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Investigation of the Effect of PKC Activation on *In vitro* Prostate Cell Metabolism using ^{13}C NMR

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PKC isozymes have been implicated in regulating everything from transformation and proliferation of prostate cancer cells to apoptosis. Many recent studies have implicated PKC- ϵ , a novel PKC, in supporting cell survival and proliferation in addition to having an anti-apoptotic effect through interactions with BAX. PKC- δ is another novel PKC that has been shown to promote apoptosis in LNCaP cells, and thus antagonizing the antiapoptotic effect of PKC- ϵ . ^{13}C -NMR and ^{13}C (3)-aspartate supplemented media were utilized to examine the metabolism of LNCaP, DU-145 and BPH cell lines with and without activation of PKC by phorbol 12-myristate 13-acetate (PMA). Equivalent amounts of ^{13}C -lactate were produced by the BPH cell line irrespective of addition of PMA (0.50 ± 0.06 mM without PMA and 0.50 ± 0.04 mM with PMA). The LNCaP cells produced significantly less ^{13}C -lactate on PMA treatment from 0.40 ± 0.04 mM to 0.25 ± 0.10 mM, while the DU-145 cells nearly doubled the production of ^{13}C -lactate on PMA treatment from 0.27 ± 0.09 mM to 0.53 ± 0.09 mM. Real-time PCR experiments showed the dramatic effect of PMA on cell metabolism could not be directly explained by the relative expression level of PKC- ϵ and PKC- δ mRNA as there was no statistically significant difference in levels of PKC- ϵ and PKC- δ mRNA. These results suggest an alternative explanation, such as 2nd messenger expression levels, need to be explored.

Introduction

Prostate cancer is the most abundant type of non-skin cancer in American men, with an estimated 230,110 new cases diagnosed in the U.S. in 2004.[1] Human peripheral prostate epithelial cells have unique metabolic properties that allow for the accumulation and secretion of large amounts of citrate at the expense of energy production via the tricarboxylic acid (TCA) cycle and oxidative phosphorylation.[2, 3] Studies have shown that prostate cancer cells do not accumulate or secrete citrate to the same degree as normal prostate epithelial cells.[2, 4, 5] A number of studies have also

shown changes in signaling protein expression between normal prostate epithelium and prostate cancer cells, most notably PKC isoforms.[6-8]

The hypothesis of increased TCA/oxidative phosphorylation activity in prostate cancer cells has been tested using the LNCaP prostate cancer cell line. This cell line is derived from an androgen-dependent prostate tumor. Wright *et al.*[9] observed that in androgen-stimulated LNCaP cells, enzymes involved in fatty acid synthesis, cholesterol synthesis and glycogen catabolism had all increased, while enzymes involved in glycolysis, the TCA cycle, oxidative phosphorylation and β -oxidation of fatty acids had decreased. However, upon androgen depletion, the exact opposite was true. Therefore, these authors concluded that de-regulation of these energy producing pathways was more likely an effect of inactivating the androgen signaling pathway, instead of an early event in neoplastic transformation. The androgen-dependent decrease in TCA cycle and oxidative phosphorylation enzymes provides some insight into a potential mechanism for citrate accumulation and secretion from prostate epithelial cells, but it doesn't offer any clues to the decrease in citrate levels observed in androgen-dependent prostate cancers. These results also open the question as to how energy is generated by normal and neoplastic prostate epithelial cells to support the catabolic processes of fatty acid and cholesterol synthesis.

In the last few years it has become apparent that PKC isoenzymes play a crucial role in prostate cancer cell survival [10], transformation [7, 11] and even apoptosis. [6, 12] While textbooks often refer to simply PKC when describing signaling pathways, this is certainly a gross oversimplification. There are currently 12 members of the PKC family of serine/threonine kinases that have been characterized, and these 12 can be divided into

3 classes based upon their cofactor requirements: classic, novel and atypical.[13] The classic PKCs (α , β_1, β_2 and γ) are activated *in vivo* by Ca^{2+} and diacylglycerol (DAG), the novel PKCs (δ , ϵ , η, θ) simply require DAG, and the atypical PKCs (ζ , λ , μ , ι) are Ca^{2+} and DAG independent. Studies in both androgen-dependent and androgen-independent prostate cancer cell lines have shown that, of all the PKC isoenzymes, down-regulation of PKC- β isoforms and upregulation of PKC- ϵ define the largest differences between malignant and benign prostate tissue.[7] In fact, an immunohistochemical study comparing benign prostate epithelium with cancerous showed that none of the benign prostate epithelial samples stained positive for PKC- ϵ , while 22 of 23 cancerous samples stained positive.[7]

