Proteomics of rat prostate lobes treated with 2-*N*-hydroxylamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine, 5α-dihydrotestosterone, individually and in combination

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Abstract. Epidemiological and preclinical studies suggest that environmental factors, hormonal responses and lifestyle, including diet and physical inactivity, are likely contributors to the initiation and progression of prostate cancer in humans. Although the effects of the food derived carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and/or testosterone (T) in the development of prostate cancer in the rat have been reported, the extent to which such compounds impact cancer related proteins is not clear. Knowledge of cancer-related proteins impacted by PhIP and/or T is pre-requisite to developing novel strategies to early-detect prostate cancer. Male F344 rats were sacrificed, the prostate tissue isolated and separated into dorsolateral, ventral, and anterior lobes. The lobes were cultured and treated with 10^{-3} M NHPhIP and/or 10^{-7} M DT for 24 h. NHPhIP is the genotoxic form of PhIP and DT is the more proliferative form of T. We used 2D-DIGE and LC/MS/MS technologies to study the proteome of the prostate lobes to determine if the compounds will trigger detectable changes in expression of cancer-related proteins. Analysis of the signals from 2D-DIGE revealed that about 10% of proteins were differentially expressed in the NHPhIP and/or DT treatments compared to controls. Eight candidate protein spots detected by 2D-DIGE in at least two out of three lobes showed ≥2-fold difference between treated and control samples. Five out of the eight spots contained single proteins; including, phospholipase Cα (PLP-Cα), Rab7, SAR1a, ribosomal protein S7 (RPS7), and nucleoside diphosphate kinase (NDPK). A survey of the literature shows that NDPK expression is altered in human cancers, including prostate cancer. Thus, we validated the altered expression of NDPK by Western blot analysis. The concordance between 2D-DIGE and Western blot analysis was 80%. The results of this study demonstrate, for the first time, that the combination of 2D-DIGE and LC/MS/MS is a powerful tool for identification of proteins in the prostate tissue that are altered by environmental carcinogens and/or hormones.

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Abbreviations: PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; NHPhIP, 2-*N*-hydroxylamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; T, testosterone; DT (DHT), 5α-dihydrotestosterone; HAA, heterocyclic aromatic amines; PLP-Cα, phospholipase Cα; RPS7, ribosomal protein S7; NDPK, nucleoside diphosphate kinase; AL, anterior lobe; DL, dorsolateral lobe; VL, ventral lobe

Key words: prostate cancer, proteomic analysis, protein expression, androgen, heterocyclic aromatic amines, environmental carcinogens, hormones

Introduction

In the USA prostate cancer represents a major clinical and public health challenge; yet the etiology of this disease remains obscured. The American Cancer Society estimates that in 2008 about 186,320 men will be diagnosed with prostate cancer and an estimated 28,660 will die (1). Epidemiological studies revealed that populations that consume more saturated fat and meat products have a greater incidence of prostate cancer (2). The World Health Organization lists meat and animal fat intake as known risk factors for the development of prostate cancer (3) and Western diets are implicated in the increase in prostate cancer incidence in some ethnic groups compared with incidences in their native countries (4,5). The food-derived heterocyclic aromatic amines (HAA), which are formed when meat is cooked at high temperatures, have been shown to cause prostate cancer in rodents and are suspected to be prostate carcinogens in humans (6-8). The most abundant and most carcinogenic HAA is 2-amino-1-methyl-6-phenyl-imidazo [4,5-*b*]pyridine (PhIP, Fig. 1) (8). PhIP has been shown to form DNA adduct in all lobes of the rat prostate and seminal vesicles by ^{32}P-postlabeling (8) as well as by immuno-histochemistry (7). Although the levels of PhIP-DNA adduct in the ventral lobes (VL) were found to be almost the same as in the dorsolateral lobes (DL) and double those in the anterior

lobes (AL) (6), no clear differences in immunohistochemical staining intensity among the accessory sex organs could be identified (7).

Testosterone (T) is the principal androgen that controls prostate function (9); however, in addition to diet, preclinical studies also support the role of androgens in prostate carcinogenesis (10). For example, castration in early life greatly reduces the development of prostate cancer in rodents whereas androgens promote chemically induced rat prostate carcinogenesis (11). In humans, androgens are linked to the development of prostate cancer because the incidence of prostate cancer is significantly lower in eunuchs and patients with 5α-reductase deficiency who have low to undetectable levels of 5α-dihydrotestosterone (DT), the more proliferative form of T (12,13). Indeed cumulative lifetime exposures to androgens or specific polymorphisms of the androgen receptor are frequently associated with increased prostate cancer risk (14-18).

Environmental factors, hormonal responses and lifestyle, including diet and physical inactivity, are likely contributors to the initiation and progression of prostate cancer in humans (4,5,19-21). Indeed hormonal responses in individuals are linked to diet since reduction in fat intake has been reported to reduce circulating T levels in man (22).

PhIP alone induced non-invasive prostate carcinomas in both the ventral and anterior lobes in rats and resulted in invasive carcinomas of the anterior prostate at a pharmacological dose of testosterone propionate (TP) in the post-initiation phase (8); these findings suggest that TP strongly promotes those PhIP-initiated AL cells to develop into malignancies. Although the combined and individual effects of PhIP and TP in the development of prostate cancer in the rat have been reported (7), the extent to which such compounds cause region-specific differential protein expression in prostate lobes or more importantly, impact cancer related proteins is not clear. Knowledge of the proteins that are impacted by PhIP and/or T is prerequisite to developing novel strategies to early-detect prostate cancer from prostate biopsies.

