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Metabolic control analysis of cellular respiration in situ in intraoperational samples of human breast cancer

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Abstract The aim of this study was to analyze quantitatively cellular respiration in intraoperational tissue samples taken from human breast cancer (BC) patients. We used oxygraphy and the permeabilized cell techniques in combination with Metabolic Control Analysis (MCA) to measure a corresponding flux control coefficient (FCC). The activity of all components of ATP synthasome, and respiratory chain complexes was found to be significantly increased in human BC cells in situ as compared to the adjacent control tissue. FCC(s) were determined upon direct activation of respiration with exogenously-added ADP and by titrating the complexes with their specific inhibitors to stepwise decrease their activity. MCA showed very high sensitivity of all complexes and carriers studied in human BC cells to inhibition as compared to mitochondria in normal oxidative tissues. The sum of FCC (s) for all ATP synthasome and respiratory chain components was found to be around 4, and the value exceeded significantly that for normal tissue (close to 1). In BC cells, the key sites of the regulation of respiration are Complex IV (FCC=0.74), ATP synthase (FCC=0.61), and phosphate carrier (FCC= 0.60); these FCC(s) exceed considerably (~10-fold) those for normal oxidative tissues. In human BC cells, the outer mitochondrial membrane is characterized by an increased permeability towards adenine nucleotides, the mean value of the apparent K_m for ADP being equal to 114.8±13.6 μM. Our data support the two-compartment hypothesis of tumor metabolism, the high sum of FCC(s) showing structural and functional organization of mitochondrial respiratory chain and ATP synthasome as supercomplexes in human BC.

Keywords Cancer · Cardiomyocytes · Regulation of respiration · Mitochondria · Two-compartment metabolism · Metabolic control analysis

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Abbreviations

ANT	Adenine nucleotide translocase
BC	Breast cancer
BSA	Bovine serum albumin
CAT	Carboxyatractyloside
CK	Creatine kinase
CM	Cardiomyocyte

COX	Cytochrome c oxidase
C_{vi}^{J} or FCC	Flux control coefficient
Cr	Creatine
PEP	Phosphoenolpyruvate
PK	Pyruvate kinase
PET	Positron emission tomography
PCr	Phosphocreatine
DTT	Dithiothreitol
GLUT	Glucose transporter
HK	Hexokinase
Km	Michaelis Menten constant
MES	2-morpholinoethanesulfonic acid
MCA	Metabolic Control Analysis
MOM	Mitochondrial outer membrane
MtCK	Mitochondrial creatine kinase
3-NP	3-nitropropionic acid
N2a	Neuro-2a
O_2	Oxygen
OXPHOS	Oxidative phosphorylation
Pi	Inorganic phosphate
PIC	Inorganic phosphate carrier
PET	Positron emission tomography
TKTL1	Transketolase like isoenzyme 1
TMPD	N,N,N′,N′-tetramethyl-p-phenylenediamine
VDAC	Voltage dependent anion channel

Introduction

In spite of significant advances in understanding the pathogenesis and biology of oncological diseases, some molecular subtypes (basal-like or "triple-negative") of breast cancer (BC, the most common cancer in women) are highly resistant to systemic treatment and BC is still one of the most prevalent causes of women death from cancer worldwide (Ferlay et al. 2007; Jemal et al. 2009). Relatively low efficiency of current chemotherapy in the treatment of patients with BC is largely related to poor understanding of mechanisms (at the cellular and molecular levels) of the neoplasm dissemination as well as the processes involved in maintenance of the cancer cell energy homeostasis.

