Effect of Estrogen Withdrawal on Energy-rich Phosphates and Prediction of Estrogen Dependence Monitored by \textit{in Vivo} \textsuperscript{31}P Magnetic Resonance Spectroscopy of Four Human Breast Cancer Xenografts\textsuperscript{1}

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\textbf{ABSTRACT}

The effect of estrogen withdrawal on energy metabolism was studied in four human breast cancer xenografts: the estrogen-dependent MCF-7 and ZR75-1 and the estrogen-independent ZR75/LCC-3 and MDA-MB-231. The tumors were grown in ovariectomized nude mice with a s.c. implanted estrogen pellet. After Gompertzian growth was verified, the estrogen pellet was removed from half of the animals. \textit{In vivo} \textsuperscript{31}P magnetic resonance spectroscopy of the tumors was performed 1 day before and on days 2, 6, and 14 after estrogen removal. Estrogen withdrawal induced a significant increase in the nucleoside triphosphate:Pi ratio in the two estrogen-dependent xenografts, whereas this ratio remained unchanged in the estrogen-independent tumors. In ZR75/LCC-3 tumors a slight decrease in nucleoside triphosphate:Pi was observed following onset of estrogen stimulation after initial growth without estrogen. Extracts of freeze-clamped tumors prepared 14 days after estrogen removal were analyzed for ATP and phosphocreatine content. Our findings suggest a correlation between estrogen withdrawal and the steady-state concentrations of ATP, phosphocreatine, and Pi in human breast cancer xenografts. Discrimination analysis of the pretherapeutic spectra enabled us to identify the tumor line and the estrogen dependence of the tumors in 80–90% of all cases.

\textbf{INTRODUCTION}

The antiproliferative effect of estrogen withdrawal or antiestrogen therapy has been used clinically for decades, but only little is known about the influence of this treatment on cellular energy metabolism. Noninvasive \textsuperscript{31}P-MRS\textsuperscript{3} studies of tumor tissue \textit{in vitro} and \textit{in vivo} provide information about energy and phospholipid metabolism in malignant cells and tissues. The data are usually expressed as ratios of metabolites (e.g., NTP:Pi, PME:Pi, and PCr:NTP). Pretherapeutic ratios as well as the course of changes after initiation of anticancer therapy have been suggested as possible parameters for early response prediction and monitoring (1, 2).

The aims of the present study were to evaluate the influence of estrogen withdrawal on energy-rich phosphates in human breast tumor xenografts, and to examine the potential of the \textsuperscript{31}P MRS profile in prediction of estrogen sensitivity. Four different human breast tumor lines were included: two estrogen receptor positive/estrogen dependent (MCF-7 and ZR75-1) and two estrogen receptor negative/estrogen independent (ZR75/LCC-3 and MDA-MB-231). ZR75/LCC-3 is a subline of the estrogen-dependent ZR75-1, selected for \textit{in vivo} estrogen independence.\textsuperscript{4}

Estrogen withdrawal induced a significant increase in the NTP:Pi ratio in both of the estrogen-dependent tumor lines. Similar effects have been observed by others in MCF-7 tumors following initiation of tamoxifen therapy and concurrent withdrawal of estrogen (3, 4). Our study suggests that the increase in NTP:Pi is due to estrogen withdrawal alone. In the estrogen-independent tumors estrogen withdrawal had no influence on NTP:Pi.

The use of a linear discriminant function in the analysis of the NTP:Pi, PME:Pi, PDE:Pi, PME:NTP, and PCr:NTP ratios enabled a correct assessment of the estrogen dependence of the tumors in 80–90% of all cases.

\textbf{MATERIALS AND METHODS}

\textbf{Tumors.} Four cell lines were established as solid tumor xenografts in nude mice: the estrogen-dependent, estrogen receptor-positive tumor lines MCF-7 and ZR75-1 as well as the corresponding estrogen-independent, receptor-negative subline ZR75/LCC-3 and the estrogen-independent, receptor-negative MDA-MB-231 line.

ZR75/LCC-3 was established by injection of ZR75-1 cells into ovariectomized nude mice. Three months later several of the animals had developed small tumors. One of the tumors was excised and reestablished in culture with IMEM without phenol red and supplemented with 5% CCS. The resulting cell line was named ZR75/LCC-3.

