Effects of Dietary Energy Restriction on Gene Regulation in Mammary Epithelial Cells

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Abstract

The objective of this study was to determine whether dietary energy restriction (DER) affects the pattern of gene expression in three interrelated energy metabolism pathways: glycolysis, gluconeogenesis, and the citric acid cycle. Mammary carcinogenesis was initiated by the i.p. injection of female Sprague-Dawley rats with 50 mg of 1-methyl-1-nitrosourea per kilogram of body weight. Five days following 1-methyl-1-nitrosourea administration, animals were fed ad libitum or 80% or 60% of the ad libitum intake. Epithelial cells were harvested from histologically confirmed adenocarcinomas (adenocarcinoma epithelial cell; ACEC) and uninvolved mammary gland (mammary gland epithelial cells; MGE) via laser capture microdissection, whereas isolated RNA was arrayed on Affymetrix R230 2.0 genome chips. Principal components analysis revealed complete separation of the patterns of gene expression between ACEC versus MGE. Further examination of the data set revealed an up-regulated pattern of expression in the ACEC of genes involved in glycolysis, whereas gluconeogenesis was suppressed. In general, genes involved in the citric acid cycle were not differentially expressed; however, pyruvate dehydrogenase expression was down-regulated and lactate dehydrogenase expression was increased in ACEC versus MGE. Collectively, the observed patterns of expression were consistent with the Warburg effect. DER exerted no effect on the Warburg pattern of gene expression or on other aspects of these energy metabolism pathways. These findings imply that efforts to target the Warburg effect for cancer prevention are mechanistically distinct from those modulated by DER and provide a rationale for the combination of approaches that target basic defects in energy metabolism and energy-sensing pathways for the prevention of breast cancer. [Cancer Res 2007;67(24):12018–25]

Introduction

The ability of dietary energy restriction (DER) to reproducibly and dose-dependently inhibit the carcinogenic process in multiple organ sites is well established (1–3). To date, efforts to elucidate the mechanisms that account for inhibitory activity have focused either on the identification of the cellular processes affected, i.e., proliferation, apoptosis, and angiogenesis, or on the systemic factors that may mediate protective activity, primarily insulin-like growth factor-I, adrenal cortical steroids, and adipokines such as leptin (4, 5). Until recently, little attention had been given to the possibility that intracellular events involving energy metabolism might be prominent in accounting for DER mediated protection despite the well-recognized changes in glucose metabolism known to occur during the development of cancer (6). Although emerging evidence indicates that DER modulates the activity of energy-sensing molecules and the signaling pathways that they regulate, there have been no investigations of its effects on the genes involved in glycolysis, gluconeogenesis, and the citric acid cycle (CAC).

In 1920, Warburg made the observation that tumor cells, unlike their normal counterparts, use glycolysis instead of mitochondrial oxidative phosphorylation for energy production even when oxygen is present (the Warburg effect; ref. 7). Although the reasons for this switch to aerobic glycolysis remain unclear, emerging evidence points to the involvement of hypoxia-inducible factor 1α as well as oncogenes such as Akt and tumor suppressor genes such as succinate dehydrogenase and fumarate hydratase (8–11). Moreover, associated defects in the regulation of genes involved in glycolysis and oxidative phosphorylation seem to contribute to both early events during tumor promotion and tumor maintenance as well as advanced changes including the development of metastatic potential (12–14).

Given the central role that changes in energy metabolism play during carcinogenesis, the present study was initiated to first determine if differential patterns of gene expression existed in epithelial cells excised from mammary glands (mammary gland epithelial cells; MGE) and mammary carcinomas (adenocarcinoma epithelial cell; ACEC) via laser capture microdissection (LCM). The focus of these analyses was on genes involved in three interrelated pathways: glycolysis, gluconeogenesis, and CAC which are diagrammed in Fig. 1. Having determined that differential patterns did exist, we then proceeded to investigate the effects of DER on these altered patterns of expression. On the one hand, we argued that DER, via limiting glucose availability, might induce the pattern of gene expression in mammary carcinomas to be more like that observed in epithelial cells from the mammary gland. Alternatively, DER might exacerbate effects causing even greater differences in the pattern of expression within or between these populations of epithelial cells.

