Fatty acid synthase inhibition results in a magnetic resonance–detectable drop in phosphocholine

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Abstract
Expression of fatty acid synthase (FASN), the key enzyme in de novo synthesis of long-chain fatty acids, is normally low but increases in cancer. Consequently, FASN is a novel target for cancer therapy. However, because FASN inhibitors can lead to tumor stasis rather than shrinkage, noninvasive methods for assessing FASN inhibition are needed. To this end, we combined 1H, 31P, and 13C magnetic resonance spectroscopy (MRS) (a) to monitor the metabolic consequences of FASN inhibition and (b) to identify MRS-detectable metabolic biomarkers of response. Treatment of PC-3 cells with the FASN inhibitor Orlistat for up to 48 h resulted in inhibition of FASN activity by 70%, correlating with 74% inhibition of fatty acid synthesis. Furthermore, we have determined that FASN inhibition results not only in lower phosphatidylcholine (PtdCho) levels but also in a 59% drop in the phospholipid precursor phosphocholine (PCho). This drop resulted from inhibition in PCho synthesis as a result of a reduction in the cellular activity of its synthetic enzyme choline kinase. The drop in PCho levels following FASN inhibition was confirmed in SKOV-3 ovarian cancer cells treated with Orlistat and in MCF-7 breast cancer cells treated with Orlistat as well as cerulenin. Combining data from all treated cells, the drop in PCho significantly correlated with the drop in de novo synthesized fatty acid levels, identifying PCho as a potential noninvasive MRS-detectable biomarker of FASN inhibition in vivo. [Mol Cancer Ther 2008;7(8):2556–65]

Introduction
Fatty acid synthase (FASN) is the key enzyme in the de novo synthetic pathway of long-chain fatty acids (1). In most normal cells, FASN expression is low and fatty acids are obtained from the diet (2). However, FASN expression is significantly increased in a wide variety of human cancers including prostate, breast, colon, and ovarian cancer (3–7). Furthermore, this overexpression is associated with poor prognosis, particularly in the case of breast and prostate cancer (4, 8).

In light of these observations, FASN has been proposed as a novel target for cancer therapy (9–11). Indeed, recent studies show that inhibition of FASN by pharmacologic (e.g., Orlistat, cerulenin, C75, and EGCG) or small interfering RNA treatments result in cell cycle arrest and apoptosis of transformed cells in vitro, whereas in vivo studies show that treatment with FASN inhibitors results in inhibition of tumor growth (12–17). Importantly, normal epithelial cells are not affected by FASN inhibition (9, 12). Consequently, the use of FASN inhibitors, as well as inhibitors of other enzymes involved in fatty acid synthesis (18–20), presents a promising therapeutic approach.

However, because response to FASN inhibitors can result in tumor stasis rather than tumor shrinkage, conventional imaging methods may not be adequate to rapidly assess therapeutic response. Consequently, additional noninvasive methods for monitoring inhibition of fatty acid synthesis are needed. Furthermore, whereas the direct consequences of FASN inhibition on fatty acid synthesis and the subsequent modulation of membrane phosphatidylcholine (PtdCho) levels have been investigated in detail (12, 13, 21), additional studies are required to assess further effects of FASN inhibitors on other aspects of cellular metabolism.

Magnetic resonance spectroscopy (MRS) is a noninvasive, nondestructive method that can provide longitudinal information regarding tumor metabolism as well as its modulation following treatment. It has been used previously to monitor choline phospholipid metabolism, glucose metabolism, and cellular energy levels as well as response to chemotherapeutic agents and therapies targeted to specific oncogenic pathways (22–28). As such, MRS can therefore provide a method for both investigating the overall metabolic consequences of FASN inhibition and noninvasively assessing the molecular action of FASN inhibitors in vivo.

The goal of the work described here was therefore twofold: (a) using MRS, to simultaneously determine the effect of FASN inhibitors on fatty acid synthesis and on other metabolic pathways, and (b) using the MRS findings, to identify a MRS-based, noninvasive, metabolic biomarker of FASN inhibition that, in the long term, could be used to monitor FASN inhibition in vivo. To this end, we have used...
$^{1}$H, $^{31}$P, and $^{13}$C MRS to investigate the metabolic consequences of FASN inhibition. Our observations are in line with previous reports showing that, in addition to inhibiting de novo synthesis of fatty acid, FASN inhibition also leads to a drop in membrane PtdCho levels. However, we also show, to our knowledge for the first time, that FASN inhibition also results in a drop in de novo synthesis of the PtdCho precursor phosphocholine (PCho) and a drop in cellular PCho levels. Importantly, the drop in PCho was correlated with the drop in de novo synthesized fatty acid levels, identifying PCho as a potential MRS-based metabolic biomarker of FASN inhibition.

