior software. Aliquots of 50.0 μg of cell extract were separated on 10% SDS-PAGE Tris-HCl Ready Gels (Bio-Rad, Hercules, CA) at 80 V for 2.5 h and electroblotted to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). Membranes were blocked in 5% milk PBST for 1 h, washed in PBST, and incubated with one of the following primary antibodies: anti-AMACR rabbit mAb at a 1 μg/ml dilution or anti-β-actin rabbit polyclonal antibody at a 1:500 dilution (Sigma) for 1 h while shaking at 4°C in a sealed Kapak bag (Kapak, Minneapolis, MN). This was followed by incubation with a horseradish peroxidase-linked secondary antirabbit antibody (Amersham Biosciences, Piscataway, NJ) at a 1:10,000 dilution for 1 h. Detection was performed using ECL-plus reagent (Amersham Biosciences) according to the manufacturer's instructions and visualized on a STORM 840 imaging system (Amersham Biosciences) at 900 V in blue fluorescence mode. Relative quantification was carried out with Kodak 1D 3.5 software (Eastman Kodak Co., New Haven, CT).

Immunocytochemistry. Treated and untreated NPrEC, LNCaP, PC3, and DU145 cells were harvested after 36 h of incubation. The cells were washed with HBSS and trypsinized. After trypsinization, a neutralizing solution was then added, and cells were transferred into 15-ml centrifuge tubes. Cells were then centrifuged at 1000 rpm for 5 min. After decanting the fluid, 10% buffered formalin was added to the cell pellet formed at the bottom of the tube. After 12 h of fixation, the

pellet was removed with a spatula, routinely processed, and embedded in paraffin. Approximately 10 unstained sections were cut at 4–6 μm and attached to glass slides. Slides were then deparaffinized in xylene for 5 min. Sections were then rehydrated by briefly immersing them in graded alcohols and, finally, in water. Antigen retrieval was carried out with 0.1 M citrate buffer in an 880 W microwave for 10 min. After rinsing, endogenous peroxidase activity was blocked with 3% hydrogen peroxide. The sections were rinsed again, and nonspecific sites were blocked with a blocking protein (Ventana, Tuscon, AZ). Sections of NPrEC cells were immunostained using a Techmate 1000 automated immunostainer (Ventana). For these studies, the following antibodies were used: mouse high molecular weight cytokeratin mAb 34βE 12 at a dilution of 1:50 (HMWC; Dako, Carpenteria, CA); mouse P63 mAb (Dako); mouse AR mAb at a dilution of 1:80 (Biogenix Laboratories, San Ramon, CA); and mouse ER-α mAb at a dilution of 1:50 (Ventana). For AMACR immunostaining we used the rabbit mAb *P504S* at a dilution of 0.5 μg/ml (Corixa Corp., Seattle, WA). The same secondary antibodies and the Ventana staining procedure were then used to stain five paraffin-embedded sections of normal prostate tissue. ER-β immunostaining was carried out manually using the GC17, a rabbit polyclonal antibody (Biogenex), as described previously (45). For all immunostains, primary antibodies were incubated with each section for 45 min. After brief buffer washes, the sections were incubated with a mixture of biotinylated antirabbit IgG or antimouse IgG/IgM (Ventana) for 30 min. Sections were then incubated in an avidin/peroxidase complex (Ventana) for 30 min and, after rinsing, treated with diaminobenzidine to visualize the end product. Finally, sections were counterstained with hematoxylin. Sections of normal human prostate or normal breast tissue served as positive controls. Negative controls were the substitution of the primary antibodies with an appropriate class-matched serum.

Real-Time Reverse Transcription-PCR. Total RNA was extracted with TRI reagent (Sigma) according to the manufacturer's instructions. RNA quantification and integrity were measured by UV spectrophotometry by the 260 nm/280 nm method and denaturing gel electrophoresis, respectively. Potential contamination by genomic DNA was removed by DNase I treatment, in which 1 μg of total RNA was incubated with RQ1 RNase-free DNase (Promega, Madison, WI) in a total reaction volume of 10 μl at 37°C for 30 min, followed by termination with the addition of 1 μl of the DNase stop solution provided by the vendor. The reverse transcription reaction was carried out with TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions in a reaction containing random hexamers prepared in a reaction volume of 60 μl and 1 μg of DNase-treated RNA. Real-time PCR was carried out, using a previously published primer set, on 5 μl of the reverse transcription reaction mixture along with SybrGreen 2× PCR master mix (Applied Biosystems) in the iCycler thermocycler (Bio-Rad; Ref. 13). The cycling conditions comprised an initial denaturing step of 94°C for 4.5 min; followed by 50 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 45 s; with a final extension at 72°C for 10 min. Experiments were performed in triplicate for each treatment.