Proteomics offers a powerful strategy for elucidating the global pattern of protein expression in biological and clinical specimens. Various proteomic technologies have been used to screen tumor and serum samples to identify cancer-specific proteins with potential for use in cancer diagnosis and prognostication, or serve as therapeutic targets (23-26). Proteomics also has the potential to unravel important cellular mechanisms associated with cancer development, such as posttranslational modifications, phosphorylation, and degradation of proteins (27). Amongst the proteomics strategies that have been applied to cancer research, the two-dimensional gel electrophoresis (2-DE) technology is still one of the most widely used for discovery of proteins and comparative proteomics (23). The variant of the 2-DE technology termed two-dimensional difference in gel electrophoresis (2D-DIGE) is emerging as the most sensitive and reproducible variant of 2-DE, because it involves the use of Cyanine fluorescent dyes and co-separation of 'treated', 'reference' and control samples in the same 2D process (23). The 2D-DIGE-MS/MS approach has been successfully used to study many cancers (28-31).

We report for the first time the use of 2D-DIGE technology in combination with tandem mass spectrometry to identify differentially expressed proteins in cultured normal rat prostate lobes treated with 2-*N*-hydroxylamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (NHPhIP, the genotoxic form of PhIP) and/or DT (Fig. 1). The active forms, NHPhIP and DT, were used to bypass the metabolic activation step of the parent compounds, and thus ensure that the active genotoxic and proliferative intermediates are delivered. The effects of NHPhIP and/or DT treatments were compared to untreated control group. The main objectives of this study are: a) to identify the proteins that are impacted by NHPhIP, DT, individually and in combination, and b) to establish if the differentially expressed proteins are related to prostate carcinogenesis.

Materials and methods

Animals, prostate lobe culture and treatment with NHPhIP and DT. A total of 12 pathogen-free male F344 rats at 6-7 weeks of age were purchased from Charles River Breeding Laboratory (Kingston, NY) and used after IACUC approval. The F344 rats were selected on the basis of previous studies by Shirai *et al* that showed that TP strongly promotes PhIP-initiated prostate gland of these rats to develop into malignancies (8). Rats were fed Teklad 2018 diet and water, quarantined for one week and housed for an additional week in an animal facility maintained on a 12-h light/dark cycle, at a constant temperature (22±2˚C) and relative humidity (55±15%). Rats were sacrificed by CO_2 asphyxiation, followed by cervical dislocation; the prostate glands were rapidly excised under sterile conditions and separated into DL, AL and VL within 10 min of sacrificing. Each lobe was divided into 4 groups and cultured as described below.

Each group of lobes was minced into ~1 mm^3 pieces with a sterile razor blade in ice-cold phenol red-free Medium 199 with Earle's salts. The medium contains 100 IU/ml penicillin-streptomycin, 100 g/ml L-glutamine, (all from Gibco BRL, Grand Island, NY), 0.08 IU/ml insulin lente (Eli Lilly, Indianapolis, IN) and 10^{-7} M corticosterone (Sigma, St. Louis, MO). The prostate lobes were cultured at 37˚C in an atmosphere of 5% CO_2/air for 10 days. The medium was changed every other day. After 10 days of culture, three groups were treated with 10^{-7} M DT, 10^{-3} M NHPhIP, individually and in combination and fourth group served as vehicle control. Following treatment, the tissues were incubated for 24 h, the media removed and the tissues rapidly frozen and stored at -80˚C until proteomic analysis.

Protein isolation. Proteins were isolated from the frozen prostate lobes using the ToPI-DIGE™ total protein isolation kit (ITSI-Biosciences, Johnstown, PA). Briefly, each frozen lobe was rapidly homogenized in about 50 μl of ToPI Buffer-2 (7 M urea, 2 M thiourea, 4% Chaps, 0.5% NP-40, 5 mM magnesium acetate, 30 mM Tris-HCl, pH 8.5) using clean disposable plastic pestles supplied with the kit. After homogenization samples were incubated on ice for 30 min, with 4 vortexes, and centrifuged at 16,000 x g for 10 min. The supernatant was transferred to a fresh tube and placed on ice until analysis on the same day. Total protein concentration