Interest in the energy metabolism of cancer cells appeared as early as in the 1920s, when Otto Warburg discovered that cancer cells have a high glycolytic rate and they produce increased amount of lactate even in the presence of oxygen, a phenomenon termed subsequently "aerobic glycolysis" or "Warburg effect". Otto Warburg proposed that the cause for cancer is linked to irreversible defects in mitochondrial oxidative phosphorylation (OXPHOS) that is associated with increased glycolytic rates of malignant cells (Warburg et al. 1924; Warburg 1956a, b). Then, it has been demonstrated in multiple investigations that the Warburg hypothesis can be applied to many cancer cell types, because one of the most infamous and well-known alterations in tumor cells is certainly an elevated glycolytic capacity, even in the presence of high O_2 concentrations (Atsumi et al. 2002; Marin-Hernandez et al. 2006; Pedersen 1978, 2008; Xu et al. 2005). Although it turned out that mitochondrial dysfunction is not the fundamental cause of cancer, aerobic glycolysis could provide a growth advantage to tumor cells (Gatenby and Gillies 2004). It enables cancer cells to adapt to hypoxic conditions as the premalignant lesion grows progressively further from the blood supply. Also, the glycolytic phenotype contributes to the acidification of tumor microenvironment, which can facilitate tumor invasion (Gatenby and Gawlinski 1996; Schornack and Gillies 2003). The observation that malignant tumors have a higher glycolytic capacity than normal cells led to the use of this phenomenon in positron emission tomography (PET) for diagnosis, monitoring, and treatment of cancer with ^{18}fluorodeoxyglucose.

Some clinical studies suggest that human BC displays a glycolytic Warburg's phenotype (Safa et al. 2010; Schmidt et al. 2010) with over-expression of glycolytic genes (Altenberg and Greulich 2004). Enhanced aerobic glycolysis was also specifically identified in human BC cells in vitro, and in vivo by means of magnetic resonance imaging (Neeman and Degani 1989; Rivenzon-Segal et al. 2003; Zhao et al. 2011) and PET (Czernin and Phelps 2002), but until now the exact molecular causes of the phenomenon remain still unclear. Currently, TKTL1 up-regulation is considered as an important factor contributing to the Warburg effect (Coy et al. 2005), and this enzyme could be a relevant target in treatment of BC (Boros et al. 1997).

Pedersen and colleagues have proposed a mechanism for explanation of the Warburg effect: they suggested that voltage dependent anionic channel (VDAC) located within the outer mitochondrial membrane and its binding partner hexokinase-2 (HK-2) are pivotal players in the "Warburg effect" in cancer (Pedersen 2007a, b, 2008). The binding of HK-2 to mitochondria strongly (almost 5-fold) increases its affinity for ATP (Bustamante and Pedersen 1980). Also, mitochondrial binding of HK-2 prevents its inhibition by the product glucose-6-phosphate (Nakashima et al. 1988), and provides it with preferred access to the substrate ATP needed to convert glucose to glucose-6-phosphate and therefore to "jump start" the glycolytic pathway and produce the Warburg effect: high glycolysis in the presence of O_2.

Our recent studies as well as some data from other laboratories strongly suggest that cytoskeletal protein, tubulin, is deeply involved in the regulation of mitochondrial respiration and the synthesis of high-energy phosphates, and that peculiarity in expression of some tubulin isotypes in cancer cells may presumably mediate their Warburg phenotype (Guzun et al. 2011; Rostovtseva and Bezrukov 2008; Rostovtseva et al. 2008). So, Guzun and coauthors (2011) have shown that the levels, localization and functional role

of β-tubulin isotypes in oxidative muscle tissues differ from those in cancer type non-beating HL-1 cells; namely, it was found that in adult normal cardiomyocytes β-tubulin class II is associated with mitochondria, and that this tubulin isotype is absent in HL-1 tumor cells having a glycolytic phenotype (Monge et al. 2009). Probably, the absence of βII-tubulin in cancer cells allows binding of HK-2 to VDAC mediating thereby the appearance of Warburg effect. It was presumed that HK-2 directly binds to mitochondria through VDAC and, according to the model proposed by Majewski and coauthors (Majewski et al. 2004), supports the VDAC open state, as a consequence, HK-2 consumes practically all ATP produced via OXPHOS mediating thereby increased rates of glycolysis even in the presence of O_2. This suggests that increased rates of aerobic glycolysis in cancer cells may take place without any disturbances in the functional capacity of their mitochondria. The described mechanism of the Warburg effect, linked with competition between βII-tubulin and HK-2 for binding to VDAC, may presumably occur in the case of BC; in fact, Hiser et al. (2006) have examined the content of various β-tubulin isotypes in several human BC cell lines having a Warburg phenotype, and have found only very low levels of βII-tubulin in such cells.