**Materials and Methods**

**Cell Culture and FASN Inhibition**

PC-3 human prostate, MCF-7 human breast, and SKOV-3 human ovarian cancer cells were routinely cultured in DMEM/F-12 (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone) and 100 units/mL penicillin-100 μg/mL streptomycin, 0.25 μg/mL amphotericin (Life Technologies), and 2 mmol/L L-glutamine (Cellgro) at 37°C in 5% CO₂. For all FASN inhibition studies, fetal bovine serum was lowered to 5% (to limit the amount of available extracellular fatty acids) and glucose in the medium was reduced by half to 8.76 mmol/L (financial reasons). To inhibit FASN, PC-3 cells were incubated for 24 and 48 h with 30 μmol/L Orlistat (treated) or with carrier DMSO at 0.5% (v/v; control cells). MCF-7 and SKOV-3 cells were incubated for 48 h with 30 μmol/L Orlistat (treated) or with DMSO at 0.5% (v/v; control cells). MCF-7 cells were also treated with 30 μmol/L cerulenin (treated) or with DMSO at 0.5% (v/v; control cells).

**FASN Activity Assay**

FASN activity was determined as described previously (29). Briefly, ~5 × 10⁶ cells were trypsinized, washed in PBS, and frozen at −80°C. Cells were resuspended in 1 mL lysis buffer containing 1 mL/L EDTA, 150 mmol/L NaCl, 100 μg/mL phenylmethylsulfonyl fluoride, and 50 mmol/L Tris-HCl (pH 7.3), subjected to vortex for 30 s at 0°C, and disrupted at 0°C by ultrasonication for 10 periods of 1 s. The lysates were centrifuged (16,000 × g, 15 min) and the supernatant was stored at −80°C and assayed within 1 week. A sample was taken to measure protein content (Bio-Rad DC protein assay, Bio-Rad Laboratories). Lyophilized supernatant (~5 μg) was added to a mixture of 200 mmol/L potassium phosphate buffer (pH 6.6), 1 mmol/L DTT, 1 mmol/L EDTA, 0.24 mmol/L NADPH, and 30 μmol/L acetyl-CoA in 0.2 mL reaction volume. After monitoring at 340 nm (Beckman Coulter DU800 UV/visible spectrophotometer) at room temperature for 3 min to measure background NADPH oxidation, 50 μmol/L malonyl-CoA was added and the reaction mixture was assayed for 10 min. Data were analyzed using Beckman Coulter DU800 System and Applications Software.

**Cell Cycle Analysis**

Flow cytometry was used to determine the effect of FASN inhibition on cell cycle as follows. PC-3 and MCF-7 trypsinized cells (~2 × 10⁶) were fixed in 95% ice-cold ethanol and then stained with a PBS solution containing 40 μg/mL propidium iodide (Sigma-Aldrich) and 100 μg/mL RNase A (Sigma-Aldrich). Cells were analyzed using a BD FACScalibur (Becton Dickinson) instrument using a 488 nm excitation wavelength. Cells of uniform width were gated and propidium iodide intensities were plotted and analyzed using ModFit LT (version 3.1) software (Top sham). In the case of PC-3 cells, the integration values of the two major G1 populations (diploid (2n) and aneuploid (2.8n)) within the culture were summed to derive the final cell cycle distribution results. The values for their corresponding G2 (4n and 5.6n) and S phase populations were also combined.

**Apoptosis Assay**

PC-3 and MCF-7 cells (1 × 10⁶) were plated in 4 mm² wells, incubated overnight, and treated with 30 μmol/L Orlistat for 3, 24, and 48 h. At each time point, the medium was removed and transferred to a 1.5 mL tube to preserve any floating cells. The cell monolayers were then washed once with PBS, trypsinized, resuspended in 0.5 mL PBS, and transferred to the corresponding media tubes. The cell suspensions were then centrifuged at 1,000 × g for 5 min, washed once with PBS, and lysed using 100 μL lysis buffer [50 mmol/L HEPES (pH 7.4), 0.1% CHAPS, 5 mmol/L DTT, 0.1 mmol/L EDTA]. The lysates were centrifuged at 10,000 × g for 10 min and stored at −20°C. Caspase-3 activity was measured by incubating 10 μL of each lysate with 30 μmol/L Ac-DEVAD-AMC substrate (Biomol) in assay buffer [50 mmol/L HEPES (pH 7.4), 100 mmol/L NaCl, 0.1% CHAPS, 10 mmol/L DTT, 1 mmol/L EDTA, 10% glycerol] in a 100 μL total volume for 1 h at 37°C. Cleavage of the substrate by caspase-3 was measured using a Safire plate reader (Tecan Group) at excitation and emission wavelengths of 360 and 460 nm, respectively.