Determination of Total Pristanic and Phytanic Acid Levels in Serum by GC-MS. The serum concentrations of pristanic and phytanic acids were determined by gas chromatography-electron capture negative ion mass spectrometry using a modified form of the method reported by Stellaard *et al.* (46). Serum (100 μl) was mixed with 40 μl of iso-octane containing 100 ng of D_3-pristanic acid and 1 μg of D_3-phytanic acid internal standards (Lorodan Lipids), 160 μl of *n*-hexane, and 2.0 ml of 0.5 n HCl in acetonitrile and heated at 100°C for 45 min to free the lipid-bound fatty acids. The fatty acids were extracted into 2.0 ml of *n*-hexane, and the solvent was evaporated. The fatty acids were then converted to their PFBz esters by reaction with a solution containing 10 μl of triethylamine and 5 μl of PFBz bromide in 50 μl of acetonitrile at room temperature for 15 min. Acetonitrile (200 μl) and *n*-hexane (1.0 ml) were added to the reaction mixture, and after vigorous mixing and separation of the phases, the *n*-hexane phase was transferred to an autosampler. The solvent was evaporated under a stream of nitrogen, and the PFBz fatty acids were redissolved in 0.5 ml of *n*-hexane for GC-MS injection. GC-MS was performed using a Waters Quattro-II triple quadrupole mass spectrometry system equipped with an Agilent 6890 GC and an Agilent 7683 autosampler (Agilent, Palo Alto, CA). One μl of samples and standards were injected in the splitless mode onto a 0.25 mm × 30 m DB-23 (phase thickness, 0.25 μm) fused silica capillary column with He as the carrier gas at 0.8 ml/min. The column temperature was programmed from 165°C to 240°C at 5°C/min. Elution peaks for PFBz pristanic and phytanic acids were at 9.1 and 10.5 min, respectively. Electron capture negative ion ionization was performed using methane as the moderator gas at 3e-4 mBar with the electron energy at 50 eV and the emission current at 300 μA. Selected ion monitoring was performed at *m/z* 297.3 (pristanic acid), 300.3 (D_3-pristanic acid), 311.3 (phytanic acid), and 314.3 (D_3-phytanic acid). Concentrations for both analytes were measured from the ratio of selected ion plot peak area of each analyte to its internal standard in comparison with three-point calibration curves run with the samples.

Cytotoxicity and Cell Number Assays. Cells were seated at 10,000 cells in 24-well plates. Cytotoxicity was quantified by the release of LDH, whereas cell number was related to the reduction of the tetrazolium dye MTS in the same assay plate. After the specified treatments, 100 μl of media was transferred to a 96-well plate for the quantification of LDH as per the manufacturer's instructions, and normalized between nontreated controls and cell lysed by three repetitions of freeze and thaw. The cell media were then refreshed with added MTS reagent as per the manufacturer's instructions and measured at 590 nm after approximately 2 h. All values were normalized to nontreated controls.

Results

Immunocytochemical Characterization of NPrEC Cells. NPrEC cells, grown without the addition of DHT, E_2, or fatty acids, were found to stain strongly positive for HMWC, P63, and ER-β (Fig. 1, *A*, *C*, and *E*, respectively). In sharp contrast, AR, AMACR (Fig. 1, *G* and *I*), and ER-α (data not shown) were undetectable by IHC analysis. Basal cells in tissue sections of morphologically normal human prostate revealed identical staining with the selective antibodies (Fig. 1, *B*, *D*, *F*, *H*, and *J*) to that observed in the NPrEC cell line. Thus, NPrEC cells expressed precisely the same pattern of phenotypic immunocytological markers that characterizes human basal cells in normal prostatic tissues (41, 45, 47, 48).