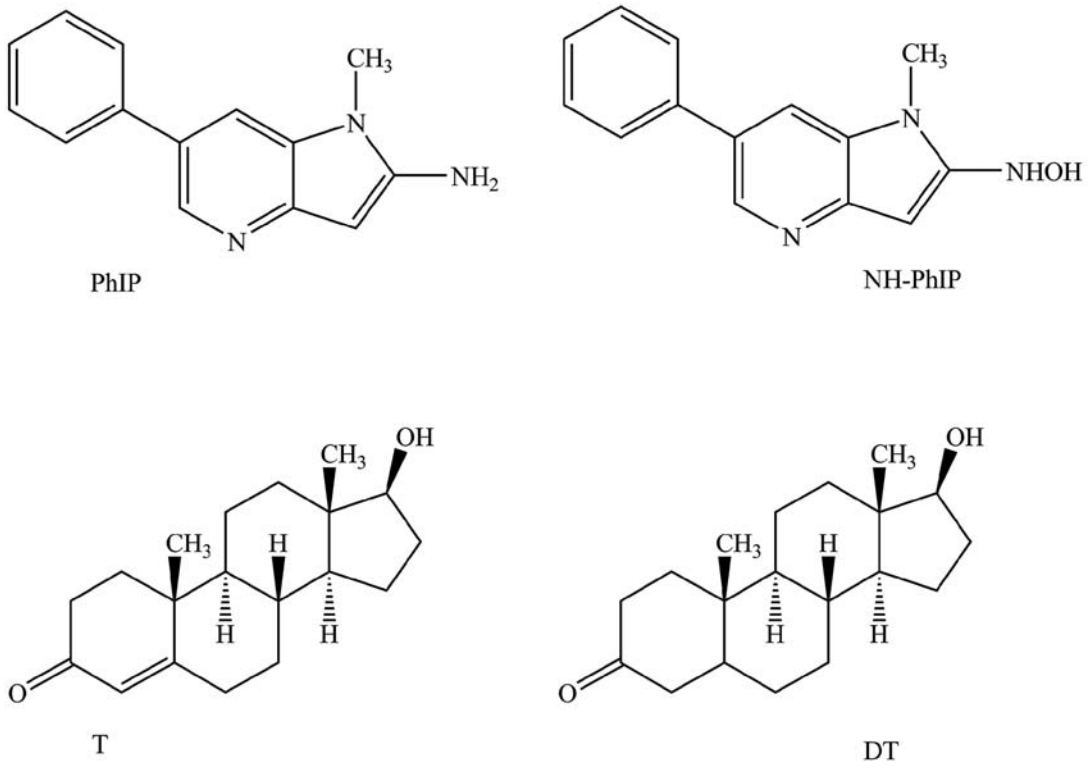

Figure 1. Chemical structures of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), 2-*N*-hydroxylamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (NHPhIP), testosterone (T) and 5α-dihydrotestosterone (DT).

of each sample was determined with the ToPA™ protein assay kit (ITSI-Biosciences) using the Bradford protocol and bovine serum albumin as standard.

2D-DIGE. For 2D-DIGE, 50 μg of protein isolated from NHPhIP-, DT-, DT plus NHPhIP-treated and control samples were transferred to clean microfuge tubes and labeled with 200 pmoles of Cy3 or Cy5, respectively, using the 'minimal-labeling' protocol (23). Dye swapping was performed to compensate for any slight difference in intensity due to batch-to-batch variations in Cy dye intensity. An aliquot of equal amount of proteins from all the samples (NHPhIP-, DT-, DT plus NHPhIP-treated and untreated control) were mixed and 50 μg labeled with Cy2 to obtain a universal internal standard that will allow gel-to-gel comparison (32). The Cy2, Cy3 and Cy5 labeled samples were mixed and co-separated by isoelectric focusing (IEF) with pH 3-10 linear Immobiline Drystrips (GE Healthcare) in the first dimension. IEF was for a total of 65,500 V hours in an IPGphor electrophoresis unit (GE Healthcare). The focused strips were equilibrated for 15 min in SDS equilibration buffer containing 1% DTT followed by a second 15-min equilibration in SDS equilibration buffer containing 2.5% iodoacetamide. The strips were then placed on 24x20 cm, 12.5% SDS-PAGE gels and electro-phoresed in an *Ettan* DALT6 (GE Healthcare) at 15 W per gel for about 4.5 h.

Image analysis. After 2nd dimension electrophoresis, all the gels were scanned on a DIGE-enabled Typhoon Trio Variable Mode Digital Imager (GE Healthcare) using the following excitation/emission wavelengths: Cy2, 488/520 nm, Cy3, 532/580 nm and Cy5, 633/670 nm. All generated images (3 per gel) were imported into the Biological Variation Analysis module of DeCyder™ software (Version 6.5, GE Healthcare) for matching, normalization and identification of differentially abundant spots, with the False Discovery algorithm enabled. The images obtained from control samples were compared to the images of the corresponding treated samples to identify spots that show 'true' differences as a function of each treatment based on the Mean Fold Difference (MFD). All protein spots automatically detected in DeCyder after normalization that displayed ≥2-fold difference in spot abundance between treated and untreated control groups and occurred in at least two out of three lobes were considered candidate differentially expressed protein spots (CPS). Those spots that passed the filter were further inspected visually for verification before inclusion in our protein-of-interest list.

Identification of differentially expressed proteins by LC/MS/MS. To obtain sufficient proteins per spot that will be amenable to in-gel digestion and sequencing by LC/MS/MS we ran a semi-preparative 2D-PAGE (no Cy dye labeling, 'picking gel') using a total of 300 μg of protein. After 1st and 2nd dimension electrophoreses using the parameters described above, the gel was stained for 1 h with SyproRuby (Invitrogen). Staining was carried out in the dark with slight shaking on a reciprocal shaker. The SyproRuby stained gel was rinsed with excess distilled water and scanned with the Typhoon Trio using 532 nm (excitation) and 610 nm (emission) filters. The resultant image was imported into the BVA module of DeCyder, matched to the 2D-DIGE images to

identify the spots of interest and a pick-list was generated. The candidate spots were picked with the Ettan Spot Picker (GE Healthcare) and in gel digested overnight with proteomics-grade trypsin using the Ettan Spot Digester (GE Healthcare). The in-gel digested samples were extracted in 50 μl of 50% acetonitrile/0.1% formic acid for 20 min (32), dried down completely at 45˚C and stored at -20˚C until sequenced by LC/MS/MS.