Today, however, there is only very limited information as to the bioenergetic function of mitochondria in human BC in situ. In addition to this, it has been shown in recent years in several important works that while all tumor cell types are characterized by enhanced glycolytic flux, not all have a diminished mitochondrial respiration: certain types of cancer cells show increased respiration and up-regulation of OXPHOS components (Jose et al. 2011; Moreno-Sanchez et al. 2007). Thus, the information presented above indicates that additional insights into the mechanisms regulating BC cells energy metabolism are needed, and they may be very helpful for further advancing our treatment regimens.

To study the important problem of the control of energy metabolism in cancer cells, Moreno-Sanchez and Westerhoff's groups have applied the Metabolic Control Analysis (MCA) with the conclusion that the role of OXPHOS in tumor cells should be re-evaluated and experimentally determined for each particular type of tumor cell (Moreno-Sanchez et al. 2007, 2010). The MCA (Gellerich et al. 1990) was developed in the period of 1970–80's by Kacser and Burns (1973) as well as by Heinrich and Rapoport (Heinrich 1985; Rapoport et al. 1974). Theoretical aspects of MCA have been subsequently analyzed by many researchers, e.g., (Kholodenko et al. 1993; Westerhoff et al. 2009a, b). MCA helps to understand the mechanisms by which a given enzyme exerts high or low control of metabolic flux and how the control of the pathway is shared by several pathway enzymes and transporters. By applying MCA it is possible to identify the steps that could be modified to achieve a successful alteration of flux or metabolite concentration in pathways. MCA has been very intensively used for analysis of the control of respiration in isolated mitochondria (Moreno-Sanchez et al. 1991; Rossignol et al. 2000). Application of MCA in our previous studies has revealed that in adult normal cardiac cells the reactions involving ADP recycling within ATP synthasome and respiratory chain complexes with activated mitochondrial creatine kinase (MtCK) are the "key points" in regulation of mitochondrial respiration and energy fluxes (Tepp et al. 2011a, b).

In the present work we applied MCA for the detection of possible OXPHOS defects in human BC cells. To determine the flux control coefficients, the flux was measured as the rate of O_2 consumption in permeabilized (by saponin pretreatment) human BC samples when all the ATP synthasome and mitochondrial respiratory chain complexes were titrated with their specific inhibitors to stepwise decrease their activity. For a better understanding, the regulation of energy metabolism in BC cells, comparative experiments were performed on murine neuroblastoma cells of the line Neuro-2a (N2a) as well as on normal tissues with well-known bioenergetics - cardiac myocytes. N2a cells are of malignant phenotype and are characterized by accelerated aerobic glycolysis (Ghosh et al. 2010; Mazzio et al. 2010).

Materials and methods

Clinical materials and patients

Immediately after surgery, anonymous paired tumor and normal breast tissue samples (0.1–0.5 g, n=35) were placed in sterile plastic vials containing pre-cooled Mitomed B solution (Kuznetsov et al. 2008) containing: 0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionate, 3 mM KH_2PO_4, 20 mM taurine, 20 mM HEPES (pH 7.1), 110 mM sucrose, 0.5 mM dithiothreitol (DTT) and 5 mg/ml fatty acid free bovine serum albumin (BSA). The tissue samples, provided by the Oncology and Hematology Clinic at the North Estonia Medical Centre (Tallinn), were obtained under general intra-tracheal anesthesia and were then within 1 h after surgery delivered to the Laboratory of Bioenergetics for corresponding studies; during transportation they were stored on ice. Pathology reports were provided by the North Estonia Medical Centre for each tissue sample. Only primary tumor samples were examined. Informed consent was obtained from all human subjects and coded identity protection was applied. All investigations were approved by the Tallinn Medical Research Ethics Committee (National Institute for Health Development) and were in accordance with Helsinki Declaration and Convention of the Council of Europe on Human Rights and Biomedicine.

All women (with ages ranging from 50 to 71 years) had local or locally advanced disease (T1-4N0-2M0, st. IA-IIIB).