AMACR Expression in Untreated Prostate Cell Lines. The prostate cancer cell lines DU145, PC3, PC3-AR (AR-transfected PC3), LNCaP, the breast cancer MCF-7 cell line, the normal immortalized NPrEC basal cell line, normal prostate tissues, and CaP tissues were all subjected to Western blot analysis (Figs. 2, *d* and *e*). Only the LNCaP cell line expressed AMACR at both the transcriptional (Fig. 2*f*) and protein levels,

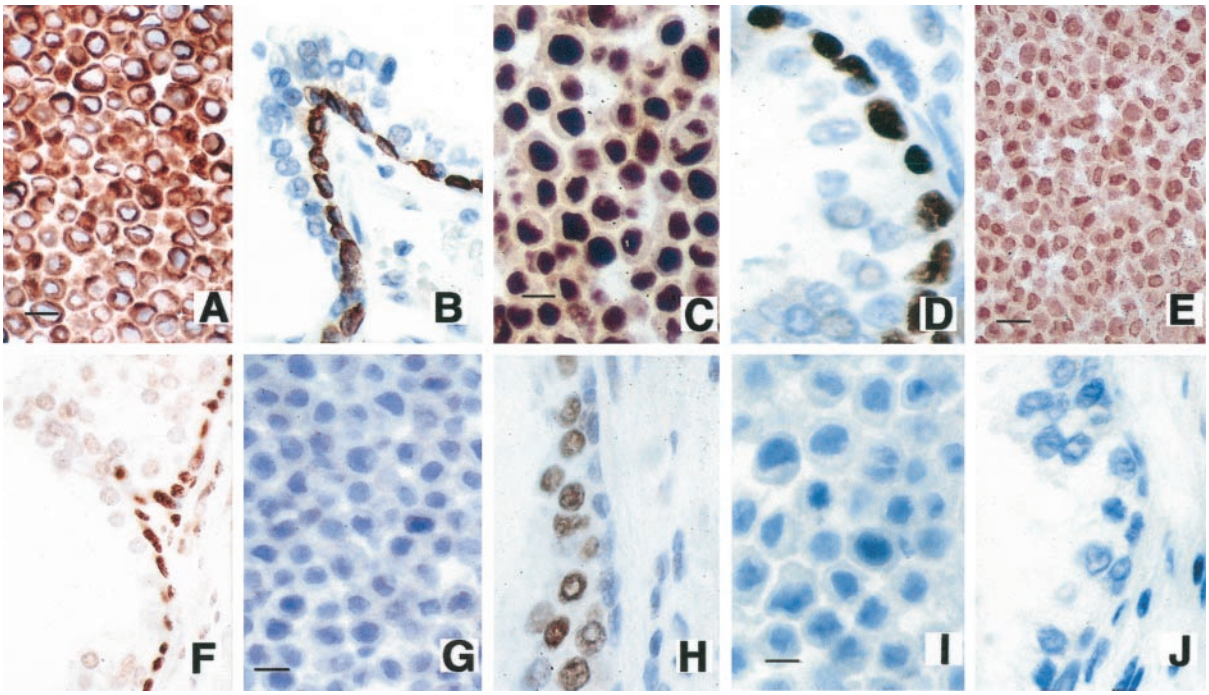

Fig. 1. NPrEC cells were immunostained for HMWC, P63, ER-β, AR, and AMACR on *A, C, E, G,* and *I,* respectively, and compared with corresponding tissue sections of normal human prostatic acinus immunostained with the same antibodies on *B, D, F, H,* and *J*. For example, strong cytoplasmic staining of NPrEC cells and tissue prostatic basal cells is evident for HMWC (*A* and *B*), whereas only nuclear staining is present with P63 (*C* and *D*) and ER-β (*E* and *F*). In contrast, immunostaining is absent for AR in NPrEC cells (*G*) and in tissue basal cells (*H*), but nuclear staining for the receptor is present in overlying tissue secretory cells (*H*). AMACR immunostaining is completely absent in NEPrEC cells (*I*) and both secretory and basal cells in prostate tissue sections (*J*). The *bar* on each panel represents 6–8 μm, and all sections were counterstained with hematoxylin.

similar to that found in CaP tissues. The NPrEC cells expressed AMACR at a level approximately 10–12-fold less than that of the LNCaP cell line at the both the transcriptional (Fig. 2*f*) and protein levels (Fig. 2*e*). The normal prostate tissues used in this study did not express measurable levels of AMACR (Fig. 2*e*). However, similar studies have illustrated that normal prostate tissues often express levels of AMACR approximately 9-fold less than that of CaP tissues, in agreement with values observed for the LNCaP and NPrEC cell lines (6, 10). The IHC findings paralleled the results of the immunoblot studies in that AMACR was strongly expressed in LNCaP cells (Fig. 1*H*; Fig. 2, *a* and *e*). In contrast, only scattered immunopositive cells (~2–3 cells/1000 cells) were observed in PC3 cells, and no staining was present in DU145 cells (Fig. 2, *b* and *c*). Whereas AMACR protein was weakly detected by Western analysis (carried out on 50 μg of total protein per lane) from NPrEC cell lysate, no immunostaining was observed (Fig. 1*H*). This was also the case for basal cells in tissue sections, which were negative for AMACR staining. In marked contrast, CaP in tissue sections and LNCaP cells stained strongly with both Western blot and IHC analysis techniques.