Studies looking at the particular effects of PKC- ϵ have shown that it induces P-glycoprotein, an ATP dependent pump encoded by the multidrug resistance gene.[14] Gene transfer experiments have also shown that overexpression of PKC- ϵ is sufficient to transform androgen-dependent LNCaP prostate cancer cells into androgen-independent cells.[11] In addition, studies have shown that PKC- ϵ is able to interact with BAX, a proapoptotic protein, and in doing so, negate its proapoptotic function.[10] From the perspective of prostate cancer cell metabolism, PKC- ϵ stimulation via prolactin or phorbol 12-myristate 13-acetate (PMA) was reported to increase mitochondrial aspartate amino transferase (m-AAT) activity and increased levels of m-AAT mRNA in both androgen-independent and androgen-dependent prostate cancer cell lines.[15-17] This is intriguing, since m-AAT is necessary for the conversion of Asp to oxaloacetate in the mitochondria for use in the TCA cycle. However, given the reported down-regulation of TCA cycle enzymes demonstrated by Wright *et al.*[9] in androgen-stimulated LNCaP

cells, the role of mAAT in this setting is unclear. What is certain is that PKC- ϵ appears to play an important role in stimulating pathways in prostate cancer cells that are distinct from those necessary to maintain benign prostate epithelial cells.

PKC- δ is another novel PKC isozyme, but unlike PKC- ϵ , it has been shown to function in a pro-apoptotic manner in LNCaP cells.[6] This isozyme works in a redundant pathway with PKC- α to oppose the proliferative effects of PKC- ϵ . Gavrielides *et al.*[18] have demonstrated that the expression of PKC- δ is androgen-dependent in LNCaP cells through androgen response elements (ARE) in the promoter region. They were also able to show that conversion of LNCaP cells to an androgen-independent phenotype via androgen withdrawal directly correlates with down-regulation of PKC- δ , and these PKC- δ depleted cells were significantly more resistant to PMA-induced apoptosis. As a corollary to these studies other groups have shown that RNAi inhibition of PKC- ϵ in androgen-independent cells made them sensitive to PMA-induced apoptosis.[19] These studies strongly suggest that the functions of PKC- δ and PKC- ϵ directly offset one another, and that the ability of prostate cancer cells to adapt and proliferate may be directly dependent upon the expression levels of these two novel PKC isozymes.

^{13}C NMR is a well-established analytical technique for the characterization of small organic molecules. The advantage of ^{13}C NMR over ^1H NMR is the enhanced sensitivity to individual species in solution, given that ^{13}C resonances range from *ca.* 0-200 ppm, while ^1H NMR resonances are generally found within 0-10 ppm. This is of particular advantage in studying cell metabolism in that there is little to no overlap between signals derived from the individual amino acid and sugar products produced by

the cell, thus reducing the ambiguity in assigning the resonances of the measured spectrum to a particular carbon in a molecule.[20] The major drawback of ^{13}C NMR is the low abundance of naturally occurring ^{13}C , with ^{13}C making up 1.1% of all C found in nature. However, this problem of abundance can be circumvented by the use of isotopically-labeled metabolites in the cell media. Due to the ready availability of ^{13}C -labeled metabolites, many groups have actually taken advantage of the extremely low abundance of naturally occurring ^{13}C to follow these exogenous ^{13}C labels through the well-characterized metabolic pathways of the cell to determine the dominant metabolic pathways at work in a variety of tissues under both normal and pathologic conditions.[20]

The studies presented herein take advantage of ^{13}C -NMR with the use of $^{13}\text{C}(3)$ -aspartate as a substrate for cell metabolism to determine the metabolic activity of *in vitro* BPH, DU-145 and LNCaP cells as a surrogate marker of cell vitality/proliferation. With this method, we have been able to show that PMA has distinct effects on the metabolism of the prostate cancer cell lines LNCaP and DU-145 which is consistent with the conclusions based upon other techniques that have been used to determine cell proliferation. In addition, real-time PCR experiments have been performed in an attempt to correlate the expression levels of PKC- δ and PKC- ϵ with the metabolic activity observed in these cells. The implications of this technique and these results for future *in vitro* and *in vivo* studies of prostate cancer therapies will be discussed.