Materials and Methods

Carcinogen administration and diets. Sprague-Dawley rats were obtained from Taconic Farms at 20 days of age. At 21 days of age, rats were injected with 50 mg of 1-methyl-1-nitrosourea/kg body weight (i.p.) as previously described (15). For the first week of the study, rats were housed three per cage in solid-bottomed polycarbonate cages equipped with a food cup. Thereafter, rats were individually housed for the duration of the study. The animal room was maintained at 22 ± 1°C with 50% relative humidity and a 12-h light/12-h dark cycle. Five days following carcinogen injection, all
Rats were randomized into three groups and meal-fed either AIN-93G control diet ad libitum (control), a modified AIN-93G diet in an amount that was 80% of the ad libitum intake of the control (20% DER) or a modified AIN-93G diet in an amount that was 60% of the ad libitum intake of the control (40% DER). All of the rats were meal-fed twice daily (8:00–11:00 a.m. and 2:00–5:00 p.m.), 7 days per week in order to reduce possible confounding due to intergroup variation in meal timing, meal number, and duration of fasting between meals. Rats in the control group were

Figure 1. Signal pathways of energy metabolism, including glycolysis, gluconeogenesis, and CAC. The numbers in parentheses represent the “Step no.” listed in Table 1.
allowed access to an unlimited amount of diet each meal whereas rats in the DER groups were given a restricted amount of their diet each meal. A modified AIN-93G diet formulation was used as previously described (16). The diet fed to 20% or 40% DER rats was formulated to insure an intake of all nutrients equivalent to the control group whereas limiting total dietary energy by reducing carbohydrate. All rats were palpated thrice a week for detection of mammary tumors beginning at 19 days post-carcinogen administration. The work reported was reviewed and approved by the Institutional Animal Care and Use Committee and conducted according to the committee guidelines.

**Experimental design and sample collection.** Preliminary studies were provided as a basis for statistical power calculations which indicated that seven chips would provide an adequate biological sample for each treatment condition of interest. To obtain the required samples, animals in each treatment group were euthanized sequentially when a palpated mammary tumor with a diameter of ~0.5 cm was detected. Tumors of this size were used to minimize the presence of necrosis, which adversely affects RNA quality. Overall, mammary gland and mammary carcinomas were evaluated from seven different animals per treatment group. This resulted in 14 samples from each group (control, 20% DER and 40% DER), i.e., seven epithelial cell samples from mammary glands and seven epithelial cell samples from mammary adenocarcinomas. The 42 samples evaluated were collected from 21 animals because a mammary gland and adenocarcinoma pair was excised from the same animal. Because of the potent inhibitory activity of DER against mammary carcinogenesis, which decreases cancer incidence, prolongs cancer latency, and slows tumor growth, it required 12 weeks (study duration) for a sufficient number of tumor-bearing animals to occur in the 40% DER group.

Following inhalation of gaseous carbon dioxide and cervical dislocation, rats were then skinned and the skin was examined under translucent light to locate mammary tumors. The tumors were excised and snap-frozen in liquid nitrogen. The contralateral abdominal inguinal mammary gland chain was excised, prepared as a whole mount on transparency film, and snap-frozen in liquid nitrogen. All samples were stored at ~80°C. A representative section of frozen mammary gland near the lymph node region in gland 4 was selected for LCM. Tissue spaces from frozen sections of both mammary gland and tumor were done to verify sample RNA integrity prior to LCM. All sections were also examined histologically before LCM. Only mammary adenocarcinomas and mammary glands confirmed to be free of pathologies were evaluated. Animals from each group were evaluated sequentially to determine if they met these criteria based on time of tumor detection; providing an unbiased approach for selecting the animals used for analysis.