To establish appropriate media for additional studies in the LNCaP cell line, the following four media variations were studied: serum-free media with and without added ITS supplement (data not shown); along with media containing either FBS or charcoal-stripped CS (Fig. 3; Refs. 23, 25, 27, 32, and 49–51). Although it is known to contain significant levels of steroid hormones, FBS has been shown to contain low levels of phytanic acid, presumably due to an inability to cross the placenta (32). On the other hand, CS is known to contain high concentrations of phytanic acid, which may not be adequately stripped with the standard dextran-coated charcoal methods commonly used to remove steroids (45, 28, 32). In this respect, charcoal-stripped FBS is not readily available for purchase, whereas charcoal-stripped CS is available from various sources. For this reason, a single lot of FBS and charcoal-stripped sera were analyzed for total pristanic and phytanic acid content by GC-MS. The FBS tested contained phytanic acid at concentrations similar to previously published reports of 0.83 μM, whereas the concentration of pristanic acid was 0.17 μM (32). Charcoal-stripping the calf sera appeared to decrease phytanic acid concentrations considerably to 2.2 μM, as compared with the reported values for non-stripped bovine sera, which range from 19 to 231 μM (32). Pristanic acid concentrations were found to be nearly 10-fold lower than phytanic acid in the stripped CS at 0.24 μM. Interestingly, LNCaP cells grown on any one of the media described expressed similar levels of AMACR, with the exception of cells grown on FBS, which showed a ~3-fold decline in message and protein expression. However, due to the presence (albeit at low levels) of phytanic acid in both the stripped CS and FBS, we chose to complete the phytol-related studies in a defined medium. In addition, for all other comparative studies involving Western blot analysis, equal amounts of protein were added to each well, and β-actin co-blotting (data not shown) was used to further assure equal loading.

Androgen Treatment. LNCaP cell cultures were supplemented with 5 nM DHT and/or CAS in charcoal-stripped CS for 36 h. Both AMACR and PSA were analyzed by Western blot and real-time reverse transcription-PCR analysis. PSA was induced by DHT at both the protein and transcriptional level