Invasive ductal carcinoma accounted for 87 % and lobular carcinoma for 13 % of these tumors. From 33 patients examined 47 % were estrogen- and 33 % progesterone-positive. HER2 protein over-expression was determined by IHC and gene amplification analysis with FISH. From 33 patients nine were 2+ positive in IHC test, but gene amplification was not found by FISH. Percentage of tumor proliferation marker Ki-67 expression varied from 3 to 90 %.

Normal tissue was taken from the same breast at a site distant (by 2 cm) from the tumor and evaluated for the presence of tumor cells. The adjacent normal tissue consisted of mixed fibrous and adipose tissue. The patients in the study had not received prior radiation or chemotherapy.

Isolation of rat cardiomyocytes, cultivation of N2a cells and permeabilization of the cells

For comparison of the data obtained on tumor samples with those on normal oxidative tissue, we used the adult cardiomyocytes as standard oxidative cells for which the respiratory parameters are very well known, including the flux control coefficients measured by using the MCA (Tepp et al. 2010, 2011a). Male Wistar-line rats weighing about 350 g were used in the experiments. The animals were housed at constant temperature (22 °C) in environmental facilities with a 12:12 h light–dark cycle and have received standard laboratory chow ad libitum. Animal procedures were approved by the Estonian National Committee for Ethics in Animal Experimentation (Estonian Ministry of Agriculture). All used chemicals and enzymes were of analytical grade or the highest purity available and were purchased from Sigma-Aldrich, Fluka and Roche.

Adult cardiomyocytes (CM) were isolated after perfusion of the rat heart with collagenase A (Roche) as described in our previous studies (Saks et al. 1991; Tepp et al. 2010). In order to examine the regulation of mitochondrial respiration in CM(s), the cells sarcolemma was permeabilized by saponin treatment. This permeabilization procedure was carried out at 25 °C with 20 μg/ml saponin for 10 min, and the saponin treated CM were then washed several times in a Ca^{2+} free medium; in detail see in (Tepp et al. 2010). It is important to note that the used parameters of saponin treatment of CM(s) keep the intactness of their mitochondrial membranes (Kuznetsov et al. 2008; Saks et al. 1998).

Stock culture of N2a cells was obtained from American Type Culture Collection, Cat. No. CCL-131. These murine neuroblastoma cells were grown as a loosely adhering monolayer on Petri dishes (Greiner Bio-One, S=58 cm^2) in high glucose DMEM with L-glutamine that was supplemented with 10 % heat inactivated fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). N2a cells were grown and maintained at 37ºC in a humidified incubator containing 5 % CO_2 in air. The cells were subcultured at 2 or 3 day intervals and had received no more than 20 cell culture passages. Prior to respiratory experiments, N2a cells were permeabilized by saponin (40 μg/ml) pretreatment for 5 min at 25 °C.

Preparation of skinned tumor fibers

Skinned fibers from human breast tissue samples were prepared according to the method described earlier (Kuznetsov et al. 1996, 2008). In order to study the regulation of mitochondrial respiration in health and tumor tissues, the sarcolemma and plasma membranes in tumor fibers was completely permeabilized by saponin treatment keeping the mitochondrial membranes intact (Kuznetsov et al. 2008; Saks et al. 1998). For this purpose, the fibers obtained from human breast tissues were transferred into plastic vials with pre-cooled (on ice) medium-A (3 mM KH_2PO_4, 0.5 mM DTT, 20 mM taurine, 5.3 mM ATP, 15 mM phosphocreatine, 9.5 mM $MgCl_2$, 1 mM K_2EGTA and 82.9 mM 2-morpholinoethanesulfonic acid (MES), pH 7.1) supplemented with 50 μg/ml saponin and incubated with mild stirring (at 40 rpm) for 30 min at 4 °C. Permeabilized (skinned) fibers were then washed in pre-cooled solution containing: 20 mM imidazole, 3 mM KH_2PO_4, 0.5 mM DTT, 20 mM taurine, 4 mM $MgCl_2$, 100 mM MES, 2.74 mM K_2Ca-EGTA, 4.72 mM K_2-EGTA, and 5 mg/ml fatty acids free BSA (medium-B, pH 7.1) for 5 min under mild stirring; this washing procedure was performed three times to completely remove saponin and all metabolites, especially trace amounts of ADP. The washed samples were placed into medium-B and kept in the same medium at 4 °C until use.