MCF-7, MDA-MB-231, and ZR75-1 were routinely grown in IMEM with phenol red containing 5% FCS, while ZR75-1/LCC-3 was routinely carried in IMEM without phenol red and supplemented with 5% CCS. Serum was stripped of endogenous steroids by treatment with dextran-coated charcoal and sulfatase as described previously (5). Cells were harvested at about 80% confluency using a cell scraper, and subsequently 2 × 10\textsuperscript{6} cells were inoculated into each flank of 8–10-week-old ovariectomized NMRI nu/nu athymic nude mice (BOMMICE, Ry, Denmark). The mice carrying estrogen-dependent tumors received an 17ß-estradiol pellet (60-day release, 0.72 mg; Innovative Research, Toledo, OH) s.c. at the time of inoculation. Growing tumors were excised, cut into 1-mm\textsuperscript{3} pieces, and each of the four tumor lines were serially transplanted to a minimum of 16 ovariectomized mice for each experiment. All four tumor lines were serially maintained as xenografts.

\textbf{Experimental Design.} A minimum of 16 tumors of each type were allowed to grow during estrogen supplementation from a s.c. pellet until they reached a size of at least 10 mm in diameter (day 0). The estrogen pellet was removed from half of the animals by skin incision on day 0. \textsuperscript{31}P MRS was performed on days −1, 2, 6, and 14. In a separate experiment, a series of ZR75/LCC-3 tumors were grown initially without 17ß-estradiol supplementation and when the tumors had reached a minimum diameter of 10 mm (day 0), half of the animals received estrogen supplementation. MRS was performed in the same intervals as described above.

\textit{In Vivo} \textsuperscript{31}P MRS. The mice were briefly anesthetized for 4–6 min with propanidide i.p. (Sombrevin, 500 mg/kg; Gideon Richter, Budapest, Hungary) and placed in a flexible PVC tube with the tumor protruding through a hole concentric with the radiofrequency coil. To provide exact fixation, the tail of the animal was taped to the tube. In this setup, the breathing movements were not transmitted to the tumor. During the spectral recording, the mice were restrained, but anesthetized. A 14-mm two-turn surface coil was located...
over the tumor in a special probe. A Vivospec spectrometer (Otsuka Electronics, Inc.) with a wide-bore 4.7 T magnet (Magnex, Inc.) was used. Sixty-four scans were acquired with a 10-s repetition time; these parameters ensured an

Table 2 Prediction strength for each of the five ratios regarding estrogen independence

<table>
<thead>
<tr>
<th>Ratio</th>
<th>Predictability of estrogen independence</th>
<th>Cumulated predictability of estrogen independence</th>
<th>( r^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>PME:P&lt;sub&gt;i&lt;/sub&gt;</td>
<td>41/48 (85%)</td>
<td>41/48 (85%)</td>
<td>0.35</td>
</tr>
<tr>
<td>PME:NTP</td>
<td>34/48 (71%)</td>
<td>41/48 (85%)</td>
<td>0.09</td>
</tr>
<tr>
<td>PDE:P&lt;sub&gt;i&lt;/sub&gt;</td>
<td>39/48 (81%)</td>
<td>43/48 (90%)</td>
<td>0.13</td>
</tr>
<tr>
<td>PCr:NTP</td>
<td>22/48 (46%)</td>
<td>45/48 (94%)</td>
<td>0.10</td>
</tr>
<tr>
<td>NTP:P&lt;sub&gt;i&lt;/sub&gt;</td>
<td>36/48 (75%)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

\( r^2 \) = squared partial correlation coefficient: relative importance of the individual variable in the discrimination function.