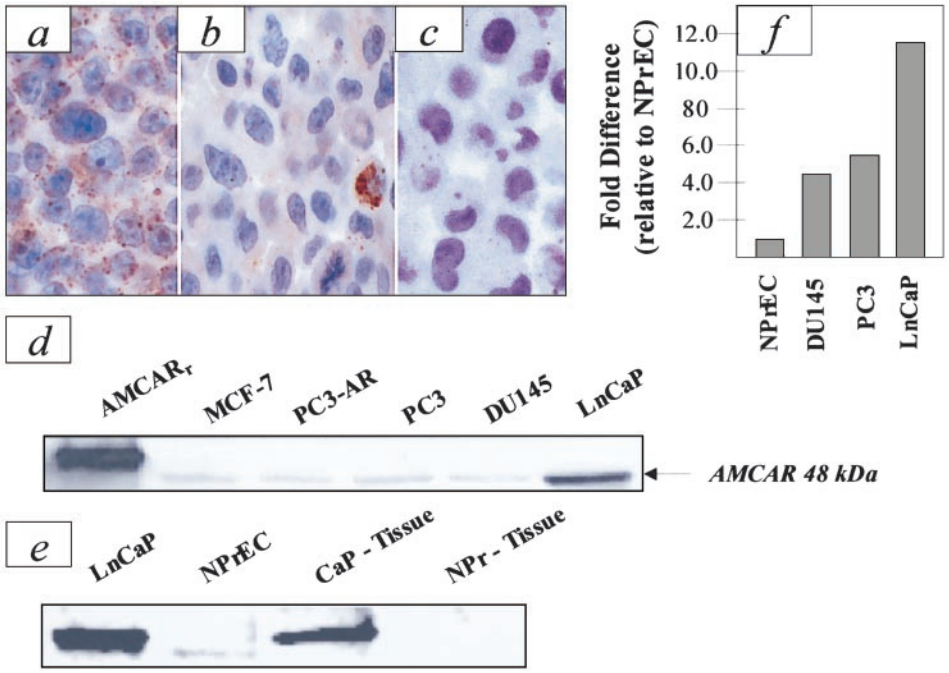

Fig. 2. Shown are the IHC results from LNCAP, PC3, and DU145 cells (*a–c*, respectively) and the immunoblotting (*d*) results on the cell lines noted with an antibody directed toward AMACR. *a*, strong and uniform cytoplasmic staining for AMACR is visualized in this pellet of LNCaP cells, compared with the absence of AMACR staining in NPrEC cells (Fig. 1*I*). *b*, positive immunostaining was consistently present in only a few (2–3 per 1000) PC3 cells. *c*, no staining for AMACR was observed in the DU145 pellet. *d*, 1.0 ng of recombinant AMACR protein and 15 μg of total cell lysate from each cell line (breast cancer cell line MCF-7 and prostate cancer cell lines PC3-AR, PC3, DU145, and LNCaP). *e*, 15 μg of total cell lysate from each (LNCaP, CaP tissue, NPrEC, and normal prostate tissue). *f*, AMACR transcript levels (average of $n = 2$); results are normalized to NPrEC cells.

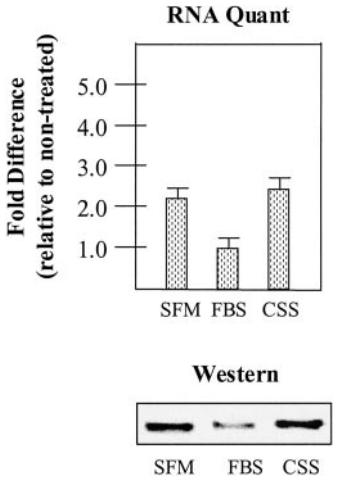

Fig. 3. LNCaP cells were grown in serum-free media (*SFM*), FBS, or charcoal-stripped sera (*CSS*) for 36 h. *Top*, AMACR mRNA levels were normalized to cells grown in FBS. *Bottom*, AMACR protein levels were measured by Western analysis in the media described.

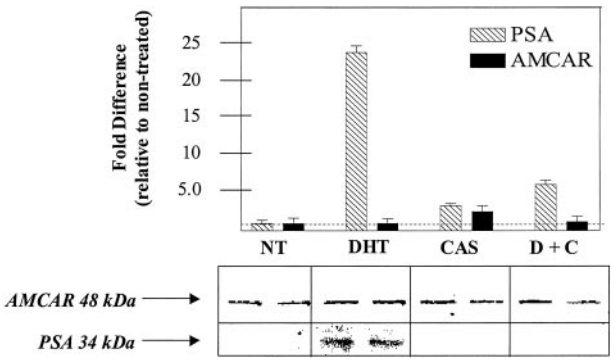

Fig. 4. LNCaP cells were treated with 5 nM DHT, 5 μM CAS, or both for 36 h. *Top*, AMACR and PSA mRNA levels were normalized to nontreated (*NT*) controls. *Bottom*, Western analysis of AMACR and intracellular PSA protein levels in LNCaP cells after the treatments described.

and was blocked by the addition of CAS. In contrast, no measurable increase in AMACR was observed with these treatments (Fig. 4). The NPrEC cell line was not treated with DHT because it was found to be free of AR by immunohistochemistry.

Estrogen Treatment. Both the LNCaP and NPrEC cell cultures were supplemented with E_2 because both cell lines express ER-β and therefore have the potential for ER-mediated regulation of AMACR. Increasing concentrations of E_2 ranging from 100 pM to 10 nM were used with no effect observed on AMACR expression at the protein or transcriptional level (Fig. 5).