**LCM.** Tumor and mammary gland cryosections were cut at 7 and 10 µm, respectively, and stored. Frozen at ~80°C in nuclease-free slide boxes. Slides were removed one at a time from a slide box stored under dry ice and dehydrated using nuclease-free HistoGene (Molecular Devices) reagents according to the following modified protocol: 75% ethanol, water, 75% ethanol, 95% ethanol, and 100% ethanol all for 30 s each followed by xylene for 1 min and air-drying for 1 min. LCM was performed using an AutoPix instrument (Molecular Devices). Dehydration and LCM was limited to 30 min or less for each sample collected. A total of four caps were collected for each tumor and eight caps for each mammary gland.

**RNA isolation.** RNA in the epithelial cells dissected from mammary gland and mammary carcinomas via LCM were isolated using PicoPure RNA Isolation Kit (Arcturus Bioscience). Briefly, the epithelial cells were incubated with 30 µL of extraction buffer in a 0.5 mL microcentrifuge tube at 42°C for 30 min before centrifuging at 800 x g for 2 min. The RNA was isolated using an RNA purification column following the manufacturer's instructions (Arcturus Bioscience).

**Total RNA quantity and integrity.** The total RNA concentration of each sample was determined using a NanoDrop ND-3300 Fluorospectrometer (NanoDrop). The assay measured the fluorescence of Ribogreen dye at 525 nm following excitation at 470 nm. RNA concentration was computed based on a standard curve. The integrity of RNA was examined using an Experion analyzer (Bio-Rad) with Experion RNA HighSens Analysis Kit.

**RNA amplification and labeling for microarray.** The Ovation Biotin RNA amplification and labeling system from NuGEN Technologies, Inc., was used for amplification and labeling. The Ovation Biotin System is powered by Ribo-SPIA Technology, a rapid, simple, and sensitive RNA amplification process. A detailed protocol is described in the users guide kit and was used without modification. The total of 3 to 5 µg of RNA per sample was amplified. Briefly, first-strand cDNA was prepared from 20 ng of total RNA using a unique first-strand DNA/RNA chimeric primer and reverse transcriptase. The primer has a DNA portion that hybridizes to the 5’ portion of the polyadenylate sequence. This resulted in a cDNA/mRNA hybrid molecule containing a unique RNA sequence at the 5’ end of the cDNA strand. Fragmentation of the mRNA within the cDNA/mRNA complex created priming sites for DNA polymerase to synthesize a second strand, which included DNA complementary to the 5’ unique sequence from the first-strand chimeric primer. This resulted in the formation of a double-stranded cDNA with a unique DNA/RNA heteroduplex at one end. SPiA amplification was achieved using a SPiA DNA/RNA chimeric primer, DNA polymerase, and RNase H in a homogeneous isothermal assay that provided highly efficient amplification of DNA sequences. RNase H was used to degrade RNA in the DNA/RNA heteroduplex at the 5’ end of the first cDNA strand. This resulted in the exposure of a DNA sequence that was available for binding a second SPiA DNA/RNA chimeric primer. DNA polymerase then initiated replication at the 3’ end of the primer, displacing the existing forward strand. The RNA portion at the 5’ end of the newly synthesized strand was again removed by RNase H, exposing part of the unique priming site for initiation of the next round of cDNA synthesis. The process was repeated, resulting in rapid accumulation of cDNA with sequence complementarity to the original mRNA. The single-stranded cDNA generated during the amplification process was fragmented by an enzymatic reaction that produced product mostly <250 bases with an average length ranging from 50 to 100 bases. The fragmented product was labeled with biotin using a chemical attachment method described in the guide. Targets containing amplified, fragmented, and biotin-labeled cDNA generated using the Ovation Biotin System were prepared for analysis on GeneChip Rat Genome 230 2.0 Arrays according to the Affymetrix GeneChip expression analysis technical manual (revision 4). Components and supply sources used in the hybridization cocktail were specified in the manual. To prepare the target for a single array, 2.2 µg of fragmented, biotin-labeled, and amplified cDNA was mixed at room temperature with the volumes of hybridization cocktail components indicated in the manual. Water was added to bring the final volume to 220 µL. The hybridization cocktail was heat denatured at 99°C for 2 min immediately before loading the solution into arrays.

