Human prostate cell lines from normal and tumourigenic epithelia differ in the pattern and control of choline lipid headgroups released into the medium on stimulation of protein kinase C

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epithelial cell lines alters the nature and control of Cho metabolites. In this study, we show that tumourigenesis in human prostate cancer cells (Ackerstaff et al., 2009; Troyer et al., 1991; de Souza et al., 2009; Yalcin et al., 2010) promotes the mitogenic activity of insulin and growth factors. Chronic treatment with 250 nM TPA for the last 9 h of [3H]-choline labelling and then used as above.

Stimulation of phosphatidylycholine (PtdCho) turnover in cells (Kiss, 1990) results in the release of Cho metabolites into the medium (Mufson et al., 1981; Hii et al., 1991; van Blitterswijk et al., 1991; Troyer et al., 1992; Morreale et al., 1997) wherein PCho can promote the mitogenic activity of insulin and growth factors (Cuadrado et al., 1993; Tomono et al., 1995; Chung et al., 1997). In this study, we show that tumourigenesis in human prostate epithelial cell lines alters the nature and control of Cho metabolites released into the medium on PKC activation.

MATERIALS AND METHODS

Cell culture

PNT1A, PNT2C2 and LNCaP prostate epithelial cell lines (up to passages 80, 150 and 50, respectively) were cultured in RPMI1640 (Gibco, Invitrogen Ltd, Paisley, Scotland, UK) with 10 mM HEPES, 2 mM glutamine and 10% fetal bovine serum (FBS) (K10). PC3 cells up to passage 50 were grown in Ham’s F12 medium (Lonza, Slough, Berkshire, UK) with 7% FBS (F7). P4E6 line cells (Maitland et al., 2001) were cultured in KSFM medium (Gibco) with epidermal growth factor and pituitary additves + 2% FBS (K2). For passage/experimentation, cells were rinsed with Tris/saline and solubilised in 100 mM NaOH. Aliquots of each phase were taken in triplicate for scintillation counting. Choline metabolites in 400 µl top phase or medium from release experiments were diluted to 5 ml in distilled water and added to Dowex-50W+ ion exchange resin columns to resolve GPCho (glycerylphosphorylcholine), PCho and/or other vesicles (Nilsson et al., 2009). Aliquots (10 µl) of supernatant medium from this step were also counted.

[3H]-Choline headgroup release into the medium

A total of 7.5 x 10⁴ cells were seeded in triplicate into wells of 44-well plates in normal growth medium (see above) and cultured overnight. For LNCaP cells, wells were coated with poly-l-lysine (20 µg ml⁻¹ in water) to aid adhesion. At 80–90% confluency, the medium was replaced with RPMI1640, F12 or KSFM containing 1% FBS and 0.5 µCi [3H]-choline (Perkin-Elmer, Beaconsfield, Buckinghamshire, UK) per well for 30 h to label Cho phospholipids to equilibrium. With this low level of serum, cells were just becoming confluent when used, and thus significant changes to metabolism or actin as loading controls.

Reverse transcriptase-PCR

Cells were grown in 75 cm² flasks, rinsed and total RNA was extracted using a Qiagen RNeasy mini kit (Qiagen, Crawley, West Sussex, UK) and QIA shredder. RNA was quantified spectrophotometrically and 1 µg taken for cDNA synthesis using the Invitrogen SuperScript II RT (Invitrogen Ltd, Paisley, Scotland, UK) protocol. This was used to prepare a master mix with

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appropriate water controls for PCR. Conditions for amplification were 94°C for 0.5 min, 54°C for 0.5 min, 72°C for 1 min for 35 cycles. Primers were from Eurogentec Ltd (Southampton, UK). Sequences for hPLD1 and hPLD2 primers were as described by Gibbs and Meier (2000). Primers for PKC\(\alpha\) and actin were as described by Myklebust et al (2000).