3<sup>1</sup>P MRS OF HUMAN BREAST CANCER XENOGRAFTS

Table 1 Classification of tumor lines according to the discrimination function

<table>
<thead>
<tr>
<th>17(^{\beta})-Estriadiol dependence</th>
<th>Tumor line</th>
<th>No. of tumors classified as MCF-7</th>
<th>No. of tumors classified as ZR75-1</th>
<th>No. of tumors classified as ZR75/LCC-3</th>
<th>No. of tumors classified as MDA-MB-231</th>
<th>Total</th>
<th>Predictability of estrogen dependence/independence</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>MCF-7</td>
<td>16/21(^{1}) (76%)</td>
<td>2</td>
<td>2</td>
<td>21</td>
<td>36/44(^{4}) (82%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ZR75-1</td>
<td>2</td>
<td>19/23(^{3}) (83%)</td>
<td></td>
<td></td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>ZR75-1/LCC-3</td>
<td>2</td>
<td>0</td>
<td>20/22(^{3}) (91%)</td>
<td>0</td>
<td>22</td>
<td>45/48(^{4}) (94%)</td>
</tr>
<tr>
<td></td>
<td>MDA-MB-231</td>
<td>1</td>
<td>0</td>
<td></td>
<td>24/26(^{2}) (92%)</td>
<td>26</td>
<td></td>
</tr>
</tbody>
</table>

\(^{1}\) The predictability of tumor line and estrogen dependence/independence using linear discrimination function is based on the five ratios: NTP:P<sub>i</sub>, PME:P<sub>i</sub>, PDE:P<sub>i</sub>, PME:NTP, and PCr:NTP.

\(^{2}\) Correctly classified tumors.

\(^{3}\) Number of tumors correctly discriminated out of the total number of tumors in the group (percentages in parentheses).

RESULTS

Estrogen Withdrawal. Estrogen withdrawal resulted in a marked increase in NTP:P<sub>i</sub> ratio in the estrogen-dependent tumors (\( P < 0.01 \)), whereas no significant differences occurred in the estrogen-independent tumors (Figs. 1–5). Typical spectra of ZR75-1 on days —1, 2, 6, and 14 are shown in Fig. 5, where the sharp increase in NTP:P<sub>i</sub> from day 2 and forward is clearly visible. When ZR75/LCC-3 was grown initially without estrogen stimulation, the growth curve did not change after estrogen supplementation, and the trend toward a decrease in the NTP:P<sub>i</sub> ratio of the estrogen-stimulated tumors was found to be statistically insignificant (0.05 \( \leq P < 0.1 \); Fig. 6).

The PCr:NTP ratio was only assessed in the ZR75-1 and ZR75/LCC-3 tumors, since most of the MDA-MB-231 and MCF-7 tumor spectra contained PCR levels below the MRS detection limit. In ZR75-1, estrogen withdrawal resulted in a constant PCr:NTP ratio of approximately 100% of the initial value during the whole 14-day time point, while the continuously estrogen-stimulated tumors had a markedly decreased PCr:NTP ratio on day 14 (\( P < 0.025 \); Fig. 2). No difference in the PCr:NTP ratio between estrogen-stimulated and estrogen-deprived ZR75/LCC-3 tumors was found; however, these tumors were not MRS analyzed on day 14 (Fig. 3).

In the tissue extracts (Table 3), no difference in tumor ATP content between the estrogen-stimulated and the estrogen-deprived ZR75-1 tumors was found. A clear increase in PCr content was observed when ZR75-1 as well as MCF-7 was estrogen deprived. This difference corresponds to the decrease in PCr:ATP during continuous estrogen stimulation demonstrated by 3<sup>1</sup>P MRS of ZR75-1 (Fig. 3) and is probably reflecting a decrease in PCr content following increasing ischemia during tumor growth. The estrogen-supplemented ZR75/
LCC-3 tumors were characterized by a significantly lower ATP concentration than the estrogen-deprived tumors (P < 0.01). This difference was also found in MCF-7, but the amount of ATP/g tumor tissue in both MCF-7 groups was between 2 and 4 μg/g, whereas in the ZR75/LCC-3 tumors the ATP concentration was approximately 10 times lower in the estrogen-deprived tumors compared to the continuously stimulated tumors. Very low levels of PCr were found and there was no difference in ATP and PCr concentrations in the MDA-MB-231 tumors when comparing ± estrogen stimulation.