**Cell Extraction, MRS Acquisition and Analysis**

For MRS studies, glucose in the medium (8.76 mmol/L) was replaced by equal concentrations of [L-1³C]glucose and unlabeled glucose (to monitor fatty acid synthesis and glycolysis) and choline in the medium was replaced with [1,2-¹³C]choline at its normal concentration of 64.1 μmol/L (to monitor choline metabolism). PC-3, MCF-7, or SKOV-3 cells (logarithmic growth) were cultured with Orlistat (treated) or DMSO (control) for 24 and/or 48 h, replenishing medium every 24 h. Spent medium was saved. To monitor cell metabolism, ~5 × 10⁶ cells were extracted using the dual-phase extraction method (25, 30). Briefly, cells were rinsed with ice-cold saline, fixed in 10 mL ice-cold methanol, and scraped from the culture flask surface. Alternatively, cells were trypsinized and centrifuged and the cell pellet was combined with 10 mL ice-cold methanol (results from both methods were indistinguishable). Following vortexing, 10 mL ice-cold chloroform was added followed by 10 mL ice-cold de-ionized water. After phase separation and solvent removal, cellular proteins were collected and stored at −80°C until measurement (see below). To acquire $^{1}$H and $^{13}$C spectra, the aqueous fraction was reconstituted in 500 μL deuterium oxide (aqueous...
phase) and the lipid fraction was reconstituted in deuterated chloroform (lipid phase). To acquire aqueous $^{31}$P MR measurements, 25 $\mu$L EDTA in deuterium oxide was further added to a final concentration of 5 mmol/L. To acquire lipid $^{31}$P MR measurements, deuterated chloroform was evaporated and the precipitate was resuspended in 500 $\mu$L of a 2:1 mixture of deuterated chloroform and 60 mmol/L methanolic EDTA adjusted to pH 7.3 with CsOH. MR spectra were acquired on an Avance DRX500 Bruker spectrometer (Bruker Biospin). $^1$H spectra: 20 ppm spectral width, 30° flip angle, and 5 s repetition time with water suppression. $^{13}$C spectra: 240 ppm spectral width, 30° flip angle, and 3.5 s repetition time with broadband proton decoupling. $^{31}$P spectra: 60 ppm (aqueous $^{31}$P) or 35 ppm (lipid $^{31}$P) spectral width, 30° flip angle, 4.5 second repetition time, and broadband proton decoupling.

Data were analyzed using Bruker Topspin 2.0a software. Changes in metabolite levels were determined relative to matched controls. Absolute metabolite quantification was obtained by determining the appropriate peak area, normalizing to an external reference (tetramethylsilane, $^1$H MRS; deuterated chloroform, $^{13}$C MRS; methylenediphosphonic acid, $^{31}$P MRS), correcting for saturation and nuclear Overhauser effects and normalizing to cellular protein. In quantifying the product of de novo fatty acid synthesis ($^{13}$C MR spectra), only the methylene carbons at 29 to 30 ppm were used and it was further assumed that fatty acids observed represented only palmitate. From $^1$H MR spectra of lipid extracts, only methylene protons at 1.2 to 1.3 ppm were used to quantify the total pool of fatty acid and all fatty acids were assumed to be palmitate.

To quantify the cellular proteins that precipitated during metabolite extraction, the pellet was resuspended in 1 mL of 1 mol/L NaOH by incubating for 1 h at 50°C. A sample was then taken for measurement of protein content (Bio-Rad assay, Bio-Rad Laboratories).

**Choline Kinase Assay**

Choline kinase (ChoK) activity was determined as described (31). Following incubation with DMSO or Orlistat for 48 h, $15 \times 10^6$ PC-3 cells were trypsinized, washed in ice-cold PBS, and resuspended in 400 $\mu$L Tris-HCl (pH 8.0) containing 10 mmol/L DTT and 1 mmol/L EDTA in deuterium oxide. Cells were homogenized for 30 s at 0°C and were disrupted at 0°C by ultrasonication for 10 periods of 1 s. Cell lysates were centrifuged (16,000 $\times$ g, 30 min) and the particle-free supernatant transferred to a NMR tube. Initial PCho levels were measured by $^1$H MRS as described above. ChoK activity was assayed by monitoring temporal accumulation of PCho by MRS following addition of choline chloride, ATP, and Mg$^{2+}$ in Tris-HCl buffer (final concentrations: 5 mmol/L choline chloride, 10 mmol/L ATP, 10 mmol/L MgCl$_2$). ChoK activity was determined from a straight-line fit to plots of PCho as nmol/mg protein versus time following addition of all substrates.

The effect of Orlistat on ChoK activity was also monitored after only 1 h of incubation. Cells were incubated for 47 h with DMSO alone and then Orlistat was added during the last hour of incubation, creating a treated group. Cells were then lysed and ChoK activity was assayed as before. To detect any direct interaction between Orlistat and ChoK, cells were incubated for 47 h with DMSO alone. Following extraction, half the cell lysates were exposed to Orlistat for 1 h at room temperature, creating a treated group, and ChoK activity was assayed as before.

