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Original Article

Tumor microenvironment promotes dicarboxylic acid carrier-mediated transport of succinate to fuel prostate cancer mitochondria

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Abstract: Prostate cancer cells reprogram their metabolism, so that they support their elevated oxidative phosphorylation and promote a cancer friendly microenvironment. This work aimed to explore the mechanisms that cancer cells employ for fueling themselves with energy rich metabolites available in interstitial fluids. The mitochondria oxidative phosphorylation in metastatic prostate cancer DU145 cells and normal prostate epithelial PrEC cells were studied by high-resolution respirometry. An important finding was that prostate cancer cells at acidic pH 6.8 are capable of consuming exogenous succinate, while physiological pH 7.4 was not favorable for this process. Using specific inhibitors, it was demonstrated that succinate is transported in cancer cells by the mechanism of plasma membrane Na^+-dependent dycarboxylic acid transporter NaDC3 (*SLC13A3 gene*). Although the level of expression of *SLC13A3* was not significantly altered when maintaining cells in the medium with lower pH, the respirometric activity of cells under acidic condition was elevated in the presence of succinate. In contrast, normal prostate cells while expressing NaDC3 mRNA do not produce NaDC3 protein. The mechanism of succinate influx via NaDC3 in metastatic prostate cancer cells could yield a novel target for anti-cancer therapy and has the potential to be used for imaging-based diagnostics to detect non-glycolytic tumors.

Keywords: Prostate cancer, mitochondria oxidative phosphorylation, acidic tumor microenvironment, Na^+-dicarboxylate transporter, succinate

Introduction

A large body of evidence indicates that the tumorigenic microenvironment makes a significant contribution to the metabolic reprogramming of tumor cells [1, 2]. The attention of researchers has been drawn to the active involvement of certain molecular constituents of the tumor surrounding tissues [3], as well as pH changes and ionic misbalance caused by or being a consequence of malignant transformations [4-7]. The abnormal distribution of potassium and other ions affects proliferation of prostate and other types of cancer [8-11]. It has been shown that local calcium deficiency could promote cancer development and metastasis [12, 13]. Moreover, acidosis has been shown to promote elevated generation of reactive oxygen species [14, 15]. The measured tumor pH values are ranges between 6.6-6.8, while in large ulcerated tumors lower pH values (5.8-6.3) were observed [16-18]. Cancer cells are able to modify the complex tumor microenvironment to enable their parasitic behaviour with respect to the surrounding tissues [19, 20]. This adaptive behavior becomes essential in solid tumors with limited access to glucose because of the lack of intensive blood circulation, or in cells such as prostate cancer cells which do not utilize glucose as a main energy substrate and are forced to use alternative energy metabolites available in interstitial fluid to support their high energy demands. The mitochondria of tumor cells deprive stromal cells of glutamate, glutamine, and TCA cycle respiratory metabolites causing degeneration of the surrounding tissues. This might explain the mechanism of development of mitophagy, autophagy and subsequent cachexia leading to increased mortality in cancer patients at meta-

static stages. However, the direct mechanisms which enable the intensive fueling of cancer cells with extracellular energetic substances have not been determined.

Limited permeability of intact cells to respiratory substrates is a tenet of bioenergetics [21]. The respirometric test on plasma membrane integrity by adding succinate to cells is a well-known methodological approach [22] in which an elevated respiration of intact cells in the presence of succinate would undoubtedly be considered as evidence of a compromised plasma membrane. The transport of succinate through the plasma membrane has interested researchers since the 1960s. The accelerated respiration upon addition of succinate and α-glycerophosphate was observed in brown fat cells [23, 24]. With respect to cancer cells, in 1976 T. Spencer specifically addressed this question and demonstrated that at acidic conditions Erhlich ascite tumor cells are capable of transporting succinate into the cytosol, although he did not focus on the mechanism of the transport [25]. Importantly, succinate along with other respiratory metabolites is generated in the mitochondria matrix and is detectable in interstisial fluids, which could be a result of metabolic misbalance leading to exessive accumulation of succinate and other metabolites and eventually their release from cells. It has been shown that the plasma level of TCA cycle intermediates varies depending on metabolic status, diet and pathological conditions [26, 27]. The concentration of succinate in normal human blood has been shown in different studies to range from 2 to 40 μM [28, 29]. At certain pathologic conditions including hypoxia [30], or under intensive exercises [31], the plasma level of succinate is elevated. Selective accumulation of succinate in tissue was shown at ischemic injury [32]. In tumors, the necrotic areas within a mass can provide additional amounts of TCA metabolites. Yet, elevated succinate content in body fluids is an atribute of cancer metabolism [33].

Since succinate is a charged substance only specific carriers can mediate its transport across the membrane. The carriers for dicarboxylic acids, such as succinate, citrate, malate, α-ketoglutarate, exist both in mitochondrial and plasma membrane forms. Inner mitochondria membrane specific phosphate-dependent dicarboxylic acid transporter is encoded by *SLC25A10 gene* [34]. The plasma membrane Na^+-dependent dicarboxylic acid transporter is presented by high (NaDC3) and low (NaDC1) affinity isoforms and sodium-dependent citrate transporter (NaCT) encoded by *SLC13A3, SLC13A2,* and *SLC13A5* genes, correspondingly [35-37]. For NaDC3 the stoichiometry of Na^+/succinate transport is determined to be 3:1 [36]. Organs such as the liver, kidney, placenta, intestine, and also brain synaptosomes, express the high affinity isoform of the transporter NaDC3, while its low affinity analogue NaDC1 is predominantly expressed in the intestine and kidney [35, 38-41]. With regards to prostate cancer it was shown that highly metastatic PC-3M cells also express an analogous Na^+-dependent transporter which provides inward transport of citrate [42], but prostate normal PNT2-C2 cells at physiological pH acquire outward K^+-mediated citrate transport [43].

This work aimed to study the respiratory activities of metastatic prostate cancer DU145 cells under acidic pH mimicking the tumorigenic microenvironment and the role of Na^+-dependent dicarboxylic acid transporter in carrying succinate and possibly other dicarboxylic acid metabolites across the cell membrane to promote prostate cancer cells' active growth and death resistivity.

Materials and methods

Cell lines and growth conditions

DU145 cells were purchased from ATCC at the available passage 60 and used up to passage 70 (Manassas, VA USA). Human primary prostate cells PrEC obtained at passage 2 from Lonza Inc. (Allendale, NJ USA) were maintained in manufacturer recommended PrEGM medium and used by passage 4. The rat aortic endothelial cells (RAEC) were provided by Dr. B. Polyak (Drexel University College of Medicine) and SKOV-3 cells were provided by Dr. W. Bowne (Drexel University College of Medicine). Cells were maintained in the corresponding growth mediums supplemented with 10% FBS at 37°C and 5% CO_2 atmosphere. Cell membrane intactness in respirometric experiments was evaluated by trypan blue exclusion assay.