Treatment with Phytanic and Pristanic Acids. Both the LNCaP and NPrEC cell cultures were supplemented with phytanic acid and its α-oxidation product, pristanic acid (22). Supraphysiological concentrations of phytanic acid are known to induce cytotoxicity in a variety of cell types (25, 52). Therefore, toxicity and cell number were determined by LDH release

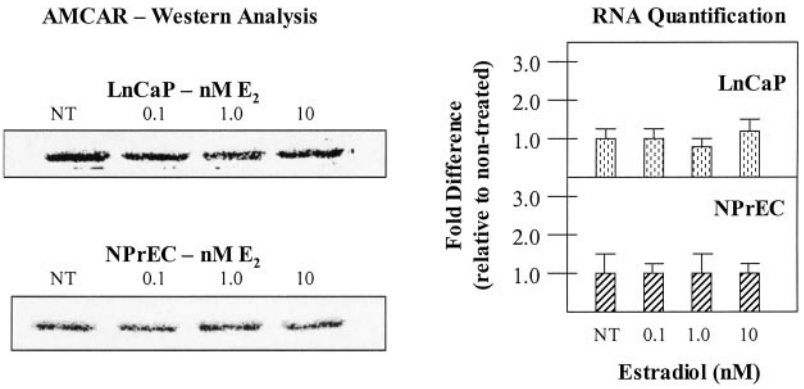

Fig. 5. LNCaP and NPrEC cells were treated with increasing levels of E_2 for 36 h. *Left panels*, Western analysis of AMACR on total cell extracts from prostate-derived cell lines. *Right panel*, AMACR mRNA levels were normalized to nontreated (*NT*) controls.

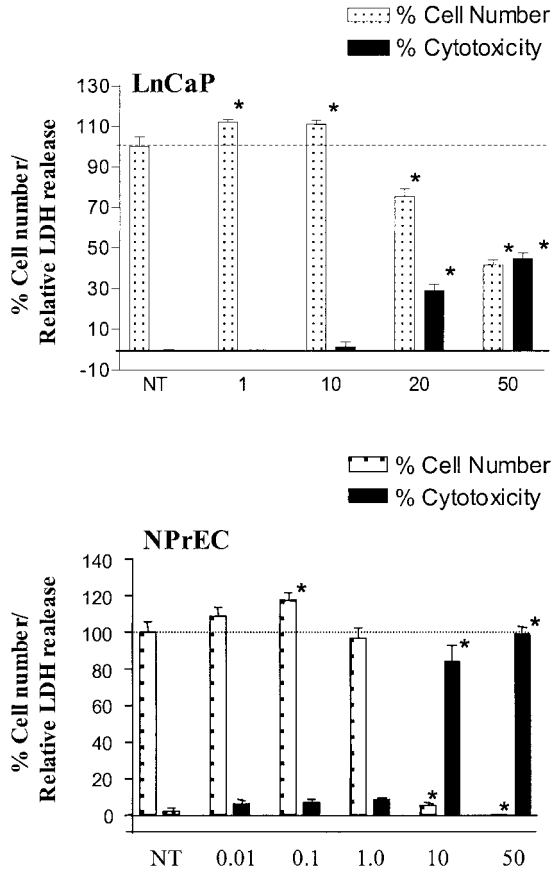

Fig. 6. LNCaP and NPrEC cells were treated with increasing levels of phytanic acid for 48 h. Cell number was quantified by the reduction of MTS, and cytotoxicity was assessed by the release of LDH into the media. All values were normalized to nontreated (*NT*) controls. *, $P < 0.05$.

and MTS reduction (respectively) after treatments with increasing amounts of phytanic acid for 48 h. Phytanic acid was found to induce nearly 100% cell death at 10 μM in NPrEC cells and 20–50% cell death at 25–50 μM in LNCaP cells grown in serum-free media (Fig. 6). These findings indicate that the immortalized basal cell line is much more susceptible to the toxic effects of phytanic acid than the prostate cancer cell line. Consistent with what would be expected of a cytotoxic mechanism, the number of cells as measured by the MTS method was inversely correlated with LDH release. Interestingly, there was a significant increase in cell number in both cell lines at only 48 h in the high nanomolar range, at a concentration that did not cause cytotoxicity (Fig. 6).

Cultured LNCaP cells were supplemented with 25 μM phytanic acid at 24, 36, and 48 h. The level of AMACR was maximally increased at the protein level after 36 h by nearly 3-fold, with no effect at the transcriptional level (Fig. 7*a*). Both the LNCaP and NPrEC cell lines were therefore treated for a set time period of 36 h with increasing concentrations of pristanic and phytanic acids from 100 nM to 3 μM for NPrEC and up to 50 μM for LNCaP cells. AMACR was maximally increased in the LNCaP cell line at the protein level by approximately 3-fold in the presence of 25 μM phytanic acid and by 5–6-fold with 25 μM pristanic acid. In marked contrast there was no effect by either phytol at the transcriptional level (Fig. 7*b*). Conversely, AMACR was slightly decreased at the protein level in NPrEC cells at the highest concentration of 3 μM, but there was no measurable change at the transcriptional level with both phytanic and pristanic acids (Fig. 8).