**Quantitative PCR Analysis**

PC-3 cells (2.5 $\times$ 10$^6$) were seeded in 10 cm$^2$ dishes for 24 h before treating with 30 mmol/L Orlistat. RNA from treated and control cells was extracted cells using TRIZOL (Invitrogen) and standardized to a concentration of 50 ng/mL. cDNA was produced using a Transcriptor First-Strand synthesis kit (Roche) according to the manufacturer’s instruction. The expression of PCho cytidylyltransferase (CCT) and ChoK following treatment was measured by quantitative PCR using an ABI 7500 instrument (Applied Biosystems). PCR primers were designed based on GenBank sequences NM_005017 (CCT; forward 5'-TGTTCAGCCAAGGCTCAATGAGG-3' and reverse 5'-TCTCTGTCTATCACCCTGAACCT-3') and NM_001277 (ChoK; forward 5'-TATCTTTGTGCTGAAGCGCGA-3' and reverse 5'-GGCGCTTAGCCATGCTACCCAAAT-3'). Glyceraldehyde-3-phosphate dehydrogenase was used as an endogenous standard and amplified using primers 5'-CATGGAAGGCTGGG-3' (forward) and 5'-CAGGTTGTGACTAGTACCAGC-3' (reverse). Expression levels of CCT and ChoK were first normalized to endogenous glyceraldehyde-3-phosphate dehydrogenase levels ($\Delta$Ct) and subsequently to the untreated control of each group ($\Delta$ACt). The results were expressed as percentages of relevant control using the equation 2$^{-\Delta\Delta Ct}$.

**Western Blot Analysis**

PC-3 and MCF-7 cells were treated with DMSO or Orlistat (30 mmol/L) for 3, 24, and 48 h and then lysed using cell lysis buffer containing 1% NP40, 1% SDS, and 1 $\mu$L/mL protease inhibitor cocktail set III (Calbiochem). Lysates were centrifuged at 12,000 rpm for 10 min at 4°C, the protein supernatant was collected, and total protein concentrations were determined using Bio-Rad DC protein assay reagents (Bio-Rad Laboratories). Proteins were separated by SDS-PAGE using 10% gels and transferred electrophoretically to 0.45 $\mu$m nitrocellulose membranes. Membranes were blocked in blocking buffer containing 5% nonfat dry milk in TBS (pH 7.6) and 0.1% Tween 20 and incubated overnight at 4°C with primary antibodies as follows: Akt (1:1,000; Cell Signaling Technology), phospho-Akt (P-Akt, 1:1,000; Cell Signaling Technology), and glyceraldehyde-3-phosphate dehydrogenase (1:5,000; Stressgen). This was followed by 1-h incubation with horseradish peroxidase–conjugated secondary anti-rabbit (Cell Signaling Technology) and anti-mouse (Cell Signaling Technology) antibodies at dilutions of 1:1,000 and 1:2,000, respectively. Membranes were washed with enhanced chemiluminescence reagents (LumiGLO & Peroxide, Cell Signaling Technology) for 1 min and exposed to Hyperfilm.
(Amersham Biosciences), which was developed on a Konica SRX-101 automatic developer (Konica). The intensity of individual bands of interest was quantified using Image J software (NIH) and normalized to the intensity of glyceraldehyde-3-phosphate dehydrogenase (loading control) and Akt and P-Akt protein expressions compared between DMSO- and Orlistat-treated groups.

**Statistical Analysis**

Unless otherwise stated, experiments were repeated three to five times. Results are expressed as mean ± SD. Two-tailed unpaired Student’s t tests were done to assess the statistical significance of results. \( P < 0.05 \) was considered significant.

**Results**

**Orlistat Treatment Leads to Inhibition of Fatty Acid Synthesis, Reduced Fatty Acid Levels, Inhibition in Cell Proliferation, and Lower Nucleotide Triphosphate Levels**

First, we investigated in detail the effect of the FASN inhibitor Orlistat on PC-3 prostate cancer cells (known to overexpress FASN; ref. 7). Cells were treated for 24 and 48 h with 30 \( \mu \)mol/L (saturating dose; ref. 12) of Orlistat. As expected, this resulted in inhibition of FASN activity by 51 ± 10\% \((P < 0.05)\) and 70 ± 21\% \((P < 0.05)\) at 24 and 48 h, respectively, when compared with controls (Fig. 1A). Figure 1B illustrates \( ^{13}C \) MRS spectra obtained from the lipid fraction of control and treated PC-3 cells grown in the presence of medium containing \( ^{13}C \)-labeled choline and glucose. From these data, it was possible to monitor fatty acids synthesized \textit{de novo} from the \( ^{13}C \)-labeled glucose present in the growth medium. As expected, fatty acid synthesis was inhibited following Orlistat treatment. Because cellular protein content \((1.9 ± 0.2 \times 10^{-7} \text{ mg/cell})\) was unaffected by Orlistat treatment, metabolite levels, determined by MRS, were normalized to protein content.