High-resolution respirometry

The activities of respiratory enzymes were analyzed by high resolution respirometry at 37°C in

a two chamber respirometer OROBOROS Oxygraph-2K (Innsbruck, Austria) [44-46]. The OROBOROS DatLab software was used for data acquisition and analysis. Cells harvested by centrifugation were rinsed with and resuspended in a modified Krebs buffer containing 137 mM NaCl, 5 mM KCl, 20 mM MOPS, pH 7.4 and 6.8, 2 mM $MgCl_2$, 1 mM KH_2PO_4, 100 nM $CaCl_2$ (0.5 mM and 0.06 mM $CaCl_2$ in the presence of 1 mM EGTA at pH 7.4 and 6.8, correspondingly). The concentration of calcium in the presence of EGTA was calculated using maxchelator program (http://maxchelator.stanford.edu/CaEGTATS.htm). The 100 nM $CaCl_2$ (which does not damage mitochondria) was used to demonstrate elevation of oxygen consumption rate in the presence of succinate upon cells' permeabilization with digitonin. Other than that, the buffers with pH 6.8 containing 0.1, 0.5, and 1 mM $CaCl_2$ were prepared with 1.1 mM, 1.5 mM and 2 mM $CaCl_2$ in the presence of 1 mM EGTA, respectively. To assess the endogenous energy capacity of cells, no glucose or pyruvate was added to the measurement chambers. Once the system was stabilized cells were challenged by 20 or 40 nM dose of FCCP (carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone), which slightly stimulated the oxygen consumption without causing membrane depolarization. Next, for maximum activity cells were stimulated by 10 mM succinate [24, 25]. Respiratory rates were expressed per million of cells, per second. Subsequently 10 μM digitonin was added to evaluate the maximum succinate oxidizing ability of mitochondria as a result of its entry into the cytosol through the detergent permeabilized membrane.

Evaluation of mitochondria membrane potential

Cells were loaded with 75 nM MitoRed (Ex/Em wavelengths 622/648 nm) (PromoCell GmbH, Heidelberg, Germany) sensitive to mitochondria membrane potential. Mitochondria membrane potential was examined on BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA). For the positive control, cells were treated with 2 μM of FCCP, the dose which uncouples mitochondria oxidative phosphorylation and results in collapse of the membrane potential.

Reverse transcription and quantitative PCR

Total RNA was extracted from the cells using an RNeasy Mini kit (QIAGEN) according to the manufacturer's instructions. The RNA was treated with Turbo DNase (Ambion(R), Gran Island, NY) according to the manufacturer's instruction to eliminate any possible genomic DNA contamination. The concentrations and the quality of the RNA sample were determined using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE). The RNA sample was adjusted to a concentration of 50 ng μl^{-1}. As an internal control, GAPDH was used. The reverse transcriptase PCR (RT-PCR) protocol was carried out in an Eppendorf Mastercycler *ProS* (Hamburg, Germany) using the High Capacity cDNA Reverse Transcriptase kit (Applied Biosystem, Grand Island, NY) and 1 μg RNA template as recommended. The reaction mixture was first incubated at 25°C for 10 min and then at 37°C for 2 h to synthesize cDNA. To inactivate the reverse transcriptase the mixture was finally heated at 85°C for 5 min, and processed for real-time PCR using Eppendorf Mastercycler EpigradientS (Hamburg). Amplification was carried out in a 10 μl final volume containing 5 μl from the SYBR green PCR Master Mix (Applied Biosystem), 1 μl of primer mix (5 μM each), 2.75 μl of water and 1.25 μl of cDNA (10 ng μl^{-1} stock). The following protocol was used: denaturation at 95°C for 10 min, followed by 40 cycles consisting of 15 s at 95°C and 1 min at 60°C/62°C. The quantitative PCR was run in triplicates and repeated at least twice. The PCR products were electrophoresed with 6% polyacrylamid gels with 1X TBE. The gel was stained with ethidium bromide and destained with water and the result was documented under a Geldoc system (Biorad, USA). Oligonucleotides used as the specific primers to amplify human dicarboxylic transporters (NaDC1, NaDC3, NaCT) cDNA were as reported in [42].

Western blotting

DU145 and PrEC cells were grown for 10 days in corresponding growth mediums, RPMI supplemented with 10% heat-inactivated FBS and PrEGM with all required supplements according to the manufacturer protocol except gentamicin. Medium pH levels were adjusted to 7.4 and 6.0 using HCl. Cells were harvested and processed for solubilization of the membrane localized dicarboxylic acid transporter proteins. First, cells ($4\text{-}5 \times 10^6$) were incubated for 15 minutes on ice in hypotonic solution containing sucrose 100 mM, MOPS 10 mM, pH 7.4, EGTA 1 mM. After centrifugation at 2000 rpm for 5 minutes the

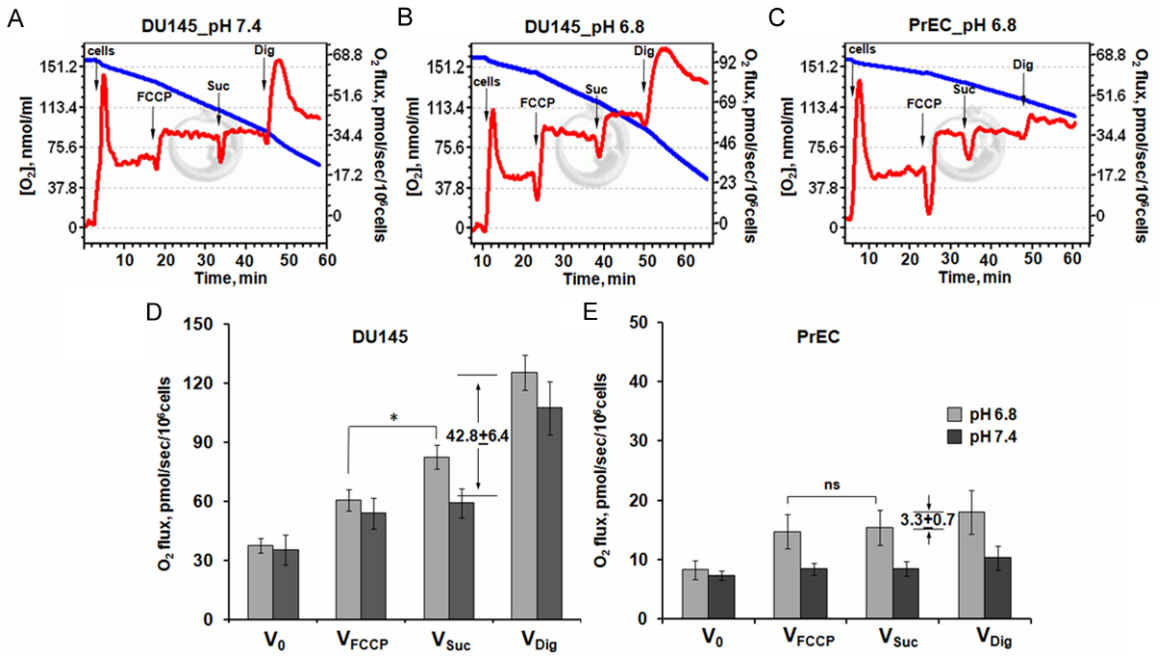

Figure 1. Succinate uptake by DU145 prostate cancer and PrEC prostate normal cells at different pH. A, B. Representative original records of DU145 cells oxygen consumption measured in the buffer with pH 7.4 and 6.8. After short period of stabilization (a basal respiration, V_0) the respiratory enzymes were activated by 40 nM FCCP (V_{FCCP}), and then cells were challenged by 10 mM succinate (V_{Suc}). At the end of the experiment, 10 μM of digitonin was added to provoke a non-transporter-mediated influx of succinate (V_{Dig}). C. Oxygen consumption by PrEC cells in the buffer with pH 6.8. D, E. Quantitative data of oxygen consumption rates of DU145 and PrEC cells presented as mean ± S.E.M. (n = 4-6), *$p < 0.0468$. In DU145 cells digitonin accelerated succinate oxidation is about 12 times higher than in PrEC cells (digit inserts).