Discussion

The recent observation that AMACR is significantly up-regulated in nearly all CaP and prostatic intraepithelial neoplasia lesions has prompted speculation regarding the role of this protein, if any, in the initiation or progression of these lesions (14). To address this question, we have searched for and identified two cell lines that could serve as *in vitro* models for CaP tissues (LNCaP) and normal prostatic basal epithelium (NPrEC). We first compared the level of AMACR expression at both the transcriptional and protein levels in a number of cell lines including DU145, PC3, PC3-AR, and LNCaP with that of normal and CaP tissues. LNCaP cells were found to be the only established CaP cell line that expressed AMACR at levels similar to those of CaP tissues. In addition, we have recently established an immortalized prostate epithelial cell line that was shown to express prototypic markers identical to those found in normal basal epithelial cells (41, 45, 47, 48). Whereas the NPrEC cells expressed very low levels of AMACR, as measured by immunoblot analysis, this protein was undetectable by IHC staining in both the normal cell line and normal tissues studied.

The addition of varying concentrations of DHT or E_2 to the culture media had no effect on AMACR expression in either cell line. While we were finalizing these experiments, a confirming report was published indicating that AMACR expression is not responsive to androgen (53). To our surprise, AMACR was highly expressed in LNCaP cells, even in growth

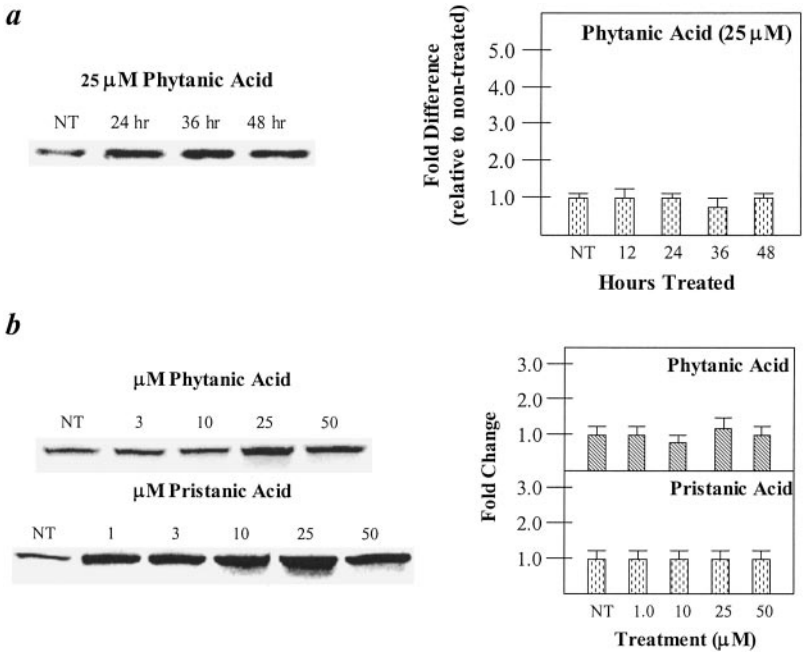

Fig. 7. LNCaP cells were treated with 25 μM phytanic acid for varied time points. *a, left panel,* Western analysis of AMACR carried out on total cell extracts. *a, right panel,* AMACR mRNA levels were normalized to nontreated controls. LNCaP cells were then treated with increasing levels of either phytanic or pristanic acid for 36 h. *b, left panels,* Western analysis of AMACR was carried out on total cell extracts. *b, right panel,* AMACR mRNA levels were normalized to nontreated controls.