To assign identities to the candidate proteins of interest, the tryptic digested samples were reconstituted with ultra pure water and sequenced by tandem mass spectrometry. LC/MS/MS was carried out with nanobore electrospray columns constructed from 360 mm o.d., 75 mm i.d. fused silica capillary with the column tip tapered to a 15-mm opening. The columns were packed with 200 Å 5 μm C18 beads (Michrom BioResources, Auburn, CA), a reverse-phase packing material, to a length of 10 cm. The mobile phase used for gradient elution consisted of: a) 0.3% acetic acid, 99.7% water, and b) 0.3% acetic acid, 99.7% acetonitrile. The flow through the column was split pre-column to achieve a flow rate of 350 nl/min. All tandem mass spectra were acquired on a Thermo LTQ ion trap mass spectrometer (Thermo Corp., San Jose, CA) with the needle voltage set at 3 kV. Ion signals above a predetermined threshold automatically triggered the instrument to switch from MS to MS/MS mode for generating fragmentation spectra. The obtained MS/MS spectra were searched against the NCBI non-redundant protein sequence database using the SEQUEST computer algorithm to establish the protein identity (33).

Western blot analysis. Polyclonal rabbit antibody against nucleoside diphosphate kinase (NDPK) (LifeSpan Biosciences, Seattle, WA) and monoclonal mouse antibody against ß-actin (Abcam Inc., Cambridge, MA), HRP conjugated secondary antibody (Millipore, Bedford, MA) were purchased and used to independently validate the relative abundance of NDPK. Western blot analysis was performed using aliquots of proteins originally isolated for the 2D-DIGE protein discovery step. Equal amounts of protein from each sample (50 μg) were diluted with Laemmli SDS-PAGE loading buffer (1 part sample to 2 parts buffer), heated for 5 min at 95˚C and electrophoresed on precast 4-15% Tris/glycine/SDS gradient gels (Bio-Rad Laboratories, Inc., Hercules, CA). The samples were electrophoresed at 200 V constant current for 35 min using Tris/lysine/SDS as the running buffer. After electrophoresis, proteins were transferred to a Hybond-low fluorescent PVDF membrane (GE Healthcare, Piscataway, NJ) using Towbin buffer which contains 20% methanol and 0.037% SDS as transfer buffer. The transfer was carried out at 43 mA for 1 h with a semi-dry blotting unit (Fisher Scientific, Pittsburgh, PA).

The blot was blocked with Tris-buffered saline containing 0.05% Tween-20 in 5% milk (TBS-T/5% milk) for 1 h. The blocking buffer was removed and the NDPK antibody diluted (1:500) in TBS-T/2.5% milk, was added and incubated for 1 h at room temperature on an orbital shaker. The blot was washed (3 times) with TBS-T by shaking for 10 min on an orbital shaker. After the third wash, the wash buffer was removed and the secondary antibody, diluted (1:3000) in TBS-T/2.5% milk, was added and the blot incubated with shaking for 30 min on the orbital shaker. To detect the signal, blots were incubated in ECL PLUS detection reagent (GE Healthcare) for 5 min (in the dark) and scanned on a Typhoon Trio Variable Mode imager (GE Healthcare) with the blue laser using 488 nm excitation and 520 nm emission filters. After capturing the signal from NDPK antibody, the same blot was rinsed with TBT-T and re-probed with ß-actin antibody diluted (1:10,000) in TBS-T/2.5% milk as described above. The signal intensities generated for NDPK were quantified with ImageQuant software (Version 2003.03, GE Healthcare) after normalization with the signal obtained for ß-actin.

Results

Effect of NHPhIP, DT, individually and in combination on global protein expression. We determined global protein expression in the rat prostate lobes following treatment with NHPhIP, DT, individually and in combination. Analysis of the 'filtered' and 'cleansed' candidate protein spot statistics generated in DeCyder revealed protein abundance differences attributable to treatment with NHPhIP and/or DT (Table I). The total number of candidate protein spots detected varied from 302 spots in the DL following treatment with NHPhIP plus DT to 846 spots in the DT-treated VL. The protein abundance difference that are likely due to NHPhIP and/or DHT treatment in AL, DL and VL ranged from 2.7 to 4.0%, 2.9 to 8.0% and 3.7 to 8.6%, respectively. In general, majority (>90%) of the proteins detected in the three lobes showed no difference in abundance following treatment with NHPhIP, DT, individually and in combination. Treatment with NHPhIP and DT apparently affected the levels of more proteins in the DL (8.0%) and VL (8.6%) compared to anterior lobes (3.0%).

The BVA module of DeCyder software clearly identified candidate proteins that displayed greater than or equal to 2-fold difference in abundance between the treated and control groups. Specifically, 3.2, 5.9 and 5.3% of the total protein spots were differentially abundant amongst the spots considered in the AL (60/1866), DL (68/1156) and VL (88/1653), respectively (Table I). The number of proteins that were elevated after treatment of AL ranged from 2 (0.4%) in lobes treated with NHPhIP plus DT to 6 (1.0%) in lobes treated with NHPhIP, and the proteins that showed lower abundance ranged from 14 (2.6%) in lobes treated with NHPhIP plus DT to 17 (3.0%) in lobes treated with NHPhIP. The number of proteins that were elevated after treatment of DL with the compounds ranged from 9 (3.0%) in lobes treated with NHPhIP plus DT to 19 (4.3%) in lobes treated with NHPhIP, whereas the proteins with lower abundance ranged from 5 (1.2%) in lobes treated with DT to 15 (5.0%) in lobes treated with NHPhIP plus DT. On the other hand, the number of proteins that were elevated in VL following treatment with the compounds ranged from 4 (1.2%) in lobes treated with NHPhIP to 10 (2.1%) in lobes treated with NHPhIP plus DT. The number of proteins that showed lower abundance ranged from 11 (3.3%) in lobes treated with NHPhIP to 31 (6.5%) in lobes treated with NHPhIP plus DT.