cell pellet was resuspended in 1 ml of Ripa buffer containing protease inhibitors cocktail (Roche, Indianapolis, IN USA). Cell suspension was processed through three freeze-thaw cycles and additionally solubilized for 30 minutes on ice. Lysate was centrifuged at 14000 rpm for 10 minutes (sample was not heated) and subjected to 12% SDS-PAGE (30 μg per sample) followed by transfer to nitrocellulose membrane and immunoblotting with corresponding antibodies, human anti-NADC1, anti-NaDC3, and anti-NaCT (1:200) (Santa Cruz Biotechnology, USA). HRP-linked secondary antibodies (anti-goat 1:50,000; anti-rabbit 1:3,000) (Santa Cruz Biotechnology, USA) were visualized by chemiluminescence. Protein concentration was determined by Bradford Protein Assay (Bio-Rad Laboratories, USA).

Statistical analysis

Statistical analyses were performed using GraphPad Prism program version 5.03 for Windows (GraphPad Software, San Diego, USA). Results are presented as mean ± S.E.M. from at least three independent experiments. Statistically significant differences between data were estimated by unpaired, two-tailed Student's *t*-test. Differences were considered significant at $p < 0.05$.

Results

Oxidation of exogenous succinate by prostate cancer cells in the buffer with acidic pH

The extracellular pH of most human tumors is consistently acidic. We therefore modified the tumorigenic microenvironment by lowering pH of the buffer for respirometric measurements from the physiological pH of 7.4 to 6.8 to evaluate the contribution of acidity on modulation of tumor energy metabolism. The cellular response obtained in more acidic pH 6.0 was similar to that obtained in the buffer with pH 6.8, at least by mean of oxygen consumption rates (data not shown). The basal non-stimulated respiration of DU145 cells did not significantly differ at both conditions being 35.3±7.6 pmolO_2/s/10^6 cells at physiological pH and 37.4±3.7

Table 1. The rates of oxygen consumption in different cell lines in the respiration buffer with pH 6.8

Cell type	Oxygen flux rates, pmol/s/10^6 cells		
	V_0	V_{FCCP}	V_{Suc}
DU145	37.4± 3.7	60.6±5.5	82.4±6.1
PrEC	8.3±1.6	14.7±2.9	15.4±3.0
RAEC	25.9±4.0	45.4±9.8	36.4±4.8
SKOV-3	26.1±3.0	31.7±4.2	49.6±8.5

The incubation conditions are the same as in legend to **Figure 1**. V_0 , rates of respiration based on endogenous substrates only, V_{FCCP}, 40 nM FCCP stimulated respiration rates, V_{Suc}, rates of respiration after addition of 10 mM succinate. Data presented as mean ± S.E.M. (n = 4-6).

pmolO_2/s/10^6 cells at acidic pH. However, cell oxidative response on FCCP stimulation in the buffer with pH 6.8 was more pronounced (60.6±5.5 pmolO_2/s/10^6 cells versus 53.9±7.7 pmolO_2/s/10^6 cells at pH 7.4). **Figure 1** demonstrates the respiration of DU145 prostate cancer and prostate primary benign PrEC cells stimulated by succinate in the buffers with near physiological and acidic pH. The rationale for FCCP pretreatment was to stimulate the respiratory enzymes and promote "cell starved" metabolic state. It can be seen that at pH 7.4 succinate does not stimulate respiration of DU145 cells. Only the addition of digitonin promotes massive influx of succinate into cytosol via compromised membrane. Importantly, the calcium content in the buffer was adjusted to be near intracellular (100 nM) that would not damage the mitochondria upon digitonin permeabilization. In contrast, at acidic pH FCCP pretreated DU145 cells respiring at a rate of 60.6±5.5 pmolO_2/s/10^6 cells actively utilize succinate resulting in elevation of oxygen consumption rate to 82.4±6.1 pmolO_2/s/10^6 cells (**Figure 1B**, **Table 1**). Addition of digitonin further increases the rate of succinate oxidation (**Figure 1B**). After a few minutes the respiration rate declined, probably due to dilution of the pool of endogenous adenine nucleotides and ionic misbalance exerted by membrane perforation. However, intactness of mitochondria membrane remains preserved within permeabilized cells, because in the presence of exogenous succinate cells kept respiring at an elevated rate for longer time. Benign prostate PrEC cells did not respond on succinate stimulation in the acidic buffer (**Figure 1C**), the response on digitonin permeabilization is also mild. The difference between succinate and digitonin-stimulated respirations consists of 42.8±6.4 pmolO_2/s/10^6 cells in DU145 and 3.3±0.7 pmolO_2/s/10^6 cells in PrEC cells, which corresponds to the higher activity of succinate dehydrogenase in DU145 cancer cells in contrast to normal PrEC cells [47]. Due to the differences between DU145 and PrEC cells in terms of their mitochondria content, amount of respiratory enzymes and their lower oxidative activities [47] in respiratory measurements we used about three times larger amount of benign cells to obtain apparent respirometric signals. The mild stimulation of DU145 cells with 40 nM FCCP increases the electron transport capacity of mitochondria and at pH 6.8 succinate further accelerates the respiration by 134% versus 110% at pH 7.4. In contrast, normal prostate epithelial PrEC cells, which are respiratory less active in resting condition (8.3±1.6 pmolO_2/s/10^6 cells) than DU145 cancer cells (37.4±3.7 pmolO_2/s/10^6 cells), after FCCP stimulation showed a very low response to succinate addition, with values of 14.7±2.9 pmolO_2/s/10^6 cells before and 15.4±3.0 pmolO_2/s/10^6 cells after succinate addition (**Table 1**). The per cent of FCCP-stimulated respiration of PrEC cells in the presence of succinate is 104% at pH 6.8 and 101% at pH 7.4, the difference was determined to be statistically non-significant (**Figure 1E**).