Classification of Tumor Lines. Pretherapeutic ratios and doubling time for the four examined tumors are presented in Fig. 7. Pretherapeutic ratios from all four tumor types were analyzed by the linear discriminant function. Table 1 shows the accuracy by which we were able to correctly classify the individual tumors and to predict the estrogen dependence and estrogen independence of the tumors. Also, the correctly classified tumors and the distribution of the misclassified tumors are given. Interestingly, there was no general pattern in the relatively infrequent misclassification of tumors: one estrogen-dependent line was not classified as the other estrogen-dependent line more frequently than as one of the estrogen-independent lines or vice versa. Since none of the 22 ZR75/LCC-3 tumors were classified as ZR75-1, there was no tendency to misclassify the estrogen-independent subline as the estrogen-dependent wild type. In Table 2 the relative importance of the five ratios with regard to their predictability of estrogen independence is given. PME:P, was the most important single contributor to the predictability of estrogen dependence on the basis of 31P MRS. NTP:P, was the second most important single parameter but, due to a very close correlation (correlation coefficient, 0.8) between these two ratios, the SAS program demands that one of the two ratios are excluded in the performance of a discrimination involving several ratios. Since PME:P, is in itself the most important ratio, we chose to exclude NTP:P,.
The mechanisms are still unclear, but spectrophotometric analyses of tumor ATP and PCr content indicate that the observed changes in NTP:P, are not caused by an increase in ATP concentration, more likely to a decrease in P, concentration or decreased NMR visibility of the P, molecules. Furthermore, we find the spectral appearance of the 31P NMR spectrum, and particularly PME:P, to be a potential predictor of breast cancer estrogen dependence. Clinical studies are warranted to further elucidate the role of NMR spectroscopy in response prediction.

The present study is the first 31P MRS report on the energy metabolic effect of estrogen withdrawal in human breast cancer xenografts on nude mice. The observed increase in the NTP:P, ratio after estrogen withdrawal was closely related to estrogen dependence, since the lack of NTP:P, increase was not only found in the de novo estrogen receptor-negative and estrogen-independent wild-type MDA-MB-231, but also in ZR75/LCC-3, selected from ZR75-1 for estrogen independence. The lack of NTP:P, increase after estrogen withdrawal from ZR75/LCC-3 further corroborates that this phenomenon is in fact related to estrogen dependence. The underlying mech-
The effect of ovariectomy on energy metabolism detected by $^{31}$P MRS in induced mammary tumors in rats has been studied by Rodrigues et al. (10), who found a significant increase in the PCr:NTP ratio in estrogen-sensitive tumors already 2 days after ovariectomy, whereas no change of this ratio was seen in response to ovariectomy in estrogen-insensitive tumors. As demonstrated in Figs. 1–6, we found a close correlation between estrogen dependence and energy metabolic (NTP:P$_r$) response to estrogen withdrawal. These findings are in concordance with those recently reported by Furman et al. (3, 4) in tamoxifen-treated MCF-7 tumors; a significant increase in the NTP:P$_r$ ratio in response to the initiation of tamoxifen therapy was detected already after 4–7 days. In these studies performed in nude mice with intact ovaries, however, the estrogen pellet was removed when tamoxifen therapy was initiated. Consequently the observed increase in the NTP:P$_r$ ratio may reflect estrogen deprivation as seen in the present study rather than initiation of tamoxifen therapy.

Whether the demonstrated changes in energy metabolic ratios after antihormonal therapy demonstrated by us and others (3, 4, 10) is caused by intracellular changes in metabolic rates or increased nutrient supply following stromal alterations has not been fully elucidated. Although a markedly decreased glycolytic rate following tamoxifen treatment has been demonstrated by $^{13}$C MRS of the estrogen-dependent cell lines MCF-7 (3) and T47D (11) in vitro, central vascular collapse, as seen on magnetic resonance images after 24 h, was proposed as the origin of the abrupt spectral changes after estrogen withdrawal and initiation of tamoxifen therapy (4). The reported central necrosis and subsequent fibrosis in MCF-7 tumors is contrasting the data of Stubbs et al. (12), who found no histologically detectable changes in the tissue structure of induced mammary tumors after 2 days of estrogen deprivation. Furthermore, previous studies have indicated that tumor cell death in response to endocrine ablation cannot be attributed to vascular deficiency (13), and it has been demonstrated that an increased number of apoptotic cells are present in MCF-7 cells already 1 day after estrogen withdrawal (14). A difference in size between estrogen-stimulated and estrogen-depleted tumors would not explain the NTP:P$_r$ increase, since in our study this observation was present already 2 days after estrogen withdrawal. Also, the acute NTP:P$_r$ increase occurred in the growth-inhibited tumors, indicating that changes in tumor volume or localization was not the reason for the observed difference between growth-inhibited and control tumors.