Oxygraphic measurements

The rates of oxygen consumption by cell and tissue preparations were determined with a high-resolution respirometer (Oxygraph-2K, from OROBOROS Instruments, Austria) in medium-B supplemented with 5 mM glutamate, 2 mM malate and 10 mM succinate as respiratory substrates. In MCA experiments, for the complex-II we used 10 mM succinate and for complex-I 5 mM glutamate together with 2 mM malate. Protein concentrations in cell and tissue extracts were routinely determined using the Pierce BCA Protein Kit as suggested by the manufacturer.

Determination of total hexokinase (HK) activity

HK activity was measured as the total glucose phosphorylating capacity of whole tissue extracts, using a standard glucose-6-phosphate dehydrogenase-coupled spectrophotometric assay (Robey et al. 2000). One milliunit (mU) of HK activity was calculated as the amount of enzyme activity required to phosphorylate 1 nmol of glucose in 1 min at 25 °C.

Metabolic control analysis (MCA). Determination of flux control coefficients

MCA is a method of study of regulatory mechanisms in complex metabolic systems. Flux control coefficient (FCC) is defined as the ratio of the fractional change in the system variable to the fractional change in the biochemical activity that caused the system change (Fell 1997). If a small change in an enzyme activity promotes a significant variation in pathway flux, then this enzyme exerts an elevated control over the regulation of pathway. In contrast, if a rather small or negligible change in flux is observed when a complexes activity is greatly varied, the enzyme has low flux control coefficient and decreased ability to influence on overall flux (Fell 1997; Tepp et al. 2010).

The FCC or C^J_{vi} is defined according to the equation (Fell 1997; Moreno-Sanchez et al. 2008):

$$C^J_{vi} = \left(\frac{dJ}{dv_i}\right) \Big/ \left(\frac{J}{v_i}\right) = \frac{d\ln J}{d\ln v_i}$$

in which the expression dJ/dv_i describes the variation in flux (J) when an infinitesimal change takes place in the enzyme i concentration or activity (v_i). Groen et al. (Groen et al. 1982) have derived a method to determine experimentally the C^J_{vi} using titration with specific enzyme inhibitors. The value of the C^J_{vi} coefficient is given by Groen et al. (1982) and Moreno-Sanchez et al. (2008):

$$C^J_E = (\Delta J/\Delta I)^*(I_{\max}/J_0)$$

where $(\Delta J/\Delta I)$ is the initial slope of the flux/inhibition graph, $I_{\max}$ is the inhibitor concentration giving complete inhibition, and J_0 is the initial steady-state flux value.

The FCC(s) for permeabilized CM(s) and saponin-skinned human BC samples were determined for all ATP synthasome and respiratory chain complexes with direct activation of respiration by ADP. C^J_{vi} values were calculated by using of graphical method described by Fell (1997), which was used in most of previous studies on isolated mitochondria or skinned fibers (Tepp et al. 2010, 2011a). This is the main method, chosen by us for determination of corresponding FCC(s).

Additionally, obtained results were compared with the computer estimated FCC(s). In this case non-linear regression analysis was used by fitting experimental data to the mathematical model, developed by Gellerich et al. (1990). The fitting was performed with the use of the MathCad Professional 2001 (MathSoft, PTC) by providing best-fit values of three parameters: K_d (dissociation constant of the enzyme-inhibitor complex), E_0 (concentration of inhibitor binding sites) and C_0 ($C_0 = (\mathrm{dlnJ/dlnE})_{E=E0}$ in the absence of the inhibitor). The following inhibitors were used to estimate the functional activity of respiratory complexes in the above mentioned biological samples: rotenone for Complex I of the respiratory chain, 3-nitropropionic acid (3-NP) for Complex II, antimycin for Complex III, sodium cyanide for Complex IV, oligomycin for complex V (ATP synthase), carboxyatractyloside (CAT) for adenine nucleotide translocase, and mersalyl for inorganic phosphate carrier, PIC. Any of the used inhibitors is considered as irreversible and non-competitive in these conditions. The inhibited flux Ji was negligible at high concentrations of all inhibitors used and was close to zero. Only small rotenone and oligomycin insensitive respiration took place.