**Hybridization, washing, and staining of the Affymetrix GeneChip Rat Genome 230 2.0 Arrays.** Hybridization was performed by incubating 200 µL of the above hybridization cocktail to the Affymetrix GeneChip arrays (Affymetrix, Inc.). Hybridization occurred at 45°C for 16 h using a GeneChip Hybridization Oven 640 (Affymetrix). After hybridization, the hybridization solutions were removed and the arrays washed and stained with streptavidin-phycocerythrin using a GeneChip Fluidics Station 450 (Affymetrix). Arrays were read at a resolution of 2.5 to 3 µm using the GeneChip Scanner 3000 (Affymetrix).

**Statistical analyses.** The effects of treatment on cancer latency were evaluated by life table analysis (17). Raw expression values from the Affymetrix Rat Genome 230 2.0 chip were analyzed using Partek software (Partek Incorporated). Raw intensity values were imported by setting up robust multianalysis background correction, quartile normalization, and log transformation. Principal components analysis (PCA) was performed as implemented in Partek as it is an excellent method for visualizing high-dimensional data. To identify pathway-specific effects, gene set enrichment analysis (GSEA) was used (the Broad Institute/Massachusetts Institute of Technology) in addition to multivariate ANOVA (18). Descriptive statistics for individual genes between MGECs and mammary tumor cells or among different DER groups were computed using Systat software (version 12).

**Results**

**Carcinogenic response.** DER resulted in highly significant inhibition of the carcinogenic response. Cancer latency was
prolonged in a dose-dependent manner. Mean time to cancer was 36.5 ± 6.4, 46.9 ± 9.7, and 78.1 ± 9.0 in control, 20% DER, and 40% DER treatment groups, respectively (P < 0.001 by life table analysis).

**PCA.** The expression data from the 42 Affymetrix chips were normalized using the robust multiarray analysis algorithm in Partek version 2.0. Those data were then subjected to PCA. As shown in Fig. 2A, at the whole-chip level, there was complete separation of expression patterns for RNA isolated from epithelial cells captured from mammary carcinomas versus mammary glands. Although differences were also discernable among levels of DER within either the mammary gland or mammary carcinomas, the distinctions were less pronounced (Fig. 2B and C, respectively).

**Figure 2.** PCA mapping of 42 samples representing all genes. Each dot represents a chip (sample). A, samples according to tissue type. B, mammary gland (MG) epithelial cells treated with various levels of DER. C, ACECs treated with various levels of DER.
GSEA. The analysis of the microarray data was broken into two components: (a) the comparison of patterns of expression between mammary glands and mammary carcinomas; and (b) the comparison of patterns of gene expression associated with DER.

Mammary gland versus mammary carcinomas. The specific hypothesis tested using GSEA was that there would be differential transcriptional regulation of genes involved in glycolysis, gluconeogenesis, and the CAC in mammary glands versus mammary carcinomas. To test this hypothesis, the genes involved in each metabolic pathway were compiled and run simultaneously in GSEA. The heat maps showing the consistency of expression for each gene on each chip for glycolysis, CAC, and gluconeogenesis are shown in Fig. 3A, B, and C, respectively. The overall P value for a differential regulation of these pathways between mammary gland and mammary carcinomas at the 10% false discovery rate was \( P < 0.037 \). That analysis indicated a dominant up-regulation of genes involved in glycolysis in mammary carcinomas relative to mammary glands; whereas gluconeogenesis was suppressed in that the principal regulatory protein, phosphoenolpyruvate carboxykinase, was dramatically down-regulated. In general, most genes involved in CAC were not differentially expressed in mammary gland versus mammary carcinomas but with two important exceptions. Transcript levels of pyruvate dehydrogenase were significantly lower and levels for lactate dehydrogenase were markedly higher in mammary carcinomas versus mammary gland. To further evaluate and quantify the differences observed, the normalized expression data were analyzed by multivariate ANOVA. Table 1 contains a list of the genes investigated and the mean level of expression for each gene. The overall multivariate Hotelling statistics for the hypothesis test for differential expression between mammary gland and mammary carcinomas were: glycolysis, \( P = 1.69 \times 10^{-12} \); CCA, \( P = 1.26 \times 10^{-11} \); and gluconeogenesis, \( P = 9.77 \times 10^{-6} \).