To assess the maximal activity of complex II-dependent respiration at designed experimental conditions we titrated the cells with increasing doses of succinate and found no effect of succinate on oxygen consumption rates of DU145 cells at pH 7.4 (**Figure 2A**) but showed a stepwise increase in complex II-mediated respiration at acidosis (**Figure 2B**) indicating on transport mechanism of succinate delivery into cytosol. No increase in respiration rates after FCCP stimulation was determined in cells in the presence of glutamate and malate (data not shown). Digitonin permeabilization resulted in a fast increase of the respiration rate due to massive uptake of succinate, but shortly thereafter the respiration rate declined as a result of disturbance of intracellular environment. Similar to **Figure 2A**, **2B** the data were obtained on cells stimulated with succinate which were pretreated with complex I inhibitor rotenone (1 μg/ml) (**Figure 2C**, **2D**). Interestingly, in acidic buffer after rotenone inhibition FCCP did not produce apparent stimulation of respiration but cells were able to

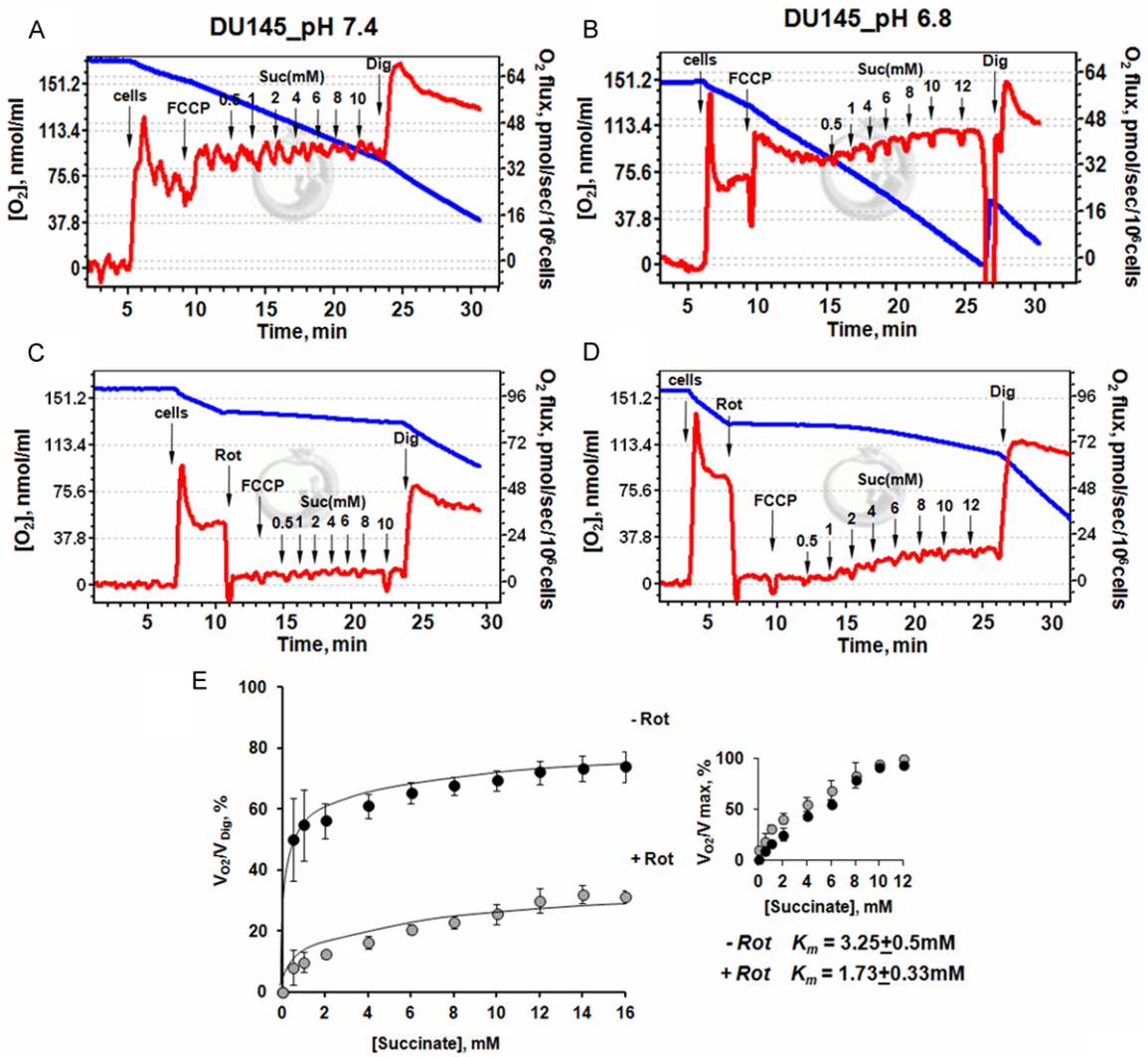

Figure 2. Substrate titration analysis of complex II-dependent respiration of DU145 cells. A, B. The representative original oxygraphic records of DU145 cells upon titration with gradually increasing doses of succinate (Suc) are presented. Succinate transport is favored only at acidic condition. The incubation conditions are the same as in legend to **Figure 1**. C, D. Similar substrate titration protocol applied after inhibition of complex I-mediated respiration with 1 μg/ml rotenone (Rot). Following 40 nM FCCP activation the cells were stimulated by increasing doses of succinate. Digitonin permeabilization of cells further increased the rates of respiration due to a massive succinate influx. Disturbance of cellular content due to digitonin permeabilization results in respiration decline. E. Kinetics of succinate transport presented as a per cent of maximum digitonin-stimulated respiration versus succinate concentrations. Inset is the transporter activity expressed as a per cent of the highest succinate-mediated respiration rates. Data are presented as mean ± S.E.M. (n = 3).

transport succinate. This effect was not observed at pH 7.4. The lowering of overall electron transport capacity by rotenone inhibition of NADH dehydrogenase complex resulted in reduced succinate-mediated respiration as it is seen from the higher rates of respiration induced by digitonin permeabilization (**Figure 2B**, **2D**, **2E**). The transport mechanism via DU145 cells plasma membrane in acidic environment was further characterized throughout determining the K_m for uptake of exogenous succinate [48]. At chosen conditions the values of K_m are shown to be 1.73±0.33 mM and 3.25±0.5 mM, with and without rotenone respectively, demonstrating that under the complex I inhibition the uptake of succinate by transporter is better promoted (**Figure 2E**).