Confocal imaging of mitochondria in human breast tissues

In order to assess the content of mitochondria in skinned human BC fibers, the prepared tissue samples were stained with mitochondrial probe - MitoTracker Red CMXRos dye (100 nM, Invitrogen) diluted in medium B for 30 min at 37ºC. After loading and washing with PBS fibers were mounted in ProLong Gold antifade reagent with DAPI between microscope slides. Cells were then imaged by a Olympus FV10i-W inverted laser scanning confocal microscope equipped with 60× water objective, using 560 nm laser excitation for MitoTracker Red.

Another way for qualitative determination the content of mitochondria in skinned human BC fibers is labeling the mitochondria with VDAC antibody. Prepared tissue samples were fixed in 4 % paraformaldehyde in PBS at 37ºC for 15 min and washed with PBS. Subsequently, fibers were incubated in Antigen Retrieval Buffer (100 mM Tris, 5 % (w/v) urea, pH 9.5) at 95ºC for 10 min, washed by PBS, permeabilized with 0.1 % Triton X-100 in PBS for 15 min, washed again in PBS and blocked in 2 % BSA dissolved in PBS for 1 h at room temperature. Fibers were then incubated overnight with primary VDAC antibody kindly provided by Dr. Catherine Brenner (Paris-Sud University, Paris, France), washed with 2 % BSA solution prepared on PBS (three times for 5 min) and incubated with fluorescent DyLight488-conjugated secondary antibody (Abcam) 2 h at room temperature. After 3-fold washing with 2 % BSA, cells were mounted with Prolong Gold Antifade reagent (Invitrogen) between coverslips and examined by a confocal microscope (Olympus Fluoveiw FV10i-W).

Data analysis

Data in the text, tables and figures are presented as mean ± standard error (SEM). Some results were analyzed by the Student's t-test. Values of P less than 0.05 were considered statistically significant. To reduce the possibility of random errors, our experiments were repeated 7–20 times and the fitting technique was used to calculate the C^J_{vi} value.

Results

Mitochondrial respiration in human breast tumors, and testing the influence of saponization on the integrity of mitochondrial membranes in BC fibers

It is well-known that malignant transformation of normal cells may be associated with mitochondrial loss and this event could mediate the appearance of a Warburg phenotype (Kulawiec et al. 2009; Seppet et al. 2006; Tseng et al. 2006). The presence of mitochondria can be easily revealed by confocal microscopic imaging (Kuznetsov et al. 2006), and because of this, prior to respiratory experiments, we, first of all, estimated by this method the content of mitochondria in ductal human breast carcinomas. Confocal microscopy with MitoTracker Red® showed that human BC tissues contain, contrary to our expectations, an increased number of mitochondria (Fig. 1a) in comparison with adjacent normal tissue (Fig. 1b), in both cases mitochondria are predominantly localized around the cell nucleus (blue fluorescence) (Fig. 1). The same results were obtained by labeling the mitochondrially localized VDAC(s) with specific fluorescent antibodies against VDAC (Fig. 1c and d). These observations suggested that malignant cells of such a histological type could be characterized by detectable rates of mitochondrial respiration.

Therefore, we performed a comparative study to characterize the respiratory activities of mitochondria in human BC cells and adjacent normal tissue in situ using the permeabilized cell technique, which allows to study the mitochondrial function under conditions close to physiological ones (Kuznetsov et al. 2008). Figure 2 shows the example of the use of this method, demonstrating the recording of respiration of permeabilized tissue samples prepared from human BC. The respiration is activated by addition of ADP with respiratory control index close to 3. It is not activated further by addition of exogenous cytochrome c, showing thereby the intactness of the mitochondrial outer membrane, and is inhibited by carboxyatractyloside to the initial level, showing that the respiration is well controlled by adenine nucleotide translocator (ANT) in the intact inner mitochondrial membrane.