Transphosphatidylation

A total of 5 \times 10^5 cells per well were cultured in duplicate in six-well plates in 2 ml normal growth medium to near confluency as mentioned above. Cells were rinsed and labelled for 6 h with 1 \muCi [\(^3\)H]-myristic acid (Amersham, GE Health Care, Chalfont St Giles, Buckinghamshire, UK) in 1 ml serum-free medium. Cells were then incubated for 30 min in fresh serum-free medium, which was then replaced with 1 ml fresh serum-free medium containing 0.3% n-butanol (Morris et al, 1997) and either 1 \muM 4z-phorbol, 1 \muM 12-O-tetradecanoylphorbol 13-acetate (TPA) or 1 \muM TPA + 1 \muM Ro31-8220. Cells were incubated for 30 min at 37°C, rinsed and lipids recovered with 1 ml methanol, followed by 1 ml each of 1:2 chloroform:methanol and 1:1 chloroform:acetate acid (9:1:1, v/v) and lipids detected with iodine. After removal of iodine, PtdBut, PtdOH and PtdCho absorbent areas were scraped into scintillation vials for total counts. Triplicate 25 \muL aliquots were applied to oxalate-impregnated silicic acid TLC plates and overlayed with authentic PtdBut, PtdOH and PtdCho standards (Lipid Products, Nutfield, UK). Plates were developed in chloroform:methanol:acetic acid (9:1:1, v/v) and scanned for radioactivity. After extraction, the chloroform phase was evaporated, redissolved in 200 \muL C/M (2:1v/v) and triplicate 10 \muL aliquots were taken into scintillation vials for total counts. Triplicate 25 \muL aliquots were applied to oxalate-impregnated silicic acid TLC plates and overlayed with authentic PtdBut, PtdOH and PtdCho standards (Lipid Products, Nutfield, UK). Plates were developed in chloroform:methanol:acetic acid (9:1:1, v/v) and lipids detected with iodine. After removal of iodine, PtdBut, PtdOH and PtdCho absorbent areas were scraped into scintillation vials for total counts. Triplicate 25 \muL aliquots were applied to oxalate-impregnated silicic acid TLC plates and overlayed with authentic PtdBut, PtdOH and PtdCho standards (Lipid Products, Nutfield, UK).

Statistical treatment

Statistical significance was determined by the Student's two-tailed t-test or by a one-way Anova and the Tukey HSD test.

RESULTS

Protein kinase C alpha expression by prostate epithelial cell lines

Reverse transcriptase–PCR with the same amount of total RNA from each cell line taken for reverse transcription confirmed that all lines express PKC\(\alpha\) mRNA. The amplified band for PKC\(\alpha\) was most prominent in PC3 cells and weakest in the P4E6 line (results not shown). Western blotting of equal cell numbers revealed that all lines express PKC\(\alpha\) protein (Figure 1A). Expression of GAPDH protein by the five cell lines was almost uniform (Figure 1B and E). Actin expression was low in LNCaP cells compared with other lines (Figure 1C and E), making it an unsatisfactory loading control for comparison between the different cell lines used, although

![Figure 1](image-url)

**Figure 1.** (A) Western blot detection of PKC\(\alpha\) protein expression in PNT2C2, PNT1A, P4E6, LNCaP and PC3 prostate epithelial cell lines as described in Materials and Methods. The PKC\(\alpha\) blot shown was stripped and reprobed for (B) GAPDH or (C) actin. Bands were quantified using Image J. (D) PKC\(\alpha\) protein content of prostate epithelial cell lines normalised to GAPDH protein. (E) Comparison of GAPDH and actin protein content of prostate cell lines showing low actin content of LNCaP cells. Blots shown are typical of several repeats. Positions of 100, 75, 50 and 37 kDa markers are shown.
satisfactory for the same cell line. Bands in the PKCζ blot were thus normalised to GAPDH (Figure 1D) to reveal differences in PKCζ protein expression.

Prostate cell lines express PKCδ, PKCε, PKCζ and MARCKS

Western blotting revealed that all five prostate cell lines express PKCα, PKCδ, PKCζ and MARCKS protein (results not shown).