Materials and Methods

Human prostate cancer cell lines LNCaP and DU-145 were obtained from American Type Culture Collection (ATCC, Manassas, VA) and BPH cells were a gift

from the laboratory of Dr. John Omdahl from a primary culture. The cells were cultured as a monolayer in a T75 vented flask under 10 mL of RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 100 U/mL penicillin-streptomycin (Invitrogen) and 2 mM L-glutamine (Sigma, St. Louis, MO). The cells were maintained in a 5% CO₂ atmosphere at 37°C, and the media was changed every 48 hours while the cells were proliferating. Upon reaching 80% confluence, the FBS supplemented media was removed, and the cells were treated with serum-free RPMI supplemented with 3.73 mM ¹³C(3)-aspartate (Cambridge Isotope Laboratory, Cambridge, MA), 1 nM DHT (Sigma, St. Louis, MO), and 100 U/mL penicillin-streptomycin. 10 µg/mL of PMA was also added to the media in half of the culture flasks. The cells were incubated in this ¹³C-aspartate labeled media for 48 hours, at which time the media was harvested, frozen and lyophilized to prepare NMR samples. The cells were harvested by treatment with 0.05% trypsin-EDTA (Invitrogen) for RNA purification.

NMR sample preparation and data analysis

NMR samples were prepared by dissolving the lyophilized media in 500 µL of D₂O (Sigma-Aldrich, St. Louis, MO) and 200 µL of 51.8 mM 2,2'-dimethyl-2-silapentane (DSS) (Sigma-Aldrich) as an internal chemical shift standard. The pH of each sample was adjusted to between 7 and 7.5 using DCl (Sigma-Aldrich) and NaOD (Sigma-Aldrich). Samples were placed in a 5 mm OD quartz NMR sample tube (Wilmad-Labglass, Buena, NJ) for NMR data acquisition.

¹³C NMR spectra were measured at 125.7 MHz on a Bruker Avance 500 MHz spectrometer using a 5 mm broadband probe using a 60° (26 µs) pulse, a delay time of 9 s

between pulses, a sweep width of 29 kHz and 16,000 data points. Each spectrum is comprised of ~1500 scans with a total data collection time of *ca.* 3.5-4 hours. All ^{13}C spectra were measured using continuous proton decoupling centered at 2762.5 Hz (5.5 ppm). Spin-lattice relaxation times (T_1) were measured on a 1 mM sample of lactic acid (Sigma) using the inversion recovery (180- τ -90) method and analyzed by a 3 parameter exponential fit (See Supporting Information, Figure 1). Chemical shifts are reported relative to DSS and referenced to $^{13}\text{C}(3)$ -aspartate (37.5 ppm).

$$M(t) = M_{\infty} \frac{(\sin \theta)(e^{t/T_1} - 1)}{e^{t/T_1} - \cos \theta} + (\eta + 1)(1 - e^{-a/T_1})e^{-t/T_1} \quad (\text{Eq. 1})$$

The ^{13}C NMR signal intensities were determined by fitting the area under the four signals ($^{13}\text{C}(3)$ -aspartate, $^{13}\text{C}(1,2, \text{ or } 3)$ -lactate) using a non-linear least squares algorithm provided with the ACD9 NMR analysis software (ACDLABS, Toronto, ON). The intensity was then adjusted for saturation with respect to T_1 and the pulse angle $\theta < 90^\circ$ as described by Sillerud and Shulman[21] (Eq 1). In equation 1, $M(t)$ is the measured intensity, M_{∞} is the true intensity of the signal, θ is the pulse angle, t is the time between pulses, η is the nuclear Overhauser effect (NOE), and a is the acquisition time. The NOE was estimated by determining the ratios of the integrated peak areas between spectra measured with gated proton decoupling and continuous proton decoupling. The largest corrections ranged from a factor of 2.3 for C3 to 5.8 for C1, with the largest contribution coming from differences in the T_1 relaxation times.

Once the NMR signal intensities had been corrected, the concentration of ^{13}C that gave rise to the individual peaks was calculated based upon the fact that the total [^{13}C] that had been added to the media came from the 3.73 mM $^{13}\text{C}(3)$ -aspartate. Since the

total [^{13}C] from the four peaks (^{13}C (3)-aspartate and ^{13}C (1,2, or 3)-lactate) in the spectra had to add up to 3.73 mM, the concentration of ^{13}C species that gave rise to each signal was calculated based upon its percentage contribution to the sum of the corrected intensities of the four NMR peaks. The spectra for each cell line both with and without added PMA were measured in triplicate, and the error in the concentration was given as the standard deviation of the replicate measurements.

Real-Time RT-PCR sample preparation data acquisition and analysis

RNA from the cells that were harvested from the tissue culture flask was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA). The quantity of RNA was determined by the absorbance at 260 nm using a Nanodrop-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). A control sample of normal prostate RNA (Ambion, Austin, TX) pooled from 9 autopsy samples was also used to generate cDNA for RT-PCR. cDNA was generated by reverse transcription using 2 μg of RNA in each sample, oligo-(dT)16 (Applied Biosystems, Foster City, CA) and the Omniscript Reverse Transcriptase kit (Qiagen) following the manufacturers instructions. The resultant cDNA was used in PKC- ϵ and $-\delta$ isotype specific real-time PCR experiments.