Activities of respiratory chain complexes in malignant human breast tumors

Table 1 shows the values of the respiration rates for different cells and tissues, including BC. It was surprisingly found that the mean value (0.363 ± 0.04 nmol O_2/min/mg dry weight) of basal respiration (V_0) in skinned preparations (fibers) of human BC exceeded substantially (more than ten times) that in nontumorous tissues and was close to the value observed for rat gastrocnemius muscle fibers (see Table 1 and Fig. 3). The addition of succinate (up to a final concentration of 10 mM) caused further and a considerable (~1.5–2 fold) increase in the V_0 value (was measured in the presence of 2 mM malate and 5 mM glutamate), both in cancerous and normal breast tissues, suggesting that Complex II of the respiratory chain is functionally active in BC

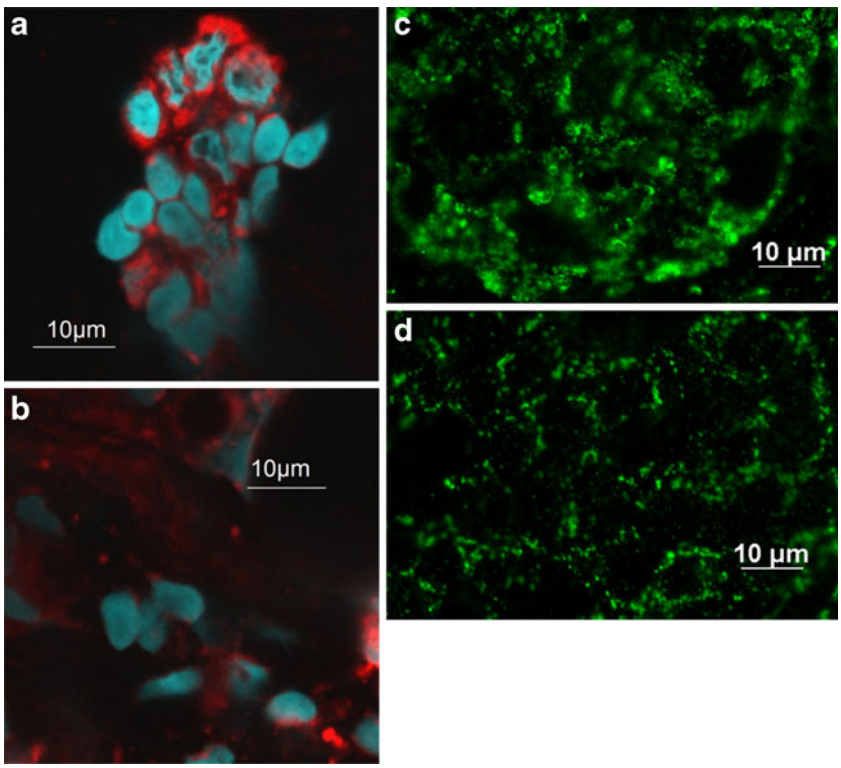

Fig. 1 Confocal microscopy images of mitochondria in breast cancer tissue (**a, c**) and human normal adjacent tissue (**b, d**). Mitochondria were visualized by using of MitoTracker Red CMXRos dye with DAPI (**a**, **b**) or VDAC antibody (**c**, **d**)