Myristoylated alanine-rich C kinase substrate phosphorylation

TPA (1 μM) stimulation of cells for 15 and 30 min increased MARCKS phosphorylation in PNT1A, P4E6 and LNCaP lines (Figure 2). Constitutively phosphorylated MARCKS was detected in PNT2C2 and PC3 cells, and this phosphorylation was not obviously increased by addition of TPA. Cells that had been exposed to 250 nM TPA for 9 h to downregulate PKCζ protein before restimulation with 1 μM TPA for 30 min (30D). In all lines, downregulation of PKCζ protein results in significantly reduced MARCKS phosphorylation. Blots were stripped and reprobed for actin as a loading control. A repeat experiment gave similar results. Positions of 80, 75, 50 and 37 kDa markers are indicated.

Prostate cell lines express mRNA for both PLD1 and PLD2

Reverse transcriptase–PCR with equal quantities of total RNA from each cell line taken for reverse transcription revealed that all five prostate epithelial cell lines express mRNA for PLD1 and PLD2 (results not shown). PLD1 mRNA expression was most prominent in PC3 cells. Phospholipase D2 mRNA expression was prominent in P4E6 and PC3 lines.

Phospholipase D activity

All cell lines showed basal (unstimulated) PtdBut formation (Figure 3) in the transphosphatidylation reaction indicating PLD activity. PtdBut formation was increased 2- to 2.5-fold when PNT2C2, PNT1A, P4E6 and LNCaP cell lines were treated with TPA to activate PKC, and by about three-fold in PC3 cells. In all cell lines, TPA-stimulated PtdBut formation was reduced to basal level by inclusion of the PKC inhibitor Ro31-8220 (Figure 3).
Prostate epithelial cell lines release choline metabolites

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Figure 5 (A–E) Prostate epithelial cell lines show different patterns of [3H]-label release into the medium on stimulation with 1 μM 12–24-phorbol [8] or 1 μM TPA [4] for up to 6 h as described in Materials and Methods. Results are means ± s.d. (n = 6). Repeat experiments (n = 3) showed similar trends. For PNT2C2 and PNT1A lines **p < 0.001, ***p < 0.0001 against basal values. For P4E6, LNCaP and PC3 lines *p < 0.05, **p < 0.01, ***p < 0.001 against basal values.

Inhibition of phorbol ester-stimulated [3H]-radioactivity release

Ro31-8220 and GF109203X reduced TPA-stimulated release of [3H]-choline metabolites from PNT2C2 and PNT1A cells to basal values as shown in results from a 3-h incubation (Figure 6A). With LNCaP cells, GF109203X reduced [3H]-label release to the medium to below basal values. Go6976, a PKC inhibitor supposedly selective for α and β1 isoforms, had only a small inhibitory effect on [3H]-label release from PNT2C2 cells and was without significant effect on label release from PNT1A cells.

Effects of hemicholinium-3 and D609

When monitored in a 3-h incubation (Figure 6B), D609 at 200 μM almost completely inhibited the TPA-stimulated release of [3H]-label from PNT2C2 and PNT1A cells. The choline transporter inhibitor hemicholinium 3 (HC-3) at 100 μM was without effect on [3H]-label released from PNT2C2 cells, and induced a partial but significant inhibition of label release from PNT1A cells. In the experiment shown (Figure 6B), activation of PKC with TPA stimulated a slight increase in label release from LNCaP cells, but this was not a consistent effect. Hemicholinium 3 reduced this stimulated release to basal levels. In this experiment, effects of TPA and inhibitors on PC3 cells were not significantly different from basal values.

Nature of the choline metabolites released into the medium

Medium from basal and phorbol ester-stimulated cells at 3 h time points was resolved into GPCho(GPC), PCho and Cho fractions on Dowex-50WH + ion exchange resin columns (Figure 7). Results for each Cho metabolite are expressed as a % of the total Cho
metabolites released (i.e., GPC, PCho + Cho). Phosphocholine was the major metabolite released into the medium by basal and phorbol ester-stimulated PNT2C2 cells, whereas Cho was the major metabolite detected in media from basal and stimulated P4E6, LNCaP and PC3 lines. PNT1A cells were intermediate in that PCho and Cho each accounted for about equal proportions of the [3H]-label released. In the results shown for PC3 cells, Cho accounted for a higher proportion of the metabolites released on TPA stimulation (68%) compared with unstimulated cells (45%). With all other lines, the proportions of GPC, PCho and Cho were the same in unstimulated and TPA-stimulated cells. Thus, in PNT2C2 cells, TPA treatment increased Cho metabolite release, but the proportions of GPC, PCho and Cho were the same as from unstimulated cells.