We found no difference in the spectrophotometrically determined ATP concentration between estrogen-depleted and estrogen-supplemented ZR-75-1 tumors (Table 3). Consequently, a decrease in the P$_r$ concentration was probably the explanation of the observed increase in the NTP:P$_r$ ratio after estrogen withdrawal. This decrease in P$_r$ could be caused by either increased washout due to improved tumor blood flow in the estrogen-depleted tumors or by a so-called compartmentation phenomenon: the intramitochondrial P$_r$ pool is probably invisible by MRS. An increased mitochondrial uptake of P$_r$ would decrease the cytoplasmatic (i.e., visible) P$_r$ concentration, and would consequently have increased the NTP:P$_r$ ratio. This phenomenon has also been demonstrated in liver tissue, where a switch from a mainly glycolytic to a mainly gluconeogenetic state was followed by a decrease in NMR-detectable P$_r$ and a corresponding increase in intramitochondrial P$_r$ concentration (15). A similar compartmentation is not affecting the intracellular ATP visibility, which is relatively high (90%) and unaffected by the metabolic state of perfused liver tissue (16).

Increased ischemia in the growth-stimulated tumors may account for the difference in PCr content of the two groups of ZR-75-1 tumors (Table 3), as also reflected in the MRS data (Fig. 2). The PCr:NTP ratio is significantly decreased in the estrogen-stimulated compared to the estrogen-depleted tumors only on day 14 (i.e., when the difference in size between estrogen-stimulated and estrogen-depleted tumors is obvious). However, the fact that also the estrogen-independent ZR75/LCC-3 subline demonstrated a tendency toward decreased PCr concentration (Table 3) during estrogen stimulation, in spite of similar tumor sizes with and without estrogen stimulation, indicates a direct estrogen-mediated effect on the turnover of high-energy phosphates, possibly by regulation of creatine kinase activity.

Surprisingly, the ATP concentration of estrogen-depleted ZR75/LCC-3 tumors was 10-fold higher than in the continuously estrogen-stimulated tumors on day 14 after estrogen withdrawal. This finding may reflect a defect in the regulatory mechanisms of phosphate transfer between P$_r$, ATP, and PCr in estrogen-independent tumor cells. Still, no difference in ATP concentration was found between estrogen-supplemented and estrogen-depleted MDA-MB-231 tumors (Table 3). Although it may be that estrogen inhibits creatine kinase activity in estrogen-dependent tumors, it should be kept in mind that a down-regulation of enzyme activity does not change the steady-state concentration of either the substrate or the product, but only the conversion rate, provided that the system is in equilibrium. Recent studies have demonstrated a marked growth inhibitory effect on rat mammary tumors of dietary creatine and cyclocreatine, and PCr has been shown to inhibit several glycolytic enzymes (17), indicating a crucial role of creatine kinase and high-energy phosphates in the regulation of tumor growth.

PME:P$_r$ turned out to be the pretherapeutic parameter most closely related to estrogen dependence (Table 2), and from the $^{31}$P MRS appearance it has been possible with great certainty to discriminate between estrogen dependence/independence and to identify the individuals. PME are precursors in the biosynthesis of phospholipids (18). Other authors have proposed PME-related ratios as indicators of cancer and sensitivity to antiestrogens (19, 20) or chemotherapy (21) due to the presumed association of PME levels to increased synthesis of membrane phospholipids and cell turnover rate (22). A receptor-mediated regulatory effect of estrogen on the phospholipid metabolism might explain the observed differences in this study (Fig. 7). Our data also indicate that it is possible to reach an even higher predictability regarding estrogen dependency by including PCr:NTP, PDE:P$_r$, and PME:NTP (Table 2) in the analysis. The NTP:P$_r$ ratio was correlated to PME:P$_r$, and also to the tumor growth rate. Slow-growing tumors expressed a high NTP:P$_r$ ratio and vice versa (Fig. 7). There was a general tendency in all tumors toward decreased NTP:P$_r$ and PCr:NTP during tumor growth (Figs. 1–6). This relationship between energy metabolism and tumor growth is consistent with other observations of a decrease in NTP:P$_r$ with increasing tumor size (2).

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