Protein identification. To assign identities to the protein spots of interest and determine if such proteins are related to

Table I. Spots statistics obtained with DeCyder software showing the total number of candidate protein spots that are altered in rat prostate lobes treated with DT and/or NHPhIP.

Rat prostate lobe	Treatment	Total no. of protein spots taken into account	No. of protein spots with similar levels (%)	No. of protein spots with higher levels in treated samples (%)	No. of protein spots with lower levels in treated samples (%)	Total no. of differentially expressed protein spots (%)
AL	DT	765	744 (97)	4 (0.5)	17 (2.2)	21 (2.7)
	NHPhIP	558	535 (96)	6 (1.0)	17 (3.0)	23 (4.0)
	NHPhIP + DT	543	527 (97)	2 (0.4)	14 (2.6)	16 (3.0)
DL	DT	409	397 (97)	7 (1.7)	5 (1.2)	12 (2.9)
	NHPhIP	447	415 (93)	19 (4.3)	13 (2.9)	32 (7.2)
	NHPhIP + DT	302	278 (92)	9 (3.0)	15 (5.0)	24 (8.0)
VL	DT	846	814 (96)	8 (0.9)	24 (2.8)	32 (3.7)
	NHPhIP	331	316 (95)	4 (1.2)	11 (3.3)	15 (4.5)
	NHPhIP + DT	476	435 (91)	10 (2.1)	31 (6.5)	41 (8.6)

Table II. Overview of proteins positively identified in the candidate protein spots (CPS) selected for LC/MS/MS.[a]

Spot code	Protein name	NCBI acc. no.	MW	PI
CPS1	Heat shock protein 1 (chaperonin)	gil11560024	60,965	5.91
	Cytoplasmic beta	gil13592133	41,737	5.29
	Alpha tubulin	gil313131	50,136	4.94
	Cytosolic 3-hydroxyl 3-methyl-glutary coenzyme-A synthase (HMG-CoA synthase)	gil55947	57,434	5.58
	Phospholipase C alpha	gil200397	56,621	5.99
	Prolyl 4-hydroxylase, beta polypeptide	gil14250251	57,058	4.77
	Chaperonin subunit 8 (theta)	gil142090480	59,555	5.44
CPS2	Phospholipase - C alpha	gil200397	56,621	5.99
CPS3	Predicted: cytosolic $NADP^+$-dependent isocitrate dehydrogenase	gil50750353	53,031	
	Acetyl-coenzyme dehydrogenase, medium chain	gil15488707	46,437	8.56
	Phosphoglycerate kinase 1	gil45384486	4,538	8.02
CPS4	Phosphoglycerate mutase type B subunit	gil8248819	28,832	6.67
	Glutamate dehydrogenase 1	gil30931187	61,337	8.05
CPS5	Rab7	gil1050551	23,490	6.39
CPS6	SAR1a gene homolog 1	gil50925795	22,399	6.22
CPS7	Ribosomal protein S7	gil55775790	60,965	5.91
CPS8	Nucleoside diphosphate kinase	gil2827446	17,553	7.11

[a]These spots were differentially expressed following treatment of rat prostate lobes with NHPhIP, DT individually and in combination.

carcinogenesis, we selected candidate protein spots (CPS) with at least 2-fold difference in abundance between treated and control samples, and filtered out those spots that were not found in at least two out of the three lobes. Eight CPS with strong signal intensities on the 'picking gel' satisfied our criteria, and were picked, in-gel digested and sequenced by tandem mass spectrometry (Fig. 2). The LC/MS/MS analyses revealed that three of the spots (CPS1, CPS3 and CPS4) contained at least two proteins per spot whereas 5 (CPS2, CPS5, CPS6, CPS7 and CPS8) contained a single protein per spot, including phospholipase Cα (PLP-Cα; CPS2), Rab7 (CPS5), SAR1a (CPS6), ribosomal protein S7 (RPS7, CPS7), and nucleotide diphosphate kinase (NDPK, CPS8) (Table II). The position of CPS7 on the gel in relation to