[3H]-Choline uptake by prostate cell lines

PNT2C2 cells at 1 × 10^5 cells per well showed a greater uptake of [3H]-Cho label over 60 min at 37°C (Figure 8) compared with the other four cell lines where levels taken up were more comparable under identical conditions.

Figure 6  (A) Protein kinase C inhibitors reduce phorbol ester-stimulated release of [3H] label into the medium from PNT2C2 and PNT1A cells. PNT2C2, PNT1A, LNCaP and PC3 cell lines labelled with [3H]-cholesterol were fractionated as described in Materials and Methods section with 1 μM 4x-phorbol (basal, B), 1 μM TPA (TP), 1 μM TPA + 1 μM Ro31-8220 (Ro), 1 μM TPA + 1 μM GF-109203X (GF) or 1 μM TPA + 1 μM Go6976 (Go) and release of [3H]-label into the medium was monitored at 3 h. In both PNT2C2 and PNT1A cells, PKC inhibitors reduced release to basal values. Results are ± s.d. (n = 6). For PNT2C2 and PNT1A cells, *p < 0.01, **p < 0.001, ***p < 0.0001 against the basal value. For LNCaP cells **p < 0.01 against the basal value. (B) D609 and hemicholinium-3 effects on [3H]-label release by prostate cell lines. PNT2C2, PNT1A, P4E6, LNCaP and PC3 cells labelled with [3H]-cholesterol were fractionated as described in Materials and Methods with 1 μM 4x-phorbol, 1 μM TPA (TP), 1 μM TPA + 200 μM HC-3 (H) or 1 μM TPA + 100 μM D609 (D) and release of [3H]-cholesterol metabolites to the medium was monitored at 3 h. Results are ± s.d. (n = 6). For PNT2C2 cells ***p < 0.0001 against the basal value. For PNT1A cells **p < 0.01 against the TPA value, ***p < 0.0001 against basal value. For LNCaP *p < 0.05 against the basal value, **p < 0.01 against the TPA value.

Figure 7  Tumourigenic prostate cell lines release mainly Cho into the medium as the principal metabolite, whereas PCho is released by non-tumourigenic PNT2C2 cells. GPC (GPC), PCho and Cho in medium from 3 h time points from basal (B) and TPA-treated (TPA) cells were fractionated as described in Materials and Methods section. Results for individual metabolites are expressed as percentage of the total GPC + PCho + Cho d.p.m. measured ± s.e.m. (n = 4).

Figure 8  Comparison of Cho uptake by non-tumourigenic and tumourigenic prostate cell lines in monolayer culture. Results are shown as d.p.m. per 25 μl from 1 × 10⁶ cells solubilised in 250 μl over 60 min at 37°C as described in Materials and Methods section and are ± s.d. (n = 6).

[3H]-Choline distribution in Cho metabolites and phospholipids after labelling

Over the 30-h labelling period, PNT1A, P4E6, LNCaP and PC3 cells contained more label into choline phospholipids than into Cho metabolites (Figure 9A). This was the opposite in PNT2C2 cells where the label detected in Cho metabolites was higher than in Cho phospholipids. These results also confirm the uptake data (Figure 8), indicating that PNT2C2 cells incorporate more label than the other cell lines under similar conditions. After labelling, most radioactivity was detected in PCho in all cell lines, especially in PNT2C2, LNCaP and PC3 lines; the least label was detected in Cho. Surprisingly, quite a high proportion of label was in GPC and, especially in P4E6 cells.

Chronic phorbol ester treatment of PNT2C2 and PNT1A cells downregulates PKCα protein and reduces choline headgroup release

Chronic exposure of PNT2C2 and PNT1A cells to 250 nM TPA for 9 h markedly reduced the PKCα protein content of cells (Figure 10A). Reprobing for actin indicated that approximately equal levels of total cell protein had been resolved, in agreement with previous results. TPA activation of PKC stimulated Cho
metabolite release into the medium from both PNT2C2 and PNT1A lines. This effect was reduced to basal levels in PKCα-depleted PNT1A cells and by about 60% in PKCα-depleted PNT2C2 cells (Figure 10B). In contrast, TPA treatment of PC3 cells failed to stimulate significant Cho metabolite release over basal levels (Figure 10B), showing the variable effect of TPA on Cho metabolite release from PC3 cells.