Evaluation of the effects of FCCP and calcium content on succinate oxidation by prostate cancer cells

The trypan blue dye exclusion assay revealed that DU145 cells retain 97.5±1.5% viability

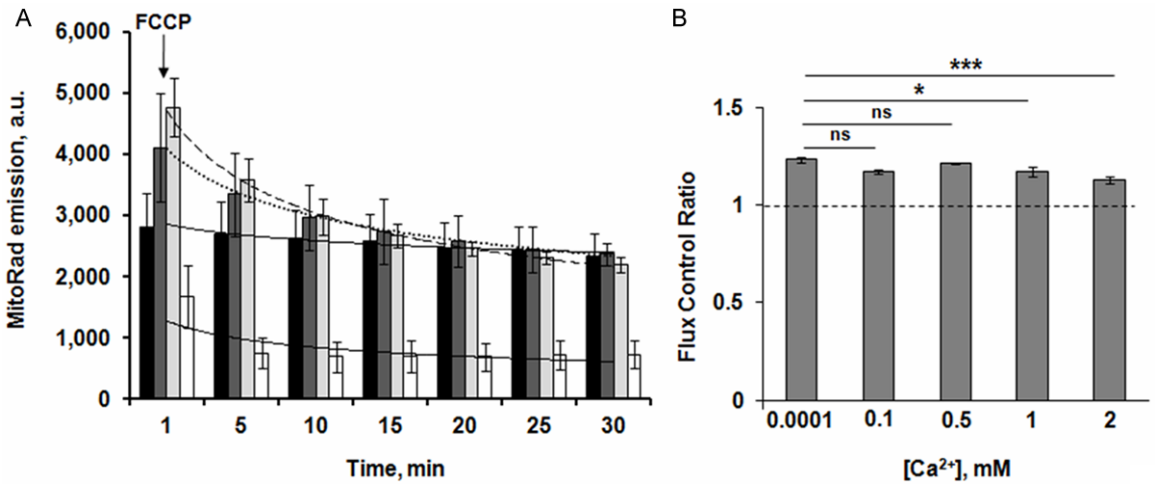

Figure 3. Evaluation of membrane stability of DU145 cells mitochondria upon treatment with FCCP and succinate oxidation by modulation of extracellular calcium content. A. DU145 cells were preloaded with mitochondria membrane potential sensitive dye MitoRed. The background MitoRed intensity is shown as black bars. Cells were exposed to 20 nM (dark grey bars) and 40 nM (light grey bars) FCCP to stimulate their oxidative activities, which restored to its initial level after 15-20 minutes. As a positive control cells were treated with 2 μM FCCP, the dose which eliminates mitochondria membrane potential (white bars). B. DU145 cells succinate oxidation activities measured in the buffers with pH 6.8 containing different concentrations of calcium. The protocol of cell pre-activation with 40 nM FCCP has been applied. The flux control ratios were normalized for FCCP-stimulated respiration. Data are presented as mean ± S.E.M. (n = 4-8). The difference in succinate oxidation rates in the presence of 0.0001 and 0.1 or 0.5 mM $CaCl_2$ was shown to be non-significant. *$p < 0.0114$, ***$p < 0.0008$.

after addition of FCCP versus 98±2.7% in FCCP non-treated cells indicating that the used FCCP doses do not compromise the cell plasma membrane. To further validate cell stability under FCCP stimulated condition the mitochondria membrane potential was measured in cells after addition of 20 nM and 40 nM FCCP using mitochondria membrane potential sensitive dye MitoRed. **Figure 3A** demonstrates that the chosen doses of FCCP do not decrease mitochondria membrane potential but enable exhaustion of the pool of endogenous substrates by intensifying the respiration [49].

Membrane stability is under the control of extracellular calcium ions [50, 51], yet succinate is capable of forming salts with calcium [52]. The low (100 nM) content of $CaCl_2$ in the respiration buffer was chosen for designed experiments to avoid mitochondria damage by high calcium concentrations after digitonin permeabilization. Therefore, it was important to evaluate the effect of different concentrations of extracellular calcium on the rate of succinate oxidation by cells at acidic pH. The basal respiration was not sensitive to alterations in extracellular calcium concentration in the range between 100 nM (near intracellular) and 2 mM (near extracellular) concentrations of calcium ions, remaining on the level of 37.4±3.7 pmol/s/10^6 cells. At lower calcium loads, namely 100 nM, 0.1 and 0.5 mM, the rates of succinate oxidation were quite similar, indicating no measurable effect of calcium ions on plasma membrane succinate transporter activity and mitochondria respiration at these concentrations of $CaCl_2$. However, in the presence of 1 and 2 mM, i.e. near physiological concentrations of extracellular calcium, the rates of succinate oxidation by DU145 cells were slightly slower, specifically 123% over FCCP stimulated respiration at 100 nM calcium versus 117% at 1 mM and 113% at 2 mM $CaCl_2$ in respiration buffer with pH 6.8. The flux control ratio values normalized for FCCP-stimulated respiration presented on **Figure 3B**.

Inhibitory analysis of the role of dicarboxylic acid transporter in succinate uptake

To validate the role of Na^+-dependent dicarboxylic acid transporters in succinate transport, we employed inhibitory analysis using specific inhibitors of dicarboxylic acid transporters, mersalyl and N-ethylmaleimide (NEM), which exert their effects through interaction with pro-

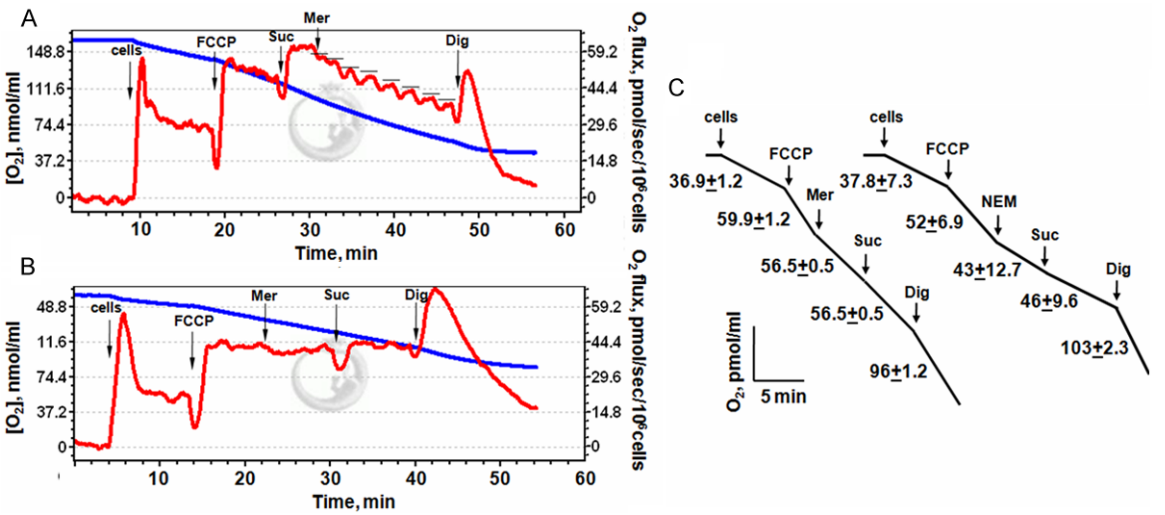

Figure 4. Effects of inhibitors on succinate uptake by DU145 prostate cancer cells in acidic buffer. The protocol of FCCP pretreatment was applied as on **Figure 1**. After short period of stabilization the respiratory enzymes were activated by 40 nM FCCP. A. After mild stimulation, cells were activated by 10 mM succinate. To determine the optimal concentration of the inhibitor, which however preserves the mitochondria intactness, the cells were titrated with gradually increasing doses of mersalyl (10, 20, 60, 120, 180, 240, 300, and 360 μM). Subsequently, 10 μM of digitonin was added to provoke a massive non-transporter-mediated influx of succinate. B. The chosen 250 μM dose of mersalyl was applied before addition of succinate, than cells were permeabilized with digitonin. C. The quantitative data of changes in oxygen consumption rates in the presence of inhibitors are presented as mean ± S.E.M. (n = 4-6). Alternative to 250 μM mersalyl (Mer) dicarboxylic acid transporter inhibitor N-ethylmaleimide (NEM) was used in concentration of 120 μM.