The effects of DER in mammary glands and mammary carcinomas. The heat maps generated within GSEA and shown in Fig. 3 also contain information about the level of DER to which each animal was exposed and consequentially from which each chip was generated. However, the current version of GSEA only

![Figure 3](image-url)
was complete distinction between patterns of gene expression and the effects of DER on glucose metabolism in the rat. The numbers in the first column “Step no.” are related to the numbers labeled in Fig. 1 for each gene.

Table 1. Expression of genes influencing the energy metabolism in MGEC and ACEC of rat

<table>
<thead>
<tr>
<th>Step no.</th>
<th>Accession no.</th>
<th>Gene name</th>
<th>Symbol</th>
<th>MGEC</th>
<th>ACEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NM_012735</td>
<td>Hexokinase</td>
<td>HK2</td>
<td>1,128 ± 75</td>
<td>2,518 ± 159</td>
</tr>
<tr>
<td>2</td>
<td>NM_013098</td>
<td>Glucose-6-phosphatase</td>
<td>G6PC</td>
<td>42 ± 1</td>
<td>44 ± 2</td>
</tr>
<tr>
<td>3</td>
<td>NM_207592</td>
<td>Glucose phosphate isomerase</td>
<td>GPI</td>
<td>217 ± 9</td>
<td>526 ± 33</td>
</tr>
<tr>
<td>4</td>
<td>NM_013190</td>
<td>Phosphofructokinase</td>
<td>PKFL</td>
<td>102 ± 4</td>
<td>190 ± 12</td>
</tr>
<tr>
<td>5</td>
<td>NM_053716</td>
<td>Fructose-1,6-bisphosphatase 2</td>
<td>FBP2</td>
<td>40 ± 3</td>
<td>70 ± 7</td>
</tr>
<tr>
<td>6</td>
<td>NM_012495</td>
<td>Aldolase A</td>
<td>ALDOA</td>
<td>631 ± 28</td>
<td>1,288 ± 89</td>
</tr>
<tr>
<td>7</td>
<td>NM_022922</td>
<td>Triosephosphate isomerase 1</td>
<td>TPI</td>
<td>935 ± 59</td>
<td>2,180 ± 139</td>
</tr>
<tr>
<td>8</td>
<td>NM_017008</td>
<td>Glyceroldehyde-3-phosphate dehydrogenase</td>
<td>GAPDH</td>
<td>518 ± 39</td>
<td>1,316 ± 89</td>
</tr>
<tr>
<td>9</td>
<td>NM_053291</td>
<td>Phosphoglycerate kinase 1</td>
<td>PGK1</td>
<td>661 ± 50</td>
<td>1,743 ± 97</td>
</tr>
<tr>
<td>10</td>
<td>NM_053290</td>
<td>Phosphoglycerate mutase 1</td>
<td>PGM1</td>
<td>1,482 ± 69</td>
<td>2,751 ± 98</td>
</tr>
<tr>
<td>11</td>
<td>NM_139325</td>
<td>Enolase 2</td>
<td>ENO2</td>
<td>190 ± 10</td>
<td>163 ± 15</td>
</tr>
<tr>
<td>12</td>
<td>NM_053297</td>
<td>Pyruvate kinase isozyme M2</td>
<td>PKM2</td>
<td>354 ± 15</td>
<td>1,092 ± 71</td>
</tr>
<tr>
<td>13</td>
<td>NM_198780</td>
<td>Phosphoenolpyruvate carboxykinase 1</td>
<td>PCK1</td>
<td>276 ± 53</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>14</td>
<td>NM_017025</td>
<td>Lactate dehydrogenase A</td>
<td>LDHA</td>
<td>953 ± 43</td>
<td>2,027 ± 102</td>
</tr>
<tr>
<td>15</td>
<td>NM_001004072</td>
<td>Pyruvate dehydrogenase α1</td>
<td>PDHA1</td>
<td>905 ± 23</td>
<td>608 ± 14</td>
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<tr>
<td>16</td>
<td>NM_017321</td>
<td>Aconitase 1</td>
<td>ACO1</td>
<td>590 ± 21</td>
<td>400 ± 15</td>
</tr>
<tr>
<td>17</td>
<td>NM_031510</td>
<td>Isocitrate dehydrogenase 1</td>
<td>IDH1</td>
<td>3,508 ± 75</td>
<td>3,688 ± 133</td>
</tr>
<tr>
<td>18</td>
<td>NM_001017461</td>
<td>α-Ketoglutarate (oxoglutarate) dehydrogenase</td>
<td>OGDH</td>
<td>46 ± 5</td>
<td>53 ± 3</td>
</tr>
<tr>
<td>19</td>
<td>NM_033752</td>
<td>Succinate-CoA ligase α</td>
<td>SUCLG1</td>
<td>487 ± 31</td>
<td>417 ± 24</td>
</tr>
<tr>
<td>20</td>
<td>BC099746</td>
<td>Succinate-CoA ligase β</td>
<td>SUCLG2</td>
<td>1,287 ± 47</td>
<td>1,292 ± 52</td>
</tr>
<tr>
<td>21</td>
<td>NM_198788</td>
<td>Succinate dehydrogenase complex D</td>
<td>SDHD</td>
<td>858 ± 34</td>
<td>781 ± 26</td>
</tr>
<tr>
<td>22</td>
<td>NM_017005</td>
<td>Fumarate hydratase 1</td>
<td>FH1</td>
<td>334 ± 22</td>
<td>401 ± 22</td>
</tr>
<tr>
<td>23</td>
<td>NM_033235</td>
<td>Malate dehydrogenase 1</td>
<td>MDH1</td>
<td>1,026 ± 52</td>
<td>1,185 ± 46</td>
</tr>
<tr>
<td>24</td>
<td>NM_130755</td>
<td>Citrate synthase</td>
<td>CS</td>
<td>527 ± 21</td>
<td>502 ± 19</td>
</tr>
<tr>
<td>25</td>
<td>NM_053826</td>
<td>Pyruvate dehydrogenase kinase 1</td>
<td>PDK1</td>
<td>151 ± 11</td>
<td>220 ± 16</td>
</tr>
<tr>
<td>26</td>
<td>NM_145091</td>
<td>Pyruvate dehydrogenase phosphatase isoenzyme 2</td>
<td>PDP2</td>
<td>139 ± 14</td>
<td>161 ± 10</td>
</tr>
</tbody>
</table>