The following PKC isoform specific primers and glyceraldehyde-phosphate dehydrogenase (GAPDH) normalization control primers reported by Furness *et al.*[22] were used in the real-time PCR experiments (Integrated DNA Technologies, Coralville, IA): PKC- ϵ (Sense 5'-3') AAGGAAAAAGCTCATTGCTGG, (Antisense 5'-3') CCGGACTTCGCCATTCTCACC; PKC- δ (Sense 5'-3') ATCAAACAGGCCAAAATCCAC, (Antisense 5'-3')

TCTGGAATATAGTGTCCCGGC; GAPDH (Sense 5'-3')

GAAGGTGAAGGTCCGAGT, (Antisense 5'-3') GAAGATGGTGATGGGATT.

Relative quantitative real-time PCR experiments were performed using the QuantiFast SYBR Green PCR kit (Qiagen). Each 25 μ L sample contained 12.5 μ L of 2x QuantiFast SYBR Green master mix, 1 μ L each of sense and antisense primers (0.4 μ M each), 2 μ L of a 5 fold dilution of cDNA template and 5.5 μ L of RNase free water. Real-time PCR was performed with an ABI Prism 7000 using the following program: 5 minute activation at 95°C, followed by 40 repetitions of two-step cycling consisting of denaturation at 95°C for 10 s and annealing/extension at 60°C for 30 s. Once the cycling was complete, a melting curve analysis in which the sample temperature was increased linearly from 60°C to 95°C over 20 minutes while the fluorescence is measured for each sample across the temperature range to assess the contribution of non-specific products (eg. primer-dimers) to the fluorescence signal. Each cDNA template was measured with each primer set in quadruplicate and each primer set was measured in quadruplicate in non-template control reactions to verify that there was no contamination in the samples.

Validation assays were performed utilizing the GAPDH reference gene primers and the PKC- ϵ and PKC- δ target gene primers on 5-fold serial dilutions of the cDNA template from the BPH cell line. The slopes of the validation assays were <0.1, demonstrating that the comparative C_t method would be valid in the analysis of the real-time PCR data.[23] The relative expression ratios to the GAPDH reference gene were calculated as $\Delta C_t = C_t(\text{GAPDH})/C_t(\text{PKC})$. The indeterminate error in this calculation is expressed as the standard deviation that is propagated as the sum of the variances. The relative expression levels of PCK- ϵ to PKC- δ in each cell type were calculated using the