Control cells synthesized 30 ± 12 nmol/mg protein of \textit{de novo} \( ^{13}C \)-labeled fatty acids during 24 h and 56 ± 18 nmol/mg protein during 48 h. In cells treated with Orlistat, \textit{de novo} \( ^{13}C \)-labeled fatty acid synthesis decreased significantly by 45 ± 21\% to 15 ± 2 nmol/mg protein \((P < 0.05)\) at 24 h and by 74 ± 12\% to 13 ± 4 nmol/mg protein \((P < 0.02)\) at 48 h. It is noteworthy that the measured decrease in \textit{de novo} fatty acid synthesis correlated with the measured decrease in FASN activity following 24 and 48 h of Orlistat treatment \((R^2 = 0.99, P < 0.05)\). Figure 1C illustrates the \( ^{1}H \) MRS spectra of the same cellular extracts monitoring the total fatty acid pool. Assuming that on average all fatty acid are 16 carbons in length, the cellular fatty acid pool was estimated as 523 ± 147 nmol/mg protein in control PC-3 cells. Following Orlistat treatment, and consistent with the inhibition of \textit{de novo} fatty acid synthesis, total cellular fatty acid levels dropped slightly to 91 ± 31\% following 24 h and significantly to 70 ± 17\% \((P < 0.04)\) following 48 h of FASN inhibition.

Orlistat treatment also led to cell cycle arrest in PC-3 cells (Fig. 2A). Cell fractions in the G\(_1\) phase increased in association with length of treatment. A matched concomitant decrease in the S and G\(_2\)--M phase fractions was also observed in both cell lines. Cell cycle arrest was associated with inhibition of cell proliferation. Thus, the number of Orlistat-treated PC-3 cells remained essentially unchanged at 111 ± 17\% at 24 h and 83 ± 11\% at 48 h compared with cell numbers before treatment. In contrast, untreated control cell numbers increased to 149 ± 38\% \((P < 0.05\) relative to treated cells) and 206 ± 62\% \((P < 0.005\) relative to treated cells) at 24 and 48 h, respectively. Apoptosis was not evident during the course of Orlistat treatment. Specifically, no significant increase in apoptotic cells was observed in treated cells, where the maximum percentage of the apoptotic cell fraction (sub-G\(_1\)) did not exceed 0.9 ± 0.3\% in treated PC-3 cells. Furthermore, an increase in

\[ \text{Control cells synthesized 30 ± 12 nmol/mg protein of de novo } ^{13}C \text{-labeled fatty acids during 24 h and 56 ± 18 nmol/mg protein during 48 h. In cells treated with Orlistat, de novo } ^{13}C \text{-labeled fatty acid synthesis decreased significantly by 45 ± 21% to 15 ± 2 nmol/mg protein (} P < 0.05) \text{ at 24 h and by 74 ± 12% to 13 ± 4 nmol/mg protein (} P < 0.02) \text{ at 48 h. It is noteworthy that the measured decrease in de novo fatty acid synthesis correlated with the measured decrease in FASN activity following 24 and 48 h of Orlistat treatment (} R^2 = 0.99, P < 0.05) \text{. Figure 1C illustrates the } ^{1}H \text{ MRS spectra of the same cellular extracts monitoring the total fatty acid pool. Assuming that on average all fatty acid are 16 carbons in length, the cellular fatty acid pool was estimated as 523 ± 147 nmol/mg protein in control PC-3 cells. Following Orlistat treatment, and consistent with the inhibition of de novo fatty acid synthesis, total cellular fatty acid levels dropped slightly to 91 ± 31% following 24 h and significantly to 70 ± 17% (} P < 0.04) following 48 h of FASN inhibition. \]

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caspase-3 activity was not detected at 3, 24, and 48 h post-treatment in contrast to the significant increase detected in a positive control, staurosporine-treated Jurkat cells (data not shown).

Levels of the high-energy metabolites phosphocreatine and nucleotide triphosphates (predominantly consisting of ATP) were determined from $^{31}$P MR spectra of the aqueous fraction of control PC-3 extracts at values measuring $16 \pm 7$ and $57 \pm 22$ nmol/mg protein, respectively. Phosphocreatine levels decreased significantly following 24 h of Orlistat treatment to below detection level, where they remained through 48 h of FASN inhibition. Nucleotide triphosphate

Figure 2. FASN inhibition results in cell cycle arrest without the induction of apoptosis in PC-3 (A) and MCF-7 (B) cells. Left, representative histograms showing cell cycle distribution of cells treated with Orlistat (30 µmol/L) for 24 and 48 h. The PC-3 cell line exhibits two major G1 populations, diploid (2n) and aneuploid (2.8n), and two corresponding G2-M peaks. Right, cell cycle phase distributions shown in histograms were quantified using ModFit LT and the results are illustrated in the bar graphs. Each value was determined from the average of three analyses. Bars, SD.
levels also decreased following treatment, with their levels falling significantly by 34 ± 23% ($P < 0.03$) and 59 ± 10% ($P < 0.002$) at 24 and 48 h, respectively (Fig. 3A).