NOTE: Values are mean ± SE (n = 21). The numbers in the first column “Step no.” are related to the numbers labeled in Fig. 1 for each gene.

permits comparisons between two treatment conditions. Therefore, the effects of DER on gene expression were evaluated by multivariate ANOVA. Figure 4 shows bar graphs for each gene in glycolysis, CAC, or gluconeogenesis grouped according to mammary gland or carcinoma for each level of DER. Visual inspection of those data provided no evidence that DER has an effect on transcriptional regulation of glycolysis in epithelial cells from the mammary gland. On the other hand, there was a pattern of decreased gene expression with increasing DER in mammary carcinomas. Nonetheless, statistical analyses of these data failed to support an effect on gene expression associated with DER.

Discussion

The work reported in this article elucidated three aspects of our understanding of mammary carcinogenesis and the effects of DER in the 1-methyl-1-nitrosourea–induced model system in the rat: (a) substantiation of marked differences in patterns of gene expression in the ACEC versus MGEC captured via LCM; (b) identification of specific alterations in transcriptional regulation of genes involved in glycolysis, gluconeogenesis, and CAC; and (c) determination of a general lack of effect of DER on these alterations at the level of transcription. These data provide a rationale for exploring distinct approaches to inhibition of carcinogenesis via DER versus the modulation of energy metabolism. Each point is discussed in turn in the following paragraphs.

Mammary carcinoma versus MGECs. As shown in Fig. 2, there was complete distinction between patterns of gene expression characterized at the chip level between ACEC and MGEC. To our knowledge, there have been no previous reports of differential patterns of gene expression using RNA from laser-captured cells from mammary tissue in the 1-methyl-1-nitrosourea model, or more generally, in chemically induced models for breast cancer in the rat. The complete separation of patterns of expression observed provided the rationale for the additional analyses that were performed.