DISCUSSION

Protein kinase C alpha expression

We studied five cell lines to span the non-tumourigenic to metastatic extremes of PCa. PNT2C2- and PNT1A-immortalised cell lines were derived from normal prostate epithelia (Cussenot et al, 1991; Berthon et al, 1995). The P4E6-immortalised line was derived from an early prostate tumour (Maitland et al, 2001). The widely studied tumourigenic LNCaP and PC3 cell lines differ in their apoptotic response to PKCα activation, formation of metastases and regulation of the PI3K–PKB pathway (Sharrard and Maitland, 2007). We focused on PKCα because it regulates PLD (Cockcroft, 2001), which is linked to tumourigenesis (Foster, 2009). When normalised to GAPDH, PKCα protein expression varied considerably between the five cell lines, being weakest in P4E6 cells derived from an early prostate tumour (Figure 1D). This was surprising as PKCα expression is reportedly increased in PCa (Koren et al, 2004; Lahn et al, 2004) and, in agreement, was 2–4 times higher in tumourigenic LNCaP and PC3 cell lines compared with P4E6 cells. This observation with P4E6 cells derived from an early tumour may indicate that PKCα protein expression is reduced in early PCa and that expression increases in later metastatic disease. However, the immortalised PNT2C2 and PNT1A cell lines from normal prostate epithelia express PKCα protein at the same level as the tumourigenic LNCaP and PC3 cell lines. A study of PKCα expression in primary prostate epithelial cells from normal and tumour tissue is in progress to determine whether immortalisation influences PKCα expression.

Protein kinase C alpha signalling to MARCKS

Protein kinase C alpha regulates cell spreading and motility through the F-actin-binding protein MARCKS (Uberall et al, 1997; Larsson, 2006). Protein kinase C activation stimulates MARCKS phosphorylation in PNT1A, LNCaP and P4E6 cells (Figure 2), indicating that a phorbol ester–PKCα–MARCKS pathway is active even in the P4E6 line with its weaker PKCα protein content. Surprisingly, some MARCKS was constitutively phosphorylated in unstimulated PNT2C2 and PC3 cells. Novel PKCα, a MARCKS
kinase in fibroblasts (Uberall et al, 1997; Rombouts et al, 2008), might contribute to MARCKS phosphorylation in these prostate cell lines. However, PKCz protein turns over much more rapidly than PKCa on chronic exposure of cells to phorbol ester (Olivier and Parker, 1992) and is barely detectable in PNT1A and PNT2C2 cells exposed to TPA for 9 h (Figure 10A). Myristoylated alanine-rich C kinase phosphorylation in prostate cell lines depleted in PKCz was reduced, indicating that PKCz is the major link to MARCKS. Phosphorylation of MARCKS causes its release from the plasma membrane exposing PI(4,5)P2 (Larsson, 2006). Thus, constitutively phosphorylated MARCKS in PC3 cells could contribute to the increased motility and invasiveness shown by this line compared with LNCaP cells (Lang et al, 2002). MicroRNA-21 (miR-21), which is overexpressed in PCa (Krchevsky and Gabrely, 2009), targets MARCKS, promoting resistance to apoptosis and increased invasiveness (Li et al, 2009). MicroRNA-21 expression is higher in PC3 cells than in the LNCap line (Li et al, 2009) contributing to their greater invasiveness.