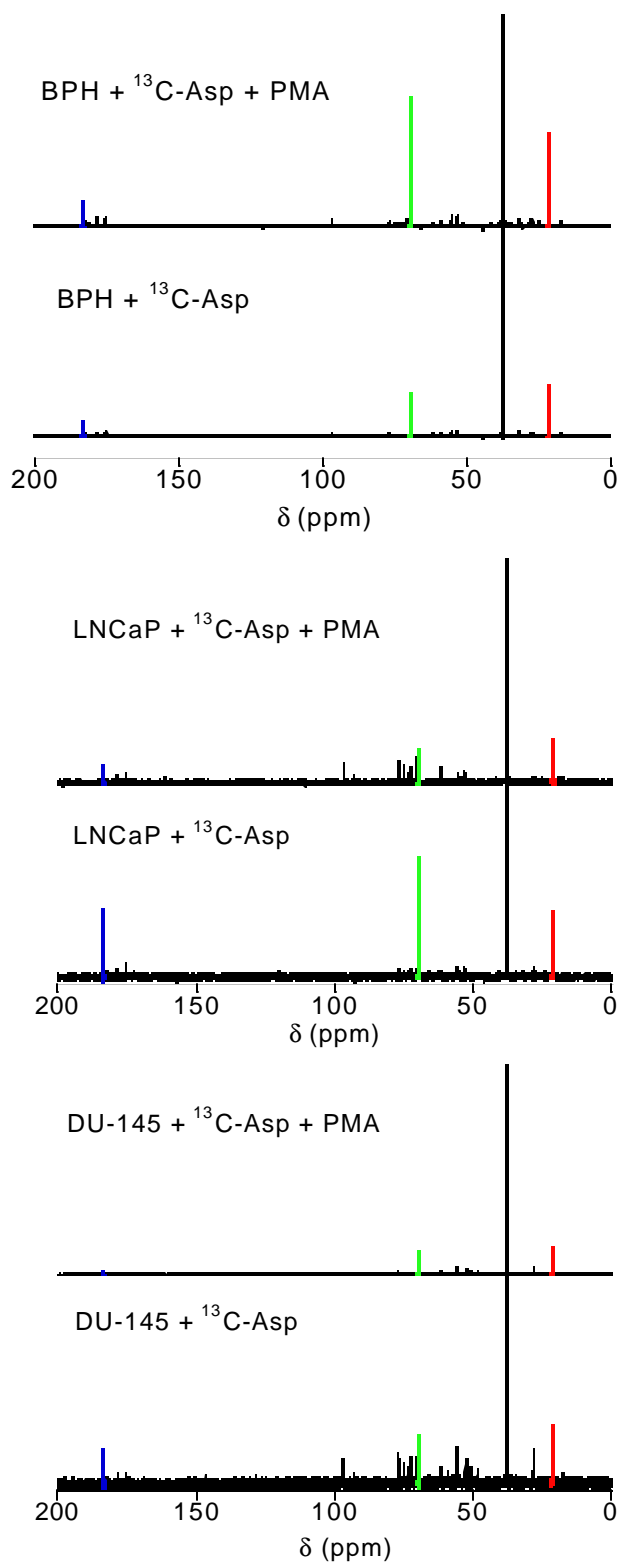


Figure 1. ^{13}C -NMR data from the culture media in which BPH cells (top), LNCaP cells (middle) and DU-145 cells (bottom) had been incubated. Four peaks are consistent throughout the spectra: $^{13}\text{C}(3)$ -lactate (red), $^{13}\text{C}(3)$ -aspartate (black), $^{13}\text{C}(2)$ -lactate (green) and $^{13}\text{C}(1)$ -lactate

formula $\Delta\Delta C_t = 2^{-(\Delta C_t(\text{PKC-}\delta) - \Delta C_t(\text{PKC-}\epsilon))}$, and the indeterminate errors in this calculation are given as the sum of the standard deviations in the C_t measurements for PKC- δ and PKC- ϵ .

Results

Representative ^{13}C -NMR data obtained from the ^{13}C -aspartate labeled media that were incubated over BPH, LNCaP, and DU-145 cells are shown in Figure 1. It is clear that there are four main peaks in the spectra that are assigned to $^{13}\text{C}(3)$ -lactate at 21.0 ppm, $^{13}\text{C}(3)$ -aspartate at 37.5 ppm, $^{13}\text{C}(2)$ -lactate at 69.5 ppm, and $^{13}\text{C}(1)$ -lactate at 183.2 ppm. Other small signals can be found in some the spectra depending on the signal-to-noise ratio of the individual spectrum. These peaks are due to the 1.1% natural abundance ^{13}C found in components of the cell culture media, since they have been concentrated 100 times in the preparation of the NMR samples (Supporting Information Figure 2). While

this small amount of ^{13}C may contribute to the overall signal intensity of the ^{13}C lactate peaks, it is a minimal contribution as one would anticipate observing J-coupling between carbon atoms in the same molecule above the noise level if there were a significant contribution from natural abundance ^{13}C signals.

While it is not surprising to observe significant amounts of lactate produced in *in*