tein sulfhydryl groups. **Figure 4A**, **4B** shows the representative oxygraph records of DU145 cells respiration in the presence of mersalyl. Gradually increasing doses of mersalyl inhibit succinate-stimulated respiration of DU145 cells (**Figure 4A**), while when added before succinate mersalyl prevents succinate oxidation and only digitonin permeabilization of DU145 cell membrane enables succinate to enter cytosol and induce complex II-dependent respiration (**Figure 4B**). The increase of respiration upon addition of digitonin confirmed the intactness of mitochondria in this experimental protocol. However, penetration of mersalyl from extracellular buffer through the perforated membrane resulted in pronounced decline of respiration rate due to inhibition of mitochondria dicarboxylic acid transporters (**Figure 4A**, **4B**). The quantitative data of the effects of both inhibitors mersalyl and N-ethylmaleimide on succinate uptake by DU145 cells are shown on **Figure 4C**.

Expression profile of dicarboxylic acid transporters in prostate cancer and normal cells

Na^+-dependent transport of dicarboxylates is well characterized for renal membranes [37, 53]. It was important to evaluate the expression profile of dicarboxylic acid transporters in prostate cells, which are physiologically not involved in fluid reabsorbtion like liver or kidney cells. We have studied the known NaDC1, NaDC3, and NaCT transporters' transcript level by qRT-PCR and the corresponding proteins level by Western blot analysis (**Figure 5A**). The pronounced level of mRNA expression of NaDC3 has been found in both DU145 prostate cancer and PrEC prostate normal cells, while no NaDC1 isoform was detected in either prostate cell lines or positive controls (data not shown). The same amplification product for NaDC3 of 200 bp size was shown to be present in liver (HepG2 cells) and kidney (HEK293T/17 cells), which are known to be positive for NaDC3 [54, 55]. Western blot analysis showed that NaDC3 was expressed only in DU145 prostate cancer cells but not in PrEC normal prostate epithelial cells. Densitometry analysis revealed that at acidic pH of the medium in which cancer cells were maintained for 10 days before harvesting, the transporter protein expression was 29% lower than in cells grown at physiologic pH. This down regulation could be a compensatory mechanism for the elevated functional activity of the transporter in acidic milieu. Another protein from the family of dicarboxylic transport-

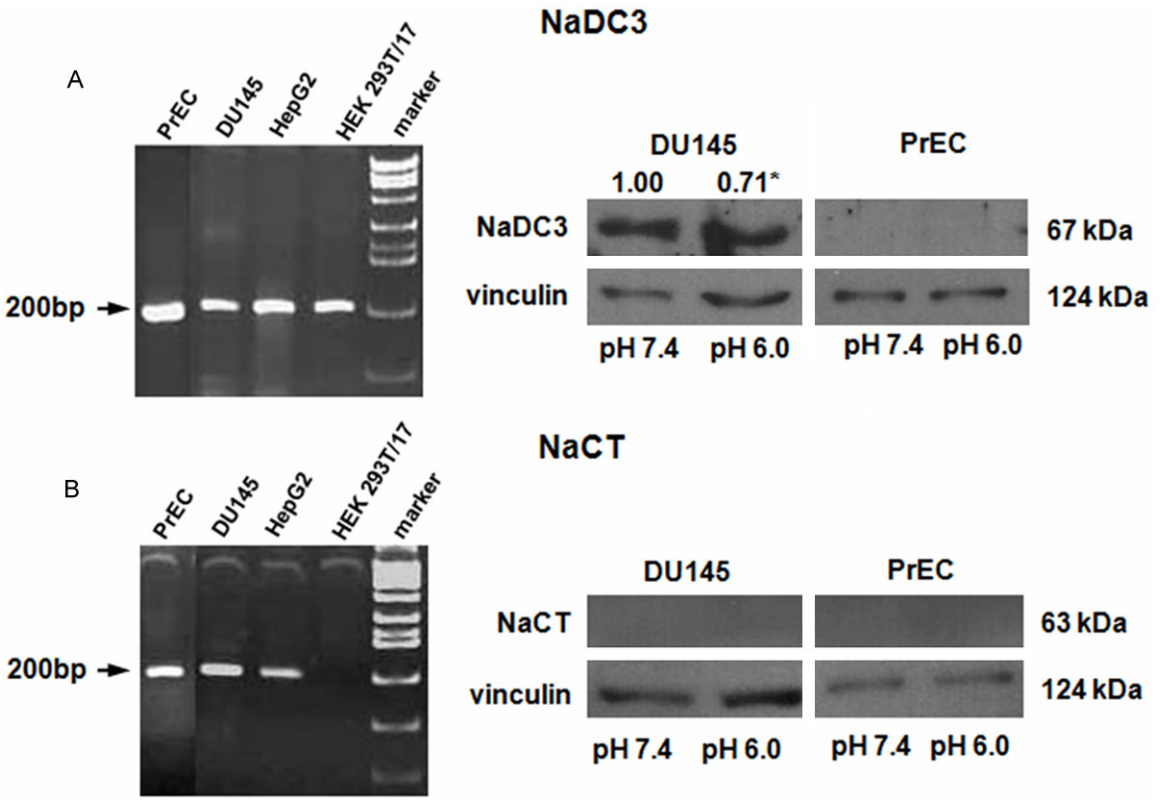

Figure 5. Analysis of dicarboxylic acid transporters transcripts and protein expression profiles in prostate cancer and normal cells. A. RT-PCR and Western blot tests of NaDC3 transporter. Images of polyacrylamide gel containing cDNA reverse-transcribes from DU145 and PrEC cell RNAs along with RNAs prepared from positive control HepG2 and HEK293T/17 cells demonstrate the presence of NaDC3 mRNA in both prostate normal and cancer cells. However, the transporter protein was expressed only in DU145 prostate cancer cells being by 29% down regulated in cells grown in acidic medium. Densitometry values of NaDC3 protein bands were normalized to a loading venculin control. To calculate a relative ratio the NaDC3 expression level at pH 7.4 was designated as 1, $*p < 0.033$. B. RT-PCR and Western blot tests of NaCT transporter. Our primers enabled detection of NaCT mRNA expression in both prostate normal and cancer cells. Western blot analysis revealed no expression of the known Na^+-dependent citrate transporter in either of investigated prostate cell lines.

ers, namely Na^+-dependent citrate transporter (NaCT), can also transport succinate to some extent [56]. We demonstrated the expression of NaCT mRNA in all studied cell lines, except HEK293T/17 kidney cells. However, further Western blot evaluation did not reveal the expression of Na^+-dependent citrate transporter in either of the examined prostate cells (**Figure 5B**).