Phospholipase D activation by PKCz

Phospholipase D activity is elevated in many cancers and transformed cell lines (Foster and Xu, 2003; Foster, 2006); thus, we were surprised that levels of basal and PKC-stimulated PLD activity (Figure 3) were similar between the five cell lines. Protein kinase C alpha is specifically linked to activation of PLD1 (Kim et al, 1999; Cockcroft, 2001) and PLD2 (Chen and Exton, 2004); however, that a PKCz link to PLD is active in cell lines derived from both normal and tumourigenic epithelia, including P4E6 cells derived from an early tumour. Activation of PKCz increases PLD activity more in PC3 cells probably because this cell line expresses PKCa, PLD 1 and PLD2 prominently. Standard PLD assay conditions with 30 mM 1-butanol released into the medium from cell lines in any membrane-bound PI(4,5)P2 (Larsson, 2006), is a key regulator of the PKCzC kinase, which functions as a reversible source of plasma membrane tumourigenic cell lines (Figure 3). Myristoylated alanine-rich C kinase, which functions as a reversible source of plasma membrane P(4, 5)P2 (Larsson, 2006), is a key regulator of the PKCz–PLD pathway (Sundaram et al, 2004).

{[H]-}label release

The centrifugation results (Figure 4) indicate that {[H]-}label is not released into the medium from cell lines in any membrane-bound prostasome or exosome form (Whiteside, 2005; Nilsson et al, 2009). Differences in levels of basal {[H]-}label release from the five cell lines (Figure 5A–E) must reflect a variation in the initial {[H]-}Cho uptake into cells by Cho transporters (Michel et al, 2006), as well as in Cho metabolism and rates of PtdCho synthesis and turnover. Our uptake results (Figure 8) indicate that PNT2C2 cells, which release the highest levels of Cho metabolites, also show the greatest uptake of Cho over 60 min. The uptake results also confirm that LNCaP cells import Cho more rapidly than do PC3 cells, as shown by Baba et al (2007) and Muller et al (2009). Choline transporter expression has been partially defined for LNCaP and PC3 cells (Hara et al, 2006; Baba et al, 2007; Muller et al, 2009). According to Muller et al (2009), Cho uptake into LNCaP and PC3 cells involves a selective Cho transporter (Michel et al, 2006). Our finding that TPA-stimulated Cho metabolite release from PNT2C2 cells is not HC-3 sensitive suggests that a CTL1 family member (Michel et al, 2006) is not involved in the release mechanism. Choline metabolite release from PNT1A cells is partially HC-3 sensitive, suggesting a contribution by a CTL1-type component (Figure 6B). Bakovic (personal communication) comments ‘CTL1 could efflux free Cho as it regulates an ATP-independent, passive transport depending on Cho concentration gradient and that CTL1, as well as OCTs are probably not involved in PCho and GPCho transport, though the efflux of such metabolites has not been tested’ (Michel et al, 2006). Our results in Figure 9B indicate that most Cho taken up by all the cell lines is converted into PCho by CK as found by Hara et al (2006) for PC3 cells. After labelling, only non-tumourigenic PNT2C2 cells had label preferentially in water-soluble Cho metabolites (mainly PCho, Figure 9B) compared with phospholipids, perhaps indicating slower membrane turnover compared with tumourigenic cell lines. Phosphocholine levels in LNCaP and PC3 cells have been measured at about 0.8 and 1.2 mm, respectively, compared with 0.1 mm for senescent normal prostate epithelial cells (Ackerstaff et al, 2001; Glunde et al, 2006).