vitro cultured cells,

particularly prostate

tumor cells which have

been shown to upregulate

lactate

dehydrogenase[24], it is

intriguing that this is at

the expense of citrate

production in BPH cells

and other immortalized

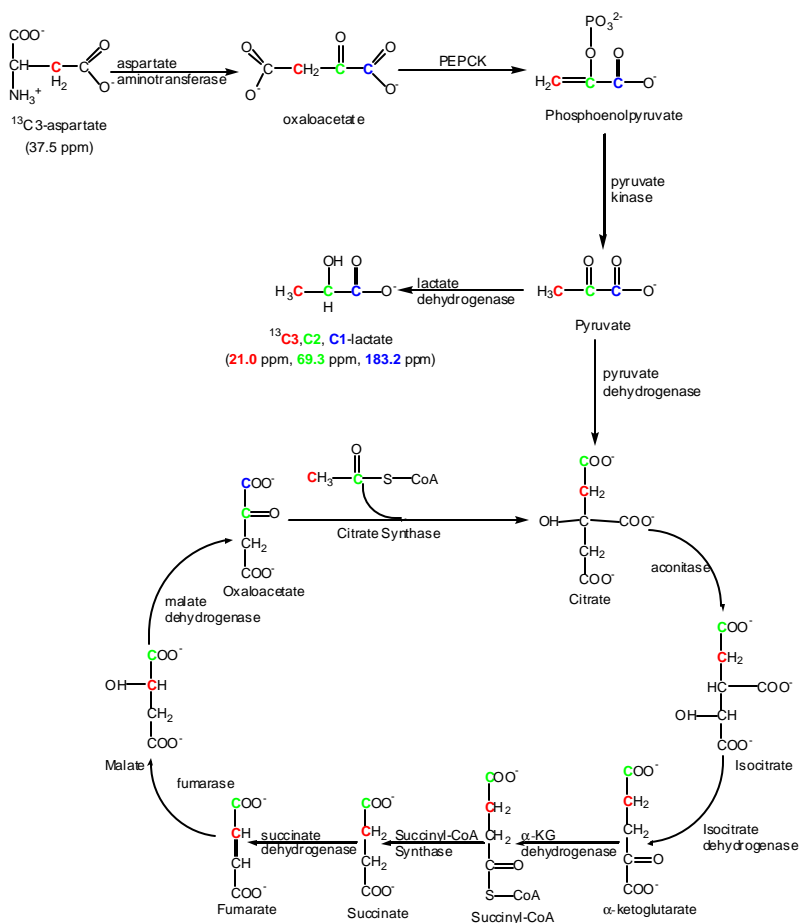
normal prostate epithelial

cell lines such as PrEC

(Clonetech) (data not

shown). It is, however,

clear that the labeled



Scheme 1. Mechanism by which $^{13}\text{C}(3)$ -aspartate label may be passed to $^{13}\text{C}(1,2, \text{ or } 3)$ -lactate.

substrate is being processed through the TCA cycle by the incorporation of ^{13}C labels in the C(2) and C(1) positions of lactate. This occurs irrespective of whether the cell lines were derived from normal prostate epithelium or prostate cancer.

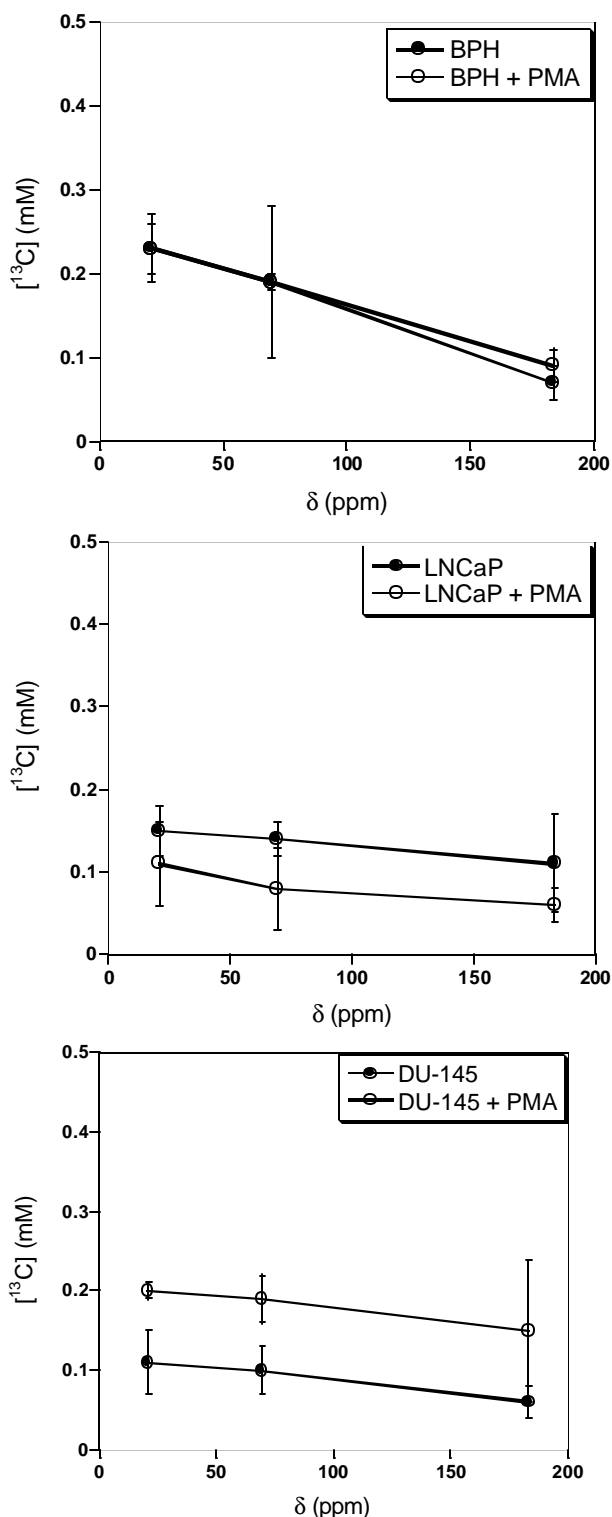


Figure 2. ^{13}C -lactate at C1 (183 ppm), C2 (69.5 ppm) and C3 (21.0 ppm) with and without the addition of PMA to the culture media with BPH (top), LNCaP (middle), and DU-145 (bottom) cell lines.