Succinate uptake is a characteristic feature of cancer cells

In addition to DU145 prostate cancer and PrEC prostate normal cells we studied the succinate uptake in other cell lines, namely ovarian cancer cells (SKOV-3) and non-human and non-epithelial origin rat aortic endothelial cells (RAEC) (**Table 1**). Only epithelial DU145 prostate and SKOV-3 ovarian cancer cells revealed an increase in succinate uptake under acidic milieu. **Figure 6** presents comparative data of respiration of different cell lines in the presence of succinate in physiological and acidic pH as per cent values of FCCP-stimulated respiration. The per cent of FCCP-activated respiration of SCOV-3 ovarian cancer cells at pH 6.8 increased upon succinate addition to 158%, which was not observed at pH 7.4. Thus, ovarian cancer cells behave similarly to the prostate cancer cells being able to transport exogenous succinate under acidosis. Similar to prostate normal cells (104% at pH 6.8 and 101% at pH 7.4) succinate did not stimulate respiration of rat aortic endothelial cells (RAEC) in acidic buffer showing 97% versus 101% at pH 6.8 and 7.4, correspondingly (**Figure 6**).

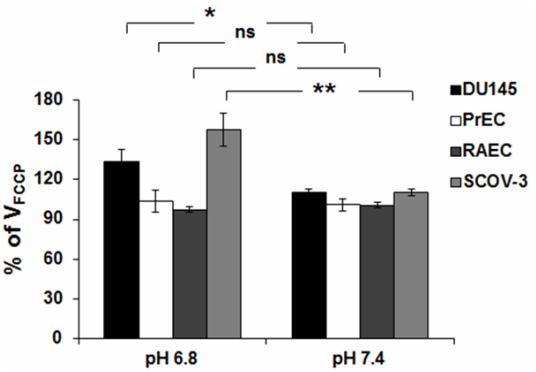

Figure 6. Succinate oxidation by different cell lines at pH 6.8 and 7.4. Incubation conditions as in the legend to **Figure 1**. Respiratory enzymes were activated with 40 nM FCCP and then stimulated with 10 mM succinate. The values of a per cent of FCCP-stimulated oxygen consumption rates presented as mean ± S.E.M. (n = 4-8). $*p < 0.038$, $**p < 0.006$.

Discussion

Since Otto Warburg first demonstrated the link between cancerogenic transformations and bioenergetic malfunctions, aerobic glycolysis has been considered as a near universal property of tumor cells. However, over the last decade, it was demonstrated that active glycolysis is tissue specific, and many tumors fuel themselves through accelerated oxidative phosphorylation by their mitochondria [20, 47, 57, 58]. While maintaining high glycolysis rates, cancer cells can also employ other energy pathways for both energy generation and for biosynthesis purposes [59] indicating on metabolic origin of cancer pathology [60]. Due to mitochondria genetic and functional plasticity, cancer cells are metabolically flexible, are less dependent on oxygen and have the ability to satisfy themselves with energy equivalents necessary for their apoptosis-resistant rapid growth [2]. Cancer cells are also capable of reorganizing their microenvironment through mitochondria reprogramming in order to support their insatiable appetites with energy-rich nutrients [61].

An often overlooked factor in the design of experimental models and conditions is accounting for cell processes in the context of cell-to-cell and cell-to-body environment cross talk. The impact of environmental perturbations, such as hypoxia, acidity, limitations in metabolites, ionic misbalance and others have to be taken in account despite their complexity. In this work we addressed the question of how cancer cells benefit from their acidic microenvironment and modulations in local calcium content for oncogenic signaling and what mechanisms they employ for their parasitic ability to consume energy reach metabolites from surrounding tissues. Studies of the functional activities of the tricarboxylic acid (TCA) cycle [62] and respiratory enzymes have changed the view on the role of metabolic alterations in cancer, and shown that cancer exhibits many of the characteristics of metabolic diseases [63]. There are a very small number of studies of plasma membrane dicarboxylate transporters of prostate and other types of cancer cells. We found that plasma membrane dicarboxylate transporter activity in prostate cancer cells is highly modulated by extracellular pH. The mechanism of succinate transport into prostate cancer cells in comparison to prostate normal cells in acidic tumorigenic microenvironment became the central focus of this study.

Succinate uptake by prostate cancer cells was studied in DU145 cells driven to the “starved” condition by stimulation with low concentrations of a protonophore (20-40 nM FCCP). Depletion of endogenous respiratory metabolites via FCCP-activated respiration enabled us to observe active oxidation of exogenous dicarboxylic acid succinate in the respiratory buffer at pH 6.8. This phenomenon was not observed in the respiration buffer with pH 7.4. The dicarboxylate transport is electrogenic and $succinate^{2-}$ in the protonated state is transported for only an inwardly directed Na^+ gradient [64]. Application of sulfhydryl agents mersalyl and NEM confirmed that in our experiments succinate uptake is promoted by Na^+-dependent dicarboxylate transporter (**Figure 4**) [25, 65]. Both inhibitors revealed similar blocking efficacy (**Figure 4C**). After exposure to inhibitors, no stimulation of respiration could be observed in the presence of succinate. Our data demonstrated that under acidosis the transport of dicarboxylates is well-suited to the uptake of TCA cycle metabolites by cancer cells from the blood and surrounding tissues.

Expression of high affinity NaDC3 transcripts was demonstrated in both prostate cancer and benign cells similar to the results reported by others for liver and kidney cells [54, 55] (**Figure 5A**). **Figure 5** also shows that mRNA information is only utilized by prostate cancer cells for

NaDC3 protein expression. Normal prostate epithelial cells do not produce the transporter proteins probably due to their physiological irrelevancy. The level of the transporter expression was shown to be controlled by its functionality. The DU145 cells cultivated at physiological pH 7.4 produced the higher level of the transporter as a compensatory adaptation for their functional quiescence. In contrast, the expression fold of the transporters which are capable of active succinate uptake was 29% lower in cells grown in acidic medium (**Figure 5A**). No transcripts for NaDC1 were detected for both prostate cancer and normal cells (data not shown). In contrast, the known NaCT transporter is presented as mRNA transcripts, but we could not detect the corresponding transporter proteins in prostate cancer or prostate normal cells (**Figure 5B**). Possibly, prostate cancer cells gain the citrate transporter isoform different from the known plasma membrane transporter as it was suggested by Mycielska's group [42]. Based on our data, we hypothesize that due to malignant transformations, cancer cells acquired NaDC3 transporters which become activated upon acidification of the tumorigenic microenvironment.

The data presented in **Figure 6** demonstrates that, similar to the prostate cancer cells, the ovarian cancer cells (SKOV-3) also uptake succinate in acidic conditions. This supports our hypothesis that an elevated uptake of succinate promoted by the acidic microenvironment is a characteristic of cancer cells, which acquire ability to transport of the metabolite via the mechanism of plasma membrane Na^+-dependent dicarboxylate transporters.