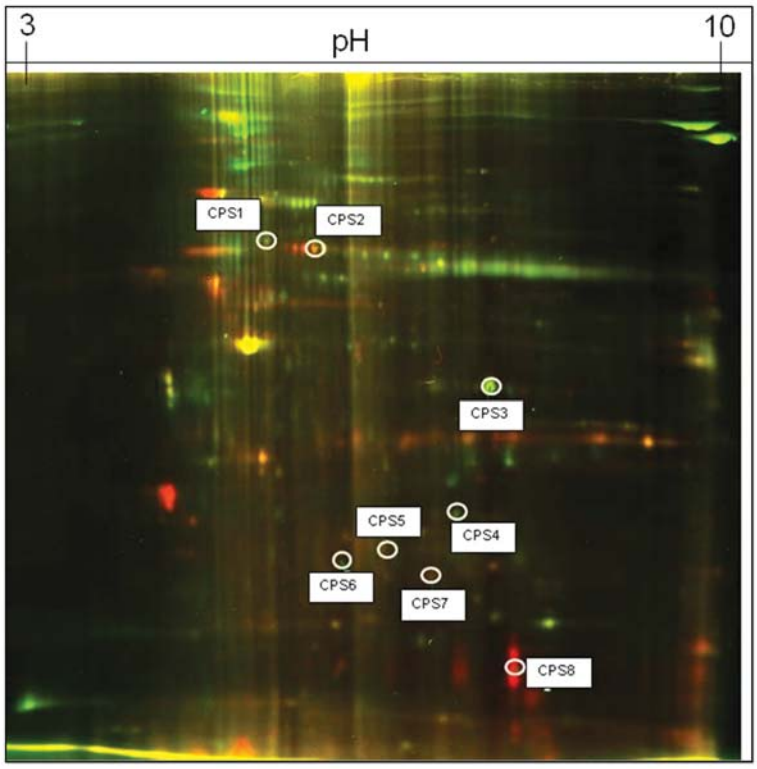

Figure 2. Representative overlaid 2D-DIGE image of Cy dye-labeled prostate lobe samples. Differentially abundant protein spots that were present in at least two of three lobes were detected by the biological variation analysis module in DeCyder, with false discovery function enabled. Eight candidate protein spots (CPS) are highlighted on the representative overlaid gel from a dorsolateral lobe.

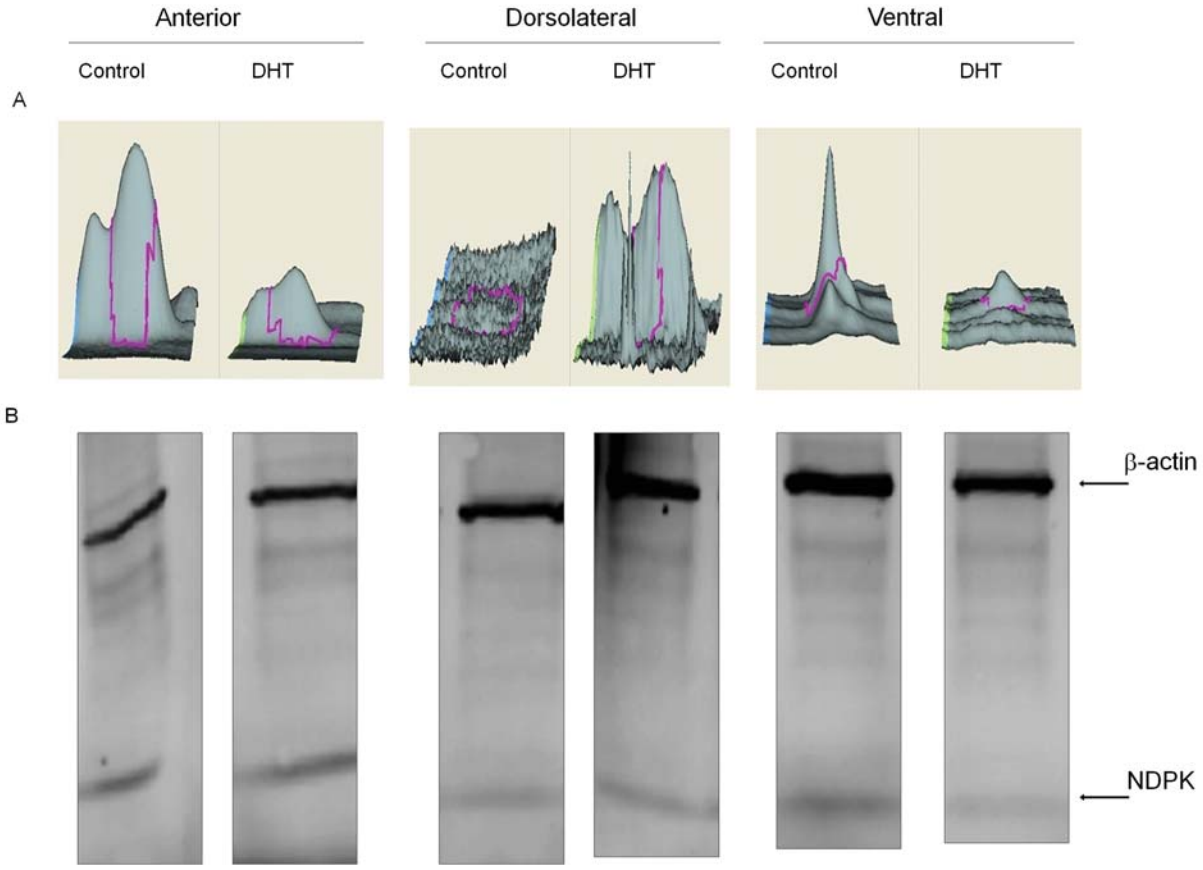

Figure 3. Representative DeCyder simulated 3D image (A) and Western blot image (B) of NDPK in anterior, dorsolateral and ventral lobes of rat prostate treated with DHT. NDPK was higher in treated sample compared to control only in the dorsolateral lobes. In all the cases, the comparison is to DMSO treated controls of the corresponding lobe. Western blot analysis was performed with polyclonal primary antibodies for NDPK and ß-actin (house-keeping control) as described in Materials and methods.

Table III. Comparison of 2D-DIGE and Western blot results for NDPK.