Stimulated Cho metabolite release

Unstimulated prostate cell lines release GPCho, PCho and Cho into the medium in varying proportions. However, the main Cho metabolite released changes from PCho to Cho with the transition to tumourigenesis (Figure 7). Significantly, tumourigenic cell lines fail to show a consistent PKC-stimulated release of Cho metabolites (Figures 5A–E, 6A and B) compared with the marked stimulation shown by PNT2C2 cells derived from normal epithelia. PNT1A cells, also from normal prostate epithelia, occupy an intermediate position in that PCho and Cho are released in about equal proportions, whereas PKC activation stimulates Cho metabolite release more weakly than is detected with PNT2C2 cells. Other cell types, for example, fibroblasts, are known to release PCho into the medium on ATP stimulation (Chung et al, 1997). The TPA-stimulated release of Cho metabolites from PNT2C2 and PNT1A cells is reduced to basal levels by the widely used PKC inhibitors Ro31-8220 and GF109203X at 1 μm concentration (Figure 6A). Neither inhibitor is specific for PKC; however, the MAPKAP kinase-1β and p70S6 kinase also inhibited GF109203X and Ro31-8220 (Alessi, 1997) are not directly involved in PtdCho turnover and Cho metabolism. These inhibitor results indicate a PKC involvement in the TPA-stimulated release pathway and are also in agreement with the observations in Figure 3 that Ro31-8220 inhibits TPA-stimulated PLD activity in all the cell lines. PNT lines depleted in PKCz protein (Figure 10A) showed reduced Cho metabolite release on restimulation (Figure 10B), further supporting the PKCz link to PLD. Therefore, in all cell lines, a TPA–PKCz–PLD pathway stimulates turnover of PtdCho to generate PtdOH and Cho. However, Cho is the main metabolite released by PNT2C2 cells, and thus Cho formed by PLD action must be converted into PCho before release. PNT1A cells release both PCho and Cho, indicating that the two non-tumourigenic cell lines differ in Cho metabolism, Cho transporter expression and PtdCho turnover. Phosphocholine may be released as a secondary signal (Cuadrado et al, 1993; Chung et al, 1997; Kiss and Mukherjee, 1997).

Phospholipase release from PNT2C2 cells

In HeLa cells, basal turnover of PtdCho occurs through phosphatidylcholine-specific phospholipase C (PC-PLC), DAG kinase and lipid phosphatases. Phosphatidylcholine does not involve PLD (Hii et al, 1991). Therefore, PCho released by PNT cell lines could be formed by PC-PLC activity, as is observed in normal and ovarian epithelial cancer cells (Spadaro et al, 2008) or in phorbol ester- or PDGF-stimulated fibroblasts (Podo et al, 1996; van Dijk et al, 1997). Involvement of a PC-PLC would explain the inhibition of Cho metabolite release from PNT lines by 100 μm D609 (Figure 6B), initially reported as a PC-PLC inhibitor (Muller-Decker, 1989). However, D609 can inhibit PLD and a group IV PLA2 (Kiss and Tomoro, 1995; van Dijk et al, 1997; Kang et al, 2008), as well as sphingomyelin synthase (Luberto and Hannun, 1998). In epithelial ovarian cancer cells and NK cells, D609 has no effect on PLD or sphingomyelin synthase (Cecchetti et al, 2007; Spadaro et al, 2008). Intriguingly, PCho could also be released by
the two PNT cell lines following translocation of a PC-PLC enzyme to the external surface of the plasma membrane (Ramoni et al., 2001, 2004). At this site, hydrolysis of PtdCho in the outer lipid leaflet would result in a direct release of PCho into the medium (Figure 11). As an example, exogenous B. cereus PC-PLC hydrolyses PtdCho in the outer lipid leaflet of fibroblasts (van Dijk et al., 1997). Such a translocation of PC-PLC may be regulated by PKC (Figure 11) as TPA can stimulate PC-PLC movement to the plasma membrane in fibroblasts (Ramoni et al., 2004). Hemicholinium 3 at 200 μM, an inhibitor of CK (Jimenez et al., 1995) and of high- and medium-affinity Cho transporters (Michel et al., 2006), has no effect on TPA-stimulated Cho metabolite release from PNT2C2 cells. This supports the possibility that PC-PLC translocated to the cell surface releases PCho directly into the medium (Figure 11). Hemicholinium 3 partially inhibits PCho release from TPA-stimulated PNT1A cells, suggesting that Cho release via a CT11 family transporter is blocked, whereas PCho release is unaffected.