In addition, the reduced level of calcium ions in tumor microenvironment could support activity of dicarboxylic acid transporters by unknown yet mechanism. In our experiments, the use of the buffer with near physiological calcium content (1-2 mM) resulted in slight reduction of succinate transport, while the $CaCl_2$ concentrations of 0.5 mM and lower were supportive for the active succinate uptake. This fact is in agreement with earlier proposed local calcium deficiency in tumor surroundings which could confer lowering cell-to-cell adhesiveness and promote tumor metastasis [12, 13].

Energy substrate fluxes regulate mitochondrial workload and their anti-apoptotic potential [47]. Along with that the TCA metabolites contribute to other malignant transformation processes [66]. It is known that succinate and fumarate stabilize hypoxia-inducible transcription factor-1α (HIF-1α)-related oncogenic signaling pathway, which is essential for tumor survival mechanisms at both hypoxic and anoxic conditions [67, 68]. Citrate is also an important hypoxic cell growth metabolite, as well as an enhancer of the mitochondrial cholesterol content, which via membrane stabilization decreases cancer cells death susceptibility [69].

Cancer cells modify their energy supply with energy equivalents and benefit from different energetic pathways, namely glycolysis, oxidative phosphorylation, β-oxidation, glutaminolysis and pentose phosphate pathway by substrates switching upon their availability. Access to alternative energy resources provides cancer cells flexibility in maintaining constant metabolic activity at any stress condition. In our previous work we demonstrated that prostate cancer mitochondria are metabolically highly active, obtaining 2 to 7 fold higher respiratory activities of NADH and succinate dehydrogenases [47], along with citrate synthase [70]. At the same time, we revealed that the affinity of respiratory complex I to its substrate NADH is lower than in normal prostate mitochondria [47]. We consider this change in enzymatic activity not as cancer mitochondria complex I defect, but rather an adaptive transformation of mitochondria respiratory system towards intensification of lactate production which requires an excess of NADH. Lactate efflux co-transported with protons acidifies the tumor microenvironment [5]. It is an important finding that prostate cancer cells in acidic ambient are capable of consuming TCA cycle intermediates, including succinate and citrate, which are available in extracellular fluids to support their elevated OxPhox. The data of this work demonstrates that succinate membrane transport and intracellular compartmentalization is a mechanism that is placed in service to support cell metabolic demands. **Figure 7** summarizes the proposed cross talk between glycolytic and oxidative fluxes in prostate normal and cancer cells emphasizing the role of NaDC3 transporter in uptake of extracellular succinate.

Other than acidity and modulations in calcium content, hyperkalemia is one of the factors per-

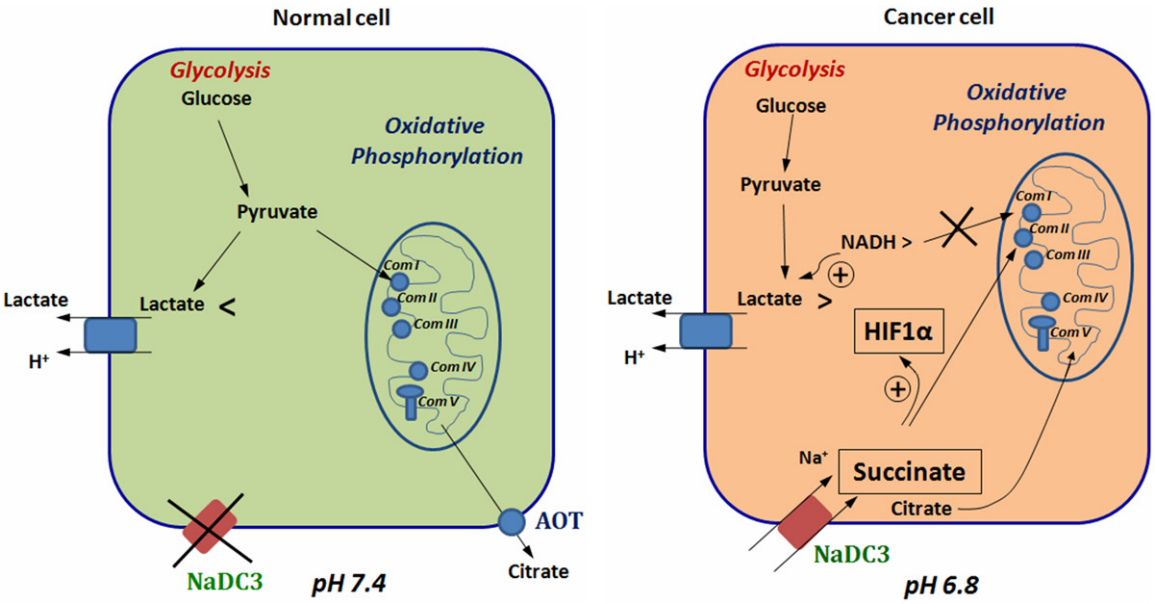

Figure 7. The schematics of distinct cross-talk mechanisms between two major energy generating pathways in prostate normal versus cancer cells. *Abbreviations:* Com I, II, II, IV, V are respiratory system enzymes and ATP synthase, AOT-plasma membrane organic anion transporter, NaDC3-plasma membrane sodium-dependent dicarboxylic acid transporter, HIF1α-hypoxia inducing factor 1α.

tinent to the tumorigenic microenvironment, which is shown to promote cancer cells invasiveness [11]. Although we did not detect any noticeable effects of K^+ ions on succinate transport and oxidation by mean of oxygen consumption changes in the KCl-based buffer (data not shown), we don't exclude the possibility of association of succinate transport with outward of K^+ ions. Earlier it has been shown that K^+ accelerates Na^+-dependent movement of citrate [71]. Our test of the potential involvement of Na^+/K^+-ATPase in mediation of succinate uptake using specific inhibitor demonstrated that 2.5 or 10 μM glybenclamide does not exert significant inhibitory effect on succinate-stimulated respiration of DU145 cells (data not shown). However, this could be also due to inhibition of Na^+/K^+-ATPase by acidic milieu.

In conclusion, the data presented in this work demonstrate that NaDC3 expression by prostate cells correlates with malignant transformation rather than being a universal mechanism gained by alterations in pH of the cellular microenvironment. We demonstrated a succinate influx via NaDC3 in prostate cancer cells. It has potential to be used for molecular imaging-based diagnostics for non-glycolytic tumors in an acidic microenvironment similar to glucose-utilization based PET. The possibility of employment of hyperpolarized succinate for imaging applications was demonstrated [72]. The use of labeled succinate could yield technology for determining the body succinate fluxes for prognosis of disease stages and chemotherapy responses. In addition, the dicarboxylate transporter-mediated succinate uptake could yield a novel prognostic biomarker and target for anticancer therapy with no effect on normal prostate cells.

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Disclosure of conflict of interest

Authors declare no conflict of interests.

Abbreviations

OxPhos, oxidative phosphorylation; TCA, tricarboxylic acid cycle; NaDC1, Na^+-dependent dicarboxylic acid transporter isoform 1; NaDC3, Na^+-dependent dicarboxylic acid transporter isoform 3; NaCT, Na^+-dependent citrate transporter; FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; NEM, N-ethylmaleimide; HIF1α, hypoxia-inducible transcription factor 1α.

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