		NDPK	
		MFD	
Prostate lobe	Treatment	2D-DIGE[a]	Western[b]
Dorsolateral	NHPhIP	8.71	ND
	DT	3.26	1.31
	DT + NHPhIP	8.50	1.82
Ventral	NHPhIP	2.04	-2.47
	DT	-6.05	-2.31
	DT + NHPhIP	NS	NS
Anterior	NHPhIP	NS	NS
	DT	-2.87	-3.57
	DT + NHPhIP	NS	NS

[a]2D-DIGE results were obtained in DeCyder software. [b]Western blot results were obtained with ImageQuant after normalization with ß-actin. NDPK, nucleotide diphosphate kinase; MFD, mean fold difference; ND, not done; NS, not significant. Positive numbers indicate higher levels in treated samples and genitive numbers indicate lower levels in treated samples. Four out of five of the spots in which 2D-DIGE showed >2-fold difference correlated with Western blot analysis.

those of the other identified proteins is not consistent with the position you would expect to find a protein that has a relative molecular weight of 60.965 and pI of 5.91. This phenomenon, which is occasionally seen on 2DE gels, is probably due to protein breakdown and/or modifications that apparently alter the physicochemical properties of the protein, and hence migratory pattern during electrophoresis. For example, the recent report in which the 2D-DIGE technology was used to study protein expression in neuromas showed that vimentin, a 51.5-kDa protein, was identified at a higher position on the 2D-DIGE gel than neurofilament L, a 61.5-kDa protein, and mTERF domain-containing protein 1, a 47.4-kDa protein was identified at a significantly lower position on the gel compared to Annexin 5, a 35.8-kDa protein (44). We included CPS7 in our candidate protein list because the spot was consistent, appearing in >50% of the gels examined.

Validation of NDPK abundance difference by Western blot analysis. NDPK was selected for Western blot analysis because it was detected in all lobes by 2D-DIGE and at least one of the treatments caused greater than or equal to 2-fold change in the level of the protein in the three lobes, has been reported by other investigators to be altered in cancer including prostate cancer (34-36) and moreover, it was the only protein among the differentially expressed proteins that has a validated antibody available commercially. The Western blot analysis was performed to independently confirm the 2D-DIGE results. Four out of five results for which the 2D-DIGE data showed a difference of at least 2-fold between treated and control groups correlated with the Western blot data. Thus the concordance between 2D-DIGE and Western blot analysis was calculated to be 80% (Table III). The 2D-DIGE and Western blot data showed that treatment with DHT caused an increase in NDPK levels in DL but a decrease in VL and AL (Table III and Fig. 3). Western blot analysis was not performed for NHPhIP treated DL because the sample was exhausted during the 2D-DIGE phase of the study. The results from the 2D-DIGE and Western blot analysis for NHPhIP treated VL did not correlate (Table III).

Discussion

This is the first report describing the proteomics of dissected and cultured rat prostate lobes treated with NHPhIP, DT, individually and in combination. This report is important because although the parent compounds, PhIP and T, have been reported to be involved in the development of prostate cancer *in vivo*, direct evidence of impact on region-specific changes in protein expression is lacking. We were motivated because identification of differentially-expressed proteins in a model of chemically-induced prostate carcinogenesis may allow the identification of cancer-specific proteins that could serve as biomarkers for prostate cancer diagnosis, monitoring, progression, and/or therapeutic and chemoprevention targets (23-26). Such prostate cancer-specific proteins or their surrogates may be detectable in body fluids and biopsy samples of individuals at high risk for developing the disease. Early stage prostate cancer usually has no symptoms and it is most frequently detected through screening tests such as prostate specific antigen (PSA) blood test and digital rectal examination. However, an augmented PSA level in the blood does not automatically point to prostate cancer, because PSA levels can be altered by other prostate circumstances including benign prostatic hyperplasia or during infection. In fact 75% of men with an elevated PSA (4-10 ng/ml) in the blood do not have prostate cancer, and prostate cancer has been diagnosed in 25% of men with normal or below normal PSA levels (≤4.0 ng/ml). Therefore there is an urgent need for more accurate biomarkers and non-invasive techniques for detection of prostate cancer.

In this study we used 2D-DIGE and LC/MS/MS technologies to study the proteome of rat prostate lobes treated with an androgen and/or a carcinogen found in cooked meat to determine if the compounds will trigger detectable changes in the pattern of protein expression. Analysis of the normalized, filtered and cleansed signals obtained after 2D-DIGE revealed <10% of proteins showed ≥2-fold differences in abundance between NHPhIP and/or DT treatment compared to controls (Table I).

Lexander and co-workers have utilized 2-DE to study protein expression in three anatomical zones of the human prostate: peripheral (PZ), transition (TZ), and central (CZ), with the aim of identifying proteins with significant zonal differential expression (37). Interestingly, 10 proteins with significant zonal differential expression were identified. Out of these, 8 proteins (arginase II, ATP synthase, cytokeratin-8, lamin A/C, peroxiredoxin-4, protein disulfide isomerase A3, tropomyosin, and vimentin) were underexpressed in the CZ compared to the PZ and the TZ, and 2 proteins (peroxiredoxin 2

and creatine kinase B) were overexpressed in the CZ. This study demonstrated that although the peripheral and transitional zones differ in terms of incidence of prostate cancer and hyperplasia, they have epithelium with highly similar major protein expression profiles.