**Orlistat Treatment Leads to a Drop in PCho Levels**

Levels of the membrane lipid PtdCho were determined from the $^{31}$P MR spectra of the lipid fraction of cell extracts (Fig. 4A). Changes in the levels of this phospholipid were below detection level during 24 h of Orlistat treatment, with values at 110 ± 44% ($P = 0.74$) relative to control. However, following 48 h of treatment, PtdCho dropped by 31 ± 4% ($P < 0.0005$) from 115 ± 31 to 80 ± 24 nmol/mg protein consistent with previous reports (12, 13, 21). $^{13}$C MRS studies (Fig. 4B) indicated that this was due to a reduction in de novo phospholipid synthesis. Control cells synthesized 21 ± 6 and 61 ± 12 nmol/mg protein PtdCho during 24 and 48 h, respectively. Following Orlistat treatment for 24 h, PtdCho synthesis decreased by 54 ± 24% to 10 ± 4 nmol/mg protein ($P < 0.03$) and by 60 ± 9% to 25 ± 9 nmol/mg protein ($P < 0.004$) following 48 h of treatment.

In addition, and somewhat unexpectedly, $^{31}$P MRS spectra of the aqueous fraction of cell extracts indicated that levels of total PCho, the precursor of PtdCho, were also affected by Orlistat treatment (Fig. 3A). PCho dropped significantly by 41 ± 24% from an average 182 ± 20 to 107 ± 44 nmol/mg protein following 24 h of Orlistat treatment ($P < 0.02$) and by 60 ± 21% from 199 ± 63 to 70 ± 26 nmol/mg protein ($P < 0.02$) following 48 h of treatment. The total choline–containing metabolite signal detected by $^1$H MRS (data not shown) also dropped by 33 ± 27% ($P = 0.05$) and by 47 ± 32% ($P = 0.06$) at 24 and 48 h, respectively, further confirming the effect of FASN inhibition on PCho levels. $^{13}$C MRS studies were used to study PCho synthesis simultaneously with fatty acid synthesis and Fig. 3B illustrates the $^{13}$C spectra of control and Orlistat-treated cells. These studies indicated that the drop in PCho levels was due to its reduced synthesis. Control cells synthesized 83 ± 13 nmol/mg protein of de novo PCho during 24 h and 131 ± 27 nmol/mg protein during 48 h. Synthesis decreased significantly on treatment.
with Orlistat by 39 ± 12% to 47 ± 11 and 59 ± 23% to 37 ± 15 nmol/mg protein after 24 and 48 h, respectively. Importantly, total as well as de novo PCho levels correlated with FASN activity (R² = 0.99, P < 0.05).

Drop in PCho Is Associated with a Drop in Cellular ChoK Activity

The data above indicate that following 48 h of Orlistat treatment the drop in total PCho levels (129 ± 89 nmol/mg protein) was comparable with the drop in PCho synthesized de novo (94 ± 42 nmol/mg protein). This indicated that the drop in PCho levels was due to reduced PCho synthesis rather than increased utilization. To confirm this hypothesis, ChoK activity was assayed in control and treated PC-3 cells using MRS as described previously (31). The rate of PCho synthesis, a measure of cellular ChoK activity, was 34 ± 6 nmol/mg protein h in control cells. Following FASN inhibition with Orlistat for 48 h, this rate dropped significantly to 21 ± 7 nmol/mg protein h (P < 0.05; Fig. 5A).

We questioned how quickly Orlistat affects choline metabolism and, to this end, also monitored ChoK activity following 1 h of Orlistat treatment. At that time point, FASN inhibition resulted in a drop in ChoK activity by 49 ± 10% (P < 0.05) relative to controls. This inhibition was comparable with that observed following 48 h of treatment and indicated that the effect of FASN inhibition on choline metabolism occurs rapidly and is sustained during treatment.

To rule out any direct inhibitory interaction between Orlistat and ChoK, Orlistat was also added directly to the extracted cell lysates. Incubation of cell lysates with 30 μmol/L Orlistat for 1 h did not alter the activity of ChoK, which remained at 110 ± 36% (P = 0.67) of control levels.

To determine the reasons behind the drop in cellular ChoK activity, we monitored enzyme expression. Quantitative PCR analysis showed that ChoK expression was unchanged at 24 h with gene expression at 106 ± 4% relative to control. However, 48 h of Orlistat treatment resulted in a drop in ChoK expression to 60 ± 28% (P < 0.05) relative to controls. The expression of CCT was not significantly altered during the same time, with quantitative PCR data showing CCT expression at 95 ± 9% and 99 ± 10% at 24 and 48 h, respectively. Thus, changes in ChoK expression can explain the drop in PCho levels following 48 h of treatment but not the drop in ChoK activity and PCho levels observed at the earlier time points following FASN inhibition.