Scheme 1 describes a potential mechanism by which these labels may be incorporated at the observed positions without multiple labels being incorporated within the same lactate molecule (label scrambling). In this scheme, $^{13}\text{C}(3)$ -lactate can be generated from the labeled $^{13}\text{C}(3)$ -aspartate by bypassing the TCA cycle altogether. However, labels in the C2- and C1-lactate positions, via this scheme, necessarily involves cycling $^{13}\text{C}(3)$ -pyruvate through the TCA cycle to produce a labeled ^{13}C -labeled carbonyl carbon within oxaloacetate that can leave the TCA cycle and pass through PEPCK and pyruvate kinase to LDH. $^{13}\text{C}(1)$ -lactate is then produced if, instead of passing to LDH, the $^{13}\text{C}(2)$ -pyruvate is shunted back to the TCA cycle and

passed out again as carboxylate labeled oxaloacetate to be passed through PEPCCK and pyruvate kinase to LDH. Since these reactions are compartmentalized within the mitochondria of the cell, it would be preferable to recycle some of the labeled products to generate more ATP equivalents than to expend the energy to transport $^{13}\text{C}(3)$ -aspartate from the external media. This scheme describes the mechanism via one anapleurotic pathway into the TCA cycle in which the labels may be placed in the ^{13}C -lactate that is excreted from the cell. It is not meant to suggest that other reaction possibilities do not exist, but this is by far the least energy intensive.

More intriguing than the mechanism of ^{13}C label incorporation is the effect of PMA on the cell metabolism. The concentration of ^{13}C -label that is incorporated into lactate in the BPH cell line shows higher levels of lactate production than either tumor cell line, but there is no difference between the samples with added PMA and those without PMA (Figure 2). The BPH cell line which is derived from cells that do not have malignant potential, demonstrates a consistently high flux of ^{13}C -labeled substrate through the LDH pathway, producing 0.50 ± 0.06 mM of ^{13}C -lactate (Table 1). The rate of substrate through the LDH pathway is not affected by the addition of PMA to the media. This is not the case for the cell lines derived from prostate cancers. The LNCaP cell line has less flux of labeled substrate through the LDH pathway than the BPH cell line, which was not unexpected as the doubling time for this cell line in culture is longer than that of the BPH cells. However, the addition of PMA to the media decreased the amount of labeled substrate produced by nearly a factor of two. The DU-145 cell line, on the other hand, had exactly the opposite response. These cells had the lowest flux of labeled substrate through LDH of all the PMA naïve cultures, with only 0.27 ± 0.09 mM

Table 1. Integrated peak areas for ^{13}C -lactate obtained from NMR measurements and the corresponding concentration of ^{13}C -labeled species.

Sample	C3	Corrected C3	[C3] (mM)	C2	Corrected C2	[C2] (mM)	C1	Corrected C1	[C1] (mM)	[$^{13}\text{C}_T$] (mM)
BPH	0.203	0.468	0.23±0.04	0.164	0.389	0.19±0.09	0.040	0.157	0.07±0.02	0.50±0.06
BPH+TPA	0.232	0.534	0.23±0.03	0.188	0.449	0.19±0.01	0.051	0.212	0.09±0.02	0.50±0.04
LNCaP	0.115	0.258	0.15±0.03	0.100	0.227	0.14±0.02	0.041	0.180	0.11±0.06	0.40±0.04
LNCaP+TPA	0.077	0.168	0.11±0.05	0.064	0.134	0.08±0.05	0.025	0.099	0.06±0.02	0.25±0.1
DU145	0.090	0.195	0.11±0.04	0.081	0.178	0.10±0.03	0.026	0.112	0.06±0.02	0.27±0.09
DU145+TPA	0.199	0.451	0.20±0.01	0.173	0.436	0.19±0.03	0.060	0.350	0.15±0.09	0.53±0.09

^{13}C -lactate produced in 48 hours. Addition of PMA to the culture media increased this amount to 0.53 ± 0.09 mM. Since both of these cell lines were derived from prostate cancer, it is not surprising that they demonstrate responses to the PKC signaling activation of PMA, but the fact that these responses are diametrically opposed is intriguing.