Choline metabolite release from tumourigenic cell lines

Our results show that, although PKC stimulates PLD activity in P4E6, LNCaP and PC3 cell lines (Figure 3), there is no consistent increase in Cho metabolite release into the medium (Figures 5C–E and 9). If the new Cho formed is not released, it must be rapidly converted into PCho by CK as detected in PC3 and LNCap cells (Ackerstaff et al., 2001; Glunde et al., 2006; Hara et al., 2006) and in other malignant cells and cancers (Glunde et al., 2006). Choline kinase activity is upregulated in tumour-derived cell lines (Ramirez de Molina et al., 2002), probably accounting for the rapid conversion of Cho to PCho in tumourigenic prostate lines and why Cho is not released on PLD activation. Phospholipase D expression and activity is also increased in several cancers and malignant cell lines (Foster and Xu, 2003), which could further increase Cho formation. Such an increase was not detected in these tumourigenic prostate cell lines perhaps because of the butanol inhibition effects discussed above. Choline transport into tumourigenic cells may be increased (Figure 11), as has been observed in several cancer cell lines (Katz-Brull et al., 2002; Yoshimoto et al., 2004; Iorio et al., 2005), although we did not observe this with the cell lines studied here (Figure 8). Protein kinase C may also influence CK activity directly (Macara, 1989; Choi et al., 2005), further explaining why Cho is not released into the medium on TPA activation of PKC. PC-PLC activity may also be upregulated in tumourigenic prostate lines (Figure 11), as detected in ovarian and breast cancer cells (Glunde et al., 2004; Iorio et al., 2005, 2010), further increasing PCho formation. Elevated levels of PCho in neoplastic cells promote growth factor-induced mitogenic signalling to Raf-1 and MAP kinases (Cuadrado et al., 1993; Jimenez et al., 1995; Yalcin et al., 2010) and will also maintain flow through the Kennedy pathway, increasing PtdCho synthesis for membrane biogenesis (Figure 11) and cell proliferation, as well as to compensate for endosome formation for growth factor signalling (Vieira et al., 1996; Li et al., 1997). The α isoform of CK also affects cell-cycle regulation promoting both cell survival and proliferation (Ramirez de Molina et al., 2008; Chua et al., 2009). PtdOH generated in transformed cells also regulates cell proliferation and survival pathways via mTOR and Raf (Foster and Xu, 2003; Foster, 2007a, b; Ramirez de Molina et al., 2008; Foster, 2009). An increase in cytidylyltransferase (CFT) activity in tumourigenic prostate cells, as has been detected in some breast cancer lines (Eliyahu et al., 2007), would further increase PCho levels. Such an increase in CHO utilisation for PtdCho synthesis. A coupling between CT and PLD turnover of PtdCho, which might further stimulate PtdCho synthesis, has been reviewed (Cornell and Northwood, 2000). As PCho levels are elevated in neoplastic cells and transformed cell lines, it is relevant to ask why this metabolite is not released into the medium from tumourigenic prostate epithelial cells as occurs

Figure 11 Summary of Cho metabolite formation and release from non-tumourigenic PNT2C2 and tumourigenic P4E6, LNCap and PC3 cell lines. PNT2C2 cells: basal PC-PLC generates PCho, some of which is reused for PtdCho synthesis for membrane biogenesis and PtdCho turnover. DAG from PC-PLC activity might sustain long-term PKC activity and generates PtdOH via DAG kinase to regulate MAPK, mTOR, PI3/Akt signalling for cell proliferation. Phosphocholine is released to function as a second messenger to promote growth factor signalling: PCho transporters are not identified. PC-PLC translocation, which would increase on addition of phorbol ester. Protein kinase C activation by phorbol ester (TPA) stimulates PLD, increasing PCho formation, and may upregulate PC-PLC activity. In tumourigenic cell lines, PLD and Cho retention is upregulated to maintain high PCho levels for PtdCho formation for increased membrane biogenesis and PtdCho turnover. PtdOH promotes cell proliferation and malignant cell survival through MAPK, PI3/K/Akt and mTOR pathways. PC-PLC activity is upregulated, increasing PCho formation. Increased Cho uptake as reported in some malignant cells (but not observed in these cell lines) would further increase PCho levels. Mechanisms of PCho release are downregulated to maintain high intracellular PCho levels in cancer cells. Phospholipase (TPA) activation of PKC does not stimulate Cho formation by PLD and its release into the medium as it is rapidly phosphorylated to PCho by CKα.
with normal PNT cell lines. Our findings with these cell lines infer that tumourigenesis in prostate epithelia results in the down-regulation of normal mechanisms of PChO release into the medium so that high intracellular levels of PChO are maintained to enhance mitogen pathway signalling and PtCho synthesis for increased cell proliferation and survival, as summarised in Figure 11.

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