Phosphatidylinositol 3-kinase (PI3K) signaling has been linked to FASN expression in clinical samples (11, 32, 33); recently, a bidirectional link between FASN and PI3K signaling has been shown, with PI3K controlling FASN expression and FASN inhibition resulting in a reduction in PI3K signaling downstream of HER-2/neu (11, 34, 35). We have shown previously that inhibition of the PI3K pathway results in a drop in PCho expression (36), and ChoK activity can be controlled by PI3K signaling (37). We therefore questioned whether PI3K signaling provided the mechanistic link between fatty acid and choline metabolism and could explain the drop in ChoK activity and PCho levels following FASN inhibition, particularly at the earlier time points. To answer this question, we monitored the effect of Orlistat on P-Akt levels in PC-3. A slight drop in P-Akt levels was observed following 3 h of Orlistat treatment in PC-3 cells (Fig. 5B), indicating some inhibition of PI3K signaling. However, 24 or 48 h of exposure to

Figure 4. Inhibition of FASN causes a drop in the synthesis and total levels of membrane phospholipids. A, representative 31P MR spectra of PC-3 lipid extracts following 48 h of treatment with Orlistat. a, cardiolipin; b, plasmologen-PtdEtn; c, PtdEtn; d, PtdS; e, sphingomyelin; f, phosphatidylinositol; g, plasmologen-PtdCho. Inset, % (relative to control) cellular PtdCho levels of PC-3 cells following treatment with Orlistat for the times indicated. B, representative 13C MR spectra of lipid extracts following 48 h of Orlistat treatment; peaks at 63 to 64 ppm have not been unequivocally identified. Inset, % (relative to control) de novo PtdCho synthesis in PC-3 cells following Orlistat treatment for the indicated times.
Orlistat did not result in a significant reduction in P-Akt levels.

**Drop in PCho Is a General Observation following FASN Inhibition**

To confirm the generality of our observations, we extended our studies of Orlistat to another two cell lines: MCF-7 human breast cancer and SKOV-3 human ovarian cancer cells. We further extended our studies to a second FASN inhibitor, cerulenin. Our findings are summarized in Table 1. MCF-7 cells were treated with 30 μmol/L Orlistat for 48 h. This led to a drop in cell numbers to 57 ± 14% compared with controls, associated with cell cycle arrest and no significant apoptosis, as in the case of PC-3 cells (Fig. 2B). As expected, FASN inhibition resulted in a drop in *de novo* fatty acid synthesis by 46 ± 9% in MCF-7 cells.

Importantly, however, the inhibition of FASN activity was associated, as in the case of PC-3 cells, with a drop in *de novo* synthesized PCho levels by 63 ± 12% resulting in a drop in total PCho levels by 38 ± 6%. Levels of total choline also dropped by 49 ± 15% in MCF-7 cells. The effect of Orlistat treatment on cellular energetics was less substantial than in PC-3 cells, with nucleotide triphosphate levels falling by 28 ± 32%. Western blot analysis of P-Akt levels in MCF-7 cells yielded similar results to PC-3 cells, with no significant inhibition of PI3K signaling observed following 48 h of Orlistat treatment.

Treatment of SKOV-3 cells with Orlistat also lead to inhibition of PCho synthesis and a reduction in PCho levels (Table 1). Specifically, FASN inhibition resulted in a drop in SKOV-3 cell number to 79 ± 7% relative to control associated with a drop in *de novo* fatty acid synthesis by 37 ± 7%, a drop in *de novo* synthesized PCho levels by 31 ± 21%, and a drop in total PCho levels by 56 ± 14%.

MCF-7 cells were also treated with 30 μmol/L cerulenin for 48 h (Table 1). The effect on cell proliferation was negligible, with cell numbers remaining at 99 ± 17% of control. Nonetheless, treatment with cerulenin was associated with a reduction in *de novo* fatty acid synthesis by 40 ± 6%. Furthermore, as in the case of Orlistat, the inhibition of FASN activity was associated with a drop in *de novo* synthesized PCho levels by 21 ± 12%; importantly, total PCho levels dropped by 32 ± 14% in cerulenin-treated MCF-7 cells, whereas total choline dropped by 48 ± 4%. Cerulenin treatment had no noticeable effects on cellular energetics, with nucleotide triphosphate levels remaining at 97 ± 32%.

**Drop in PCho Levels Correlates with the Drop in Fatty Acid Synthesis following FASN Inhibition**

Finally, we wanted to confirm that PCho levels were correlated with FASN activity. As mentioned above, FASN activity tightly correlates with *de novo* synthesis of fatty acid. We therefore used changes in *de novo* synthesis of fatty acid as an indicator of FASN activity in the exact same experiment in which PCho levels were measured. Our findings are summarized in Fig. 3C in which we combined the data obtained from the different experiments summarized in Table 1. We find that the drop in PCho levels significantly correlates with the drop in *de novo* fatty acid synthesis ($R^2 = 0.73; P < 0.03$), validating PCho as an indicator of FASN inhibition.