As mentioned previously, immunofluorescence staining for the PKCs in resected sections of prostate tumors have shown that one of the novel PKC, PKC- ϵ , produces strong immunofluorescence signals in cancerous prostate epithelium, but is virtually absent in normal prostate epithelium.[7] In addition, it has been demonstrated that PKC- δ promotes apoptosis in LNCaP cells, and may actually function to counteract the proliferative effect of PKC- ϵ . [6] Since these novel PKCs only require DAG or PMA for activation[13], one explanation for the increased cell metabolism in the DU-145 cell line upon PMA treatment while the LNCaP cell line shows the opposite effect may have to do with the relative expression levels of these novel PKC isoforms. Real time PCR experiments were performed on these cell lines in addition to the BPH cell line and an RNA sample pooled from normal prostate cells of 9 donors (Table 2). The first result of note is that PKC- ϵ mRNA is present in all of the templates, even that derived from the sample of pooled normal prostates. This is in stark contrast to the previous

Table 2. Real-time PCR data.

Sample	Mean C _t	DC _t ^a	DDC _t
Normal prostate PKC-ε	25.69±1.15	0.98±0.04	0.95±0.11
Normal prostate PKC-δ	23.66±0.28	1.06±0.07	
BPH PKC-ε	33.14±1.65	0.89±0.05	0.95±0.08
BPH PKC-δ	30.81±0.66	0.95±0.03	
LNCaP PKC-ε	28.36±1.51	0.85±0.08	0.99±0.16
LNCaP PKC-δ	28.22±1.17	0.86±0.08	
DU-145 PKC-ε	28.72±1.25	0.71±0.03	0.94±0.06
DU-145 PKC-δ	25.56±0.95	0.79±0.03	

immunofluorescence results showing that PKC-ε is not present in normal prostate cells.

In addition, these data show that all of these cells may have a slight preponderance of PKC-δ mRNA, but that it is not significantly greater than the level of PKC-ε mRNA regardless of the template origin.

Discussion

The data presented within demonstrate the feasibility of using *in vitro* ¹³C-NMR to determine the major metabolic pathways utilized in the cell and gauge the metabolic activity of cells in culture by measuring the quantity of excreted byproducts. In this study, we were able to demonstrate not only the basal metabolism of these cells in culture, but also monitor the effect of PKC activation by PMA on the metabolism. While it was not surprising, given previously published studies that activation of PKC in the two cell lines derived from malignant cells had nearly equal and opposite effects on the metabolism[6], the real-time PCR experiments suggest that this is not indicative of the levels of PKC-ε and PKC-δ present in these cells. The demonstration of nearly

equivalent levels of PKC- ϵ and PKC- δ mRNA transcripts in all of the cell lines and the pooled normal template requires an alternative explanation for the effect of PMA on the metabolism of these *in vitro* prostate cells.

The first possible reason the expression levels from the real-time PCR results fail to correlate with the metabolic changes found on PMA treatment lies in the limitations of mRNA levels to necessarily reflect the level of protein expression. While other groups have demonstrated a correlation between the quantity of PKC- ϵ mRNA and the amount of protein ascertained through immunohistochemistry[25], no reports have been published looking at both PKC- ϵ and PKC- δ isoforms over a number of cell lines in these types of correlations. This leaves open the possibility that PKC isozymes are not only regulated at a transcriptional level via AREs [18] and at an allosteric level by substrate binding[13], but also on a translational level through an, as yet, undefined mechanism.

Another possibility is that these cell lines may express other PKC isozymes that participate in redundant pathways much like that described for PKC- α and PKC- δ . [6] If PKC- ϵ and PKC- δ are present in quantities that would provide for a direct offset between the pro- and anti-apoptotic effects, another pro-apoptotic (in the case of LNCaP) or anti-apoptotic/pro-proliferative (in the case of DU-145) PKC isozyme would dominate the phenotype. Along these lines, it is also possible that the phenotype is driven more by the expression levels of the 2nd messenger proteins in these cell lines than the level of PKC- ϵ or PKC- δ that is expressed. This hypothesis has been examined in recent studies that have shown that downstream signaling pathways from PKC- δ modulate myosin light chain activity, thus promoting the migration and invasiveness of DU145WT and PC3 cells.[26]

While any of these possible mechanisms may explain the phenotype observed in both previous cell survival experiments and the ^{13}C -NMR metabolism studies described here, these studies suggest that ^{13}C -NMR metabolic studies are a good surrogate for overall cell viability. This has significant implications for the future study of potential prostate cancer therapies, both *in vitro* and *in vivo*. This technique has obvious applicability as a screening tool for potential prostate cancer therapeutics. It would enable potential therapies to be studied in the laboratory, where the unique metabolic markers could be characterized using both ^{13}C - and ^1H -NMR spectroscopy. This information could subsequently be translated to clinical trials via the use of ^1H magnetic resonance spectroscopy, such that the laboratory results would be used to guide the *in vivo* interpretation of whether a therapy was causing a decrease in cell metabolism as a surrogate marker for stimulation of a pro-apoptotic pathway. Future studies will be directed toward establishing how robust this surrogate marker will be with known effective prostate cancer therapeutics.

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