Energy metabolism. Although the Warburg effect has been reported to be observed in the majority of human tumors (14), there has been limited investigation of this topic in autochthonous model systems for breast cancer. Hence, the data presented shows that 1-methyl-1-nitrosourea–induced mammary carcinogenesis models the human disease in previously unappreciated ways. Specifically, not only was glycolysis observed to be up-regulated in ACEC versus MGEC, but there were also shifts in expression of both pyruvate dehydrogenase and lactate dehydrogenase that were consistent with effects observed in the human disease. Moreover, these patterns were consistently observed across a large number of animals. In addition, there has been limited study of effects of carcinogenesis on other genes involved in either gluconeogenesis or CAC. As shown in Fig. 4 and Table 1, the expression of phosphoenolpyruvate carboxykinase, which catalyzes a key regulatory step in gluconeogenesis was down-regulated in ACEC. Although this is consistent with the effects of DER on glucose metabolism in this model system (19), these differences have not been previously reported. Levels of DER that have marked cancer-inhibitory activity have been reported to exert many effects; however, even in studies...
in other model systems in which expression arrays have been used, there has been no mention of whether DER modulates the Warburg effect. As shown in Fig. 4 and Table 1, little evidence was found to support the hypothesis that DER affects this aspect of energy metabolism at the level of gene transcription. Initially, this finding was somewhat surprising in view of the well-known effects of DER on circulating levels of glucose as we have previously reported; however, these findings do not rule out the possibility of regulation via effects on either mRNA translation or posttranslational modification of proteins and consequent effects on their activities. In this regard, the present findings in fact help eliminate candidate molecular processes and serve to focus research on key proteins and their activities.

**Combinatorial approach to cancer prevention.** As noted in refs. (20, 10), there is currently considerable interest in the potential targeting of alterations in energy metabolism observed in tumors cells therapeutically. Specifically, efforts to target pyruvate dehydrogenase kinase and lactate dehydrogenase in order to induce tumor cell death and to inhibit the development of metastatic potential have been proposed (21, 22). However, as noted in ref. (14), emerging evidence has indicated that these changes may occur at an early stage in tumor development. Hence, we propose that this needs to be investigated and that agents such as dichloroacetate, which is already in clinical use for the treatment of lactic acidosis (23), might have potential use to prevent the development of cancer and not just to kill established tumor cells.

**Figure 4.** Gene expression of glycolysis, CAC, and gluconeogenesis in MGECs and ACECs. Columns, mean; bars, SE. MG-CTL, control MGECs; MG-20% DER, 20% dietary energy–restricted MGECs; MG-40% DER, 40% dietary energy–restricted MGECs; TUM-CTL, control ACECs; TUM-20% DER, 20% DER ACECs; TUM-40% DER, 40% DER ACECs. The full gene name of each symbol is listed in Table 1.
Similarly, applying the lessons learned in the cancer chemoprevention field suggests the potential value of a multipronged approach to blocking tumor development. Hence, investigating agents like oxamic acid, which inhibits the activity of lactate dehydrogenase, should be considered in combination with agents that target the metabolic disregulation resulting from the inhibition of pyruvate entry into mitochondrial oxidative phosphorylation (24). We have dubbed agents that reverse the induction of the Warburg effect to prevent cancer: energy metabolism modulatory agents (EMMA).

The work reported here is important in another way. Our laboratory has been engaged in identifying energy restriction mimetic agents (ERMA), for the prevention of cancer. Before the work reported herein, we argued that ERMA might be preventing cancer via targeting events associated with altered energy metabolism, specifically the Warburg effect. At least at the transcriptional level, this does not seem to be the case, but rather, prototypic ERMA such as 2-deoxyglucose seem to be inhibiting carcinogenesis by activating AMP-activated protein kinase (6). If the pathways by which ERMA and EMMA inhibit carcinogenesis are distinct, it will offer the previously unappreciated opportunity to combine these approaches to target fundamental and highly conserved facets of energy metabolism to prevent the development of cancer.

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