**Discussion**

FASN is overexpressed in several cancers, and inhibition of FASN leads to cell cycle arrest, apoptosis, and inhibition of tumor growth, presenting a novel therapeutic approach for cancer treatment (9–17). Here, we monitored the metabolic consequences of FASN inhibition with the goal of determining the effects of this inhibition on cellular metabolism and identifying MR-based metabolic biomarkers of the molecular action of FASN inhibitors. We used $^1$H, $^{31}$P, and $^{13}$C MRS, combined with $^{13}$C-labeled glucose and choline, to assess fatty acid synthesis, choline metabolism, and

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**Figure 5.** FASN inhibition is associated with a drop in ChoK activity (A), but not in long-term inhibition of PI3K signaling (B). A, $^1$H MR spectrum of cell lysates showing endogenous PCho and added Cho. Inset, representative plot of PCho synthesized by ChoK extracted from control cells and 48 h Orlistat-treated cells. Lines represent the straight-line fits to the data used to obtain rates of PCho synthesis ($R^2 = 0.89$, control; $R^2 = 0.96$, Orlistat) indicating inhibition of ChoK following Orlistat treatment. B, Western blot analysis of P-Akt, Akt, and glyceraldehyde-3-phosphate dehydrogenase (loading control) expression in control and treated PC-3 cells following 3, 24, and 48 h of Orlistat treatment.
cellular energetics. First, using Orlistat, we determined in detail the MRS-detectable consequence of FASN inhibition in PC-3 cells. We then confirmed the generality of our findings by investigating the effect of Orlistat on two other cell lines and the effect of an additional FASN inhibitor.

As expected, FASN inhibition in all three cell lines resulted in inhibition of de novo fatty acid synthesis and a drop in total fatty acid levels, which was generally associated with inhibition in cell proliferation. These findings are consistent with previous reports (12, 13, 15–17, 38–41). Our MRS data also showed that FASN inhibition affects cellular energetics, with levels of phosphocreatine (when detectable) and nucleotide triphosphates generally decreasing with FASN inhibition. However, this effect was not always large enough to be deemed a reliable indicator of FASN activity.

Because this study used MRS, where the metabolic fate of different precursors can be monitored simultaneously, it was possible to monitor choline metabolism at the same time as fatty acid synthesis. Choline is the precursor of the main membrane phospholipid PtdCho in the Kennedy (CDP-choline) pathway. A drop in PtdCho levels has been reported previously (12, 13, 21) and others have shown a link between fatty acid synthesis and the Kennedy pathway through transcriptional and posttranscriptional control of the rate limiting enzyme CCT (42). Our studies also showed a drop in PtdCho levels. However, in this study, we observed, to our knowledge for the first time, that inhibition of fatty acid synthesis also results in inhibition of the first step in this pathway, namely synthesis of PCho by ChoK. As a result, PCho levels were lower following treatment; importantly, PCho levels correlate with fatty acid synthesis levels. Thus, we propose that a reduction in levels of the MRS-detectable metabolite PCho can be used as a noninvasive diagnostic indicator of FASN inhibition in vivo.

Regarding the mechanism for the observed drop in PCho, we hypothesized that FASN inhibition and inhibition of fatty acid synthesis lead to upstream inhibition of the first enzyme in the Kennedy pathway, namely ChoK. To test this hypothesis, we monitored the cellular activity of ChoK and our data confirm that cellular ChoK activity is indeed significantly reduced following FASN inhibition. This modulation of ChoK activity results, at least in part, from the reduction in CholK expression observed following 48 h of Orlistat treatment. However, the fact that the activity of ChoK was reduced before this time point (within 1 h of treatment) indicates that other factors are also involved in modulating ChoK activity following FASN inhibition. We thus considered the possibility that cellular ChoK activity was also modulated by inhibition of PI3K signaling, reported to occur downstream of FASN inhibition (11, 35, 37). In line with previous reports, our data indicated that PI3K signaling was indeed reduced following FASN inhibition. However, this effect was only observed following FASN inhibition for a relatively short time (3 h) and was not sustained for 24 or 48 h. Our data therefore indicate that most likely multiple translational and posttranslational factors are involved in modulating ChoK activity following FASN inhibition. It is possible that modulation of ChoK activity could be mediated by PI3K shortly after FASN inhibition, whereas the effects of longer-term treatment could be mediated by altered ChoK expression.

In conclusion, this study highlights the value of MRS for simultaneously monitoring the effect of one inhibitor on several metabolic pathways. Furthermore, our data in three different cell lines and using two FASN inhibitors indicate that a drop in PChol levels is correlated with inhibition of fatty acid synthesis following FASN inhibition. MRS is a noninvasive method that can be used in patients in vivo to monitor PCho either by monitoring the phosphomonester peak in the 31P spectrum or, more commonly, by monitoring the total choline signal in the 1H spectrum. Consequently, we propose that this previously unreported metabolic change could serve as a noninvasive in vivo MRS metabolic pharmacodynamic biomarkers of FASN inhibition.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Wendy Schober (The University of Texas M. D. Anderson Cancer Center Core Facility) for help in flow cytometry analysis.

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Molecular Cancer Therapeutics

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