An important goal of this study was to determine if the differentially expressed proteins are related to cancer. We thus selected a subset of the proteins for sequencing by mass spectrometry. Eight candidate spots that showed differential abundance between control and treated samples, and appeared to contain enough proteins (based on visual inspection of the signal intensity of the gel image) were selected for LC/MS/MS analysis. The MS/MS spectra were searched against the NCBI non-redundant protein sequence database (rodent) using the SEQUEST computer algorithm. All the candidate protein spots (CPS) selected contained sufficient peptides which allowed unequivocal assignment of identities to about 63% of the spots selected for LC/MS/MS. Five of the spots contained one protein per spot, including PLP-Cα (CPS2), Rab7 (CPS5), SAR1a (CPS6), RPS7 (CPS7), and NDPK (CPS8) (Table II). A survey of the literature revealed that some of these proteins have been reported as differentially expressed in human cancers by other laboratories (38-40). For example, noradrenaline-stimulated PLP-Cα activity is significantly reduced in tumor tissues of cholangiocarcinoma liver (38). Rab7 gene was found to be overexpressed in diffuse malignant peritoneal mesothelioma but not in ovarian/peritoneal serous carcinoma; both are highly aggressive tumors that are closely related morphologically and histogenetically (39). Rab7 and SAR1a are small GTPases which are up-regulated in hepatocellular carcinoma (40).

The other three candidate protein spots (CPS1, CPS3, CPS4) contained multiple proteins per spot (Table II). Specifically, CPS 1 contained heat shock protein 1, cytoplasmic ß, α-tubulin, cytosolic 3-hydroxyl 3-methylglutarylcoenzyme A synthase, prolyl 4-hydroxylase, ß polypeptide (protein disulfide isomerase), and chaperonin subunit 8. CPS3 contained cytosolic $NADP^+$-dependent isocitrate dehydrogenase, acetyl-coenzyme dehydrogenase, and phosphoglycerate kinase 1 and CPS 4 contained phosphoglycerate mutase type B subunit and glutamate dehydrogenase 1. The observation that some spots on a 2DE gel contain more than one protein per spot is not unusual. The relative abundance of peptides from one protein to the others found in the same spot has been used to determine which protein in a mixture is the most abundant (41). In this study, the peptide information we obtained was not considered sufficient to allow unequivocal determination of the particular protein in the mixture that most likely accounted for the difference in abundance detected by 2D-DIGE. Since we did not have sufficient samples to repeat the 2D-DIGE experiment with narrow range IPG strips to better resolve the spots we are unable to say which protein accounted for the difference in signal intensity observed for CPS1, CPS3 and CPS4.

The result of this study is noteworthy because the level of one of the identified proteins, NDPK, was independently validated by Western blot analysis. NDPK was chosen from the list of candidate proteins of interest because literature suggested that NDPK levels are altered in cancer, including prostate cancer and moreover, it was the only protein that has a validated antibody available commercially. NDPK (EC 2.7.4.6) are products of the nm23 gene family (42). They are ubiquitous enzymes that can catalyze the transfer of γ-phosphates, via a phosphohistidine intermediate, between nucleoside and deoxynucleoside tri- and diphosphates. According to the Gene Ontology annotated database (http://www.ebi.ac.uk/GOA) NDPK functions in ATP binding, magnesium ion binding, nucleotide binding, and exhibit both kinase and transferase activity. They are also involved in CTP, GTP, and UTP biosynthetic metabolic processes, and are components of mitochondrial inner membrane, mitochondrion inter membrane space and mitochondrion.

Hsu *et al* reported that NDPK enhances drug sensitivity and has anti-metastatic activity (43). It is postulated to play a key role in breast tumor angiogenesis, because it apparently interacts with proteins that are involved in cellular signal transduction in angiogenesis and tumorigenesis (34). Of particular significance is the demonstration by Western blot analysis and immunohistochemistry that NDPK level is significantly elevated in human pancreatic ductal adenocarcinoma tissue (35) and in metastatic prostate cancer (36). This suggests that the combination of 2D-DIGE and LC/MS/MS is an effective strategy for discovery and identification of differentially expressed proteins that are related to prostate cancer. The finding that the treatment of rat prostate lobes in culture with DT resulted in elevation of NDPK level in DL, the target lobe, but suppression in AL and VL is of interest (10,11). This is because it is known that the incidence of prostate cancer is significantly lower in eunuchs, and in patients with 5α-reductase deficiency who have low levels of DT (12).

In conclusion, we have provided data, for the first time, demonstrating that rat prostate lobes can be cultured and used as models for investigation of the effects of suspected etiological agents such as androgens and dietary carcinogens on protein expression. We have also shown that 2D-DIGE and LC/MS/MS technologies can be used to identify proteins that show altered expression as a function of NHPhIP and/or DT treatment, and independently validated the level of NDPK by Western blot analysis. We are encouraged by the results because some of the differentially expressed proteins identified, including NDPK have been reported by other laboratories as differentially-expressed in other cancers, including prostate cancer. We recognize that this study needs to be validated especially in an *in vivo* model, with statistics, before far reaching conclusions can be made. It would be of interest to ascertain if any of the protein biomarkers are altered in biopsy samples of humans on the Western diets as well as in those at high risk of developing prostate cancer. Such a study is prerequisite to determining the extent to which the results obtained in rodents can be applicable to humans. By elucidating distinctions between anterior, dorsolateral and ventral lobes at the protein level, we have provided the basis for further work aimed at developing biomarkers for prostate cancer diagnosis and/or therapies that target cancer-susceptible regions of the prostate gland.

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