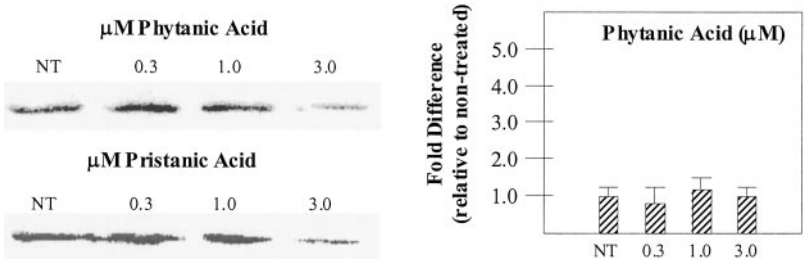

Fig. 8. NPrEC cells were treated with increasing levels of either phytanic or pristanic acid for 36 h. *Left panels,* Western analysis of AMACR carried out on total cell extracts. *Right panel,* AMACR mRNA levels were normalized to nontreated (*NT*) controls.

factor-starved media, indicating that insulin, the primary growth factor in defined media, was not required to maintain expression of this gene. However, it is noteworthy that an unknown factor(s) in FBS consistently down-regulated AMACR by approximately 3-fold at both the transcriptional and protein levels in LNCaP cells. Our data therefore indicate that AMACR is primarily expressed in a constitutive manner, not influenced by systemic hormones, in prostate cancer cells.

Because phytanic acid and its α-oxidation product, pristanic acid, are the common prosubstrate/substrate for this enzyme, respectively, we investigated the possibility that these fatty acids play a role in regulating AMACR expression (21, 22). Our interest in this possibility was prompted by past reports indicating that these fatty acids are present at high levels in beef and dairy products and that consumption of these foods is associated with an increased risk for CaP (22–24, 30, 31, 54). The addition of pristanic and phytanic acids to the LNCaP cell line resulted in a marked increase in AMACR expression at the protein level, with no effect at the transcriptional level. Most interestingly, AMACR was unaffected by similar treatments in the NPrEC cell line. This type of response in LNCaP cells is indicative of a substrate-induced protein stabilization mechanism reported for other metabolizing enzymes such as cytochrome P450 2E1 (55). In agreement with this hypothesis, we found that 1–3 μM of the substrate pristanic acid enhanced AMACR protein to a greater extent than phytanic acid at concentrations between 25 and 50 μM. This indicates either that the prosubstrate is capable of weak interaction with AMACR or that the α-oxidation pathway is significantly active in LNCaP cells, such that an appreciable level of pristanic acid is produced.

From these studies alone, it would be difficult to rule out the possibility that phytanic acid can affect AMACR expression in the NPrEC cell line. This is because 10 μM phytanic acid induced nearly 100% cell death in this cell line, well below that required to enhance AMACR in LNCaP cells. However, pristanic acid was also unable to enhance AMACR expression in the NPrEC cell line at 1.0 μM, but this concentration was sufficient to enhance AMACR expression in LNCaP cells. Moreover, we found that LNCaP cells were at least 20-fold less sensitive to the cytotoxic actions of phytanic acid than the NPrEC cell line. It may be inferred from this that AMACR acts in part as a survival factor. This may be supported in part by previous reports that phytanic acid is cytotoxic; as a result, cellular defenses have evolved in select tissues to help keep this molecule out of the cell (56–58). For example, fatty acid-binding proteins, which are induced by phytanic acid through peroxisome proliferator-activated receptor α, act in a protective fashion by binding to, thus inhibiting the cytotoxic effects of this fatty acid (56–58). In this respect, fatty acid-binding protein V has recently been reported to be expressed at appreciable levels in CaP tissues, thus indicating a need to protect against

this fatty acid in the prostate (59, 60). Finally, it is important to note that phytanic acid also induced a minor yet significant increase in cell number in both cell lines at high nanomolar concentrations within as little as 48 h. Whereas this observation has not been reported previously, it is not entirely surprising, considering the multitude of biological effects that branched-chain fatty acids including phytanic and pristanic acid have been reported to exhibit (24, 52, 61–65).

In summary, AMACR protein, but not its transcript, is expressed at significantly higher levels in CaP cells as compared with NPrEC cells through a mechanism unaffected by common serological factors including DHT, E_2, and insulin. The level of AMACR protein is further enhanced specifically in CaP cells by fatty acid substrates found in foods associated with an increased risk for CaP. These same fatty acids, while having no observed effects on AMACR expression in NPrEC cells, are capable of inducing proliferative and cytotoxic effects at physiological and supraphysiological concentrations, respectively. Although these studies do not rule out the possibility that normal prostatic "secretory" cells may respond to treatments with pristanic acid, these results indicate that enhanced metabolism of branched-chain fatty acids, such as pristanic acid, may contribute to the progression of CaP. Hence, therapeutic strategies aimed at selectively inhibiting this pathway in CaP may prove effective in the treatment and prevention of this disease.

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