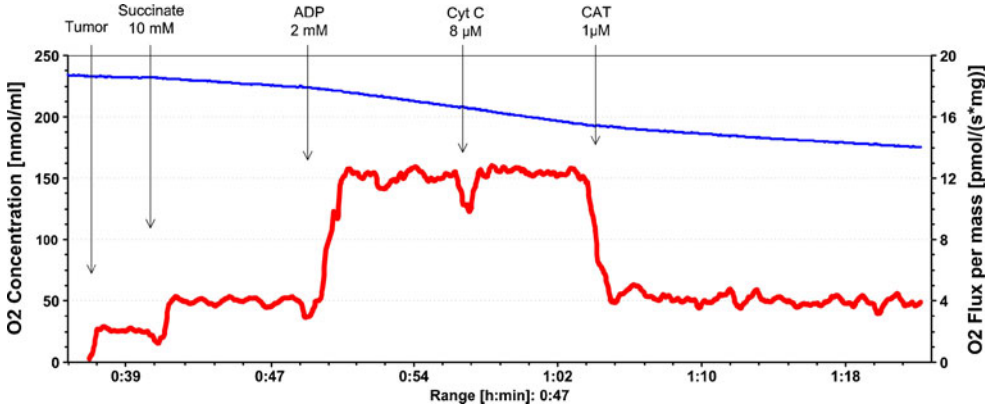

Fig. 2 Quality test of intactness of mitochondrial membranes in permeabilized human breast cancer fibers. This experiment was performed in medium-B with 5 mM glutamate, 2 mM malate, and 10 mM succinate as respiratory substrates. Respiration was activated with 2 mM ADP; addition of cytochrome c (Cyt C, up to a final concentration of 8 μM) did not cause any marked increase in the rate of oxygen consumption, indicating the intactness of the outer mitochondrial membrane. Addition of carboxyatractyloside (CAT, up to a final concentration of 1 μM) decreased the respiration rate back to the V_0 level showing the intactness of the mitochondrial inner membrane

cells. The finding was confirmed by the results of analysis of the activities of mitochondrial respiratory chain segments in human BC tissues shown in Fig. 3.

Figure 3 and Table 1 show that mitochondrial respiration in permeabilized (by saponin pre-treatment) BC preparations substantially increased over the basal levels (in the presence of malate and glutamate) after addition of 2 mM MgADP, and the ADP simulated respiration was inhibited by rotenone; this indicates that Complex I is active in cancerous tissues. The addition of 10 mM succinate abrogated the inhibitory effect of rotenone, suggesting that in BC cells the mitochondrial Complex II may be more effective in comparison with Complex I. The ADP stimulated respiration was suppressed by addition of 10 μM antimycin-A (in mitochondria, it inhibits the electron flow from complex III to cytochrome c); the finding indicates that the mitochondrial respiratory chain Complex III is also functionally active in human BC cells.

To activate cytochrome c oxidase, COX, 1 mM N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD) jointly with 5 mM

Table 1 The values of basal respiration rate (V_o), maximal rate of respiration (V_{max}; in the presence of 2 mM ADP), and apparent K_m values for ADP in permeabilized breast cancer samples, tumor cells of another histological type, and some health tissues; these measurements were performed in medium-B with 2 mM malate and 5 mM glutamate as respiratory substrates

Tissue	V_o	$K_m^{app}{}_{ADP}$, μM	V_{max}	Origin
Rat heart fibers[a]	6.45±0.19	297±35	28.7±1.1	(Kuznetsov et al. 1996; Monge et al. 2009)
Rat cardiomyocytes[b]	9.3±1	360±51	134±6	(Anmann et al. 2006)
Rat soleus[a]	2.19±0.30	354±46	12.2±0.5	(Kuznetsov et al. 1996; Monge et al. 2009)
Rat gastrocnemius white[a]	1.23±0.13	14.4±2.6	7.0±0.5; 4.10±0.25	(Kuznetsov et al. 1996; Monge et al. 2009)
HL-1-NB[b]	7.64±1.25	25±4	21.3±2.4	(Anmann et al. 2006; Eimre et al. 2008)
HL-1-B[b]	6.8±0.80	47±15	18.2±3.2	(Anmann et al. 2006; Eimre et al. 2008)
Neuro-2a[b]	1.09±0.28 1.53±0.374[c]	28.9±7.4	3.43±0.49	our data
Control breast tissue	0.02±0.01 0.10±0.02[c]	–	0.02±0.01 0.10±0.02[c]	our data
Breast cancer[a]	0.33±0.03 (range 0.04–0.96) 0.56±0.04[c]	114.8±13.6 (range, 10.5±6.3 to 304±51)	1.09±0.04 (range, 0.10 ±0.01 to 2.02±0.15)	our data

[a] rate of respiration - nmol O_2/min/mg dry weight

[b] rate of respiration was expressed as nmol O_2/min/mg protein

[c] V_o in the presence of 5 mM glutamate, 2 mM malate and 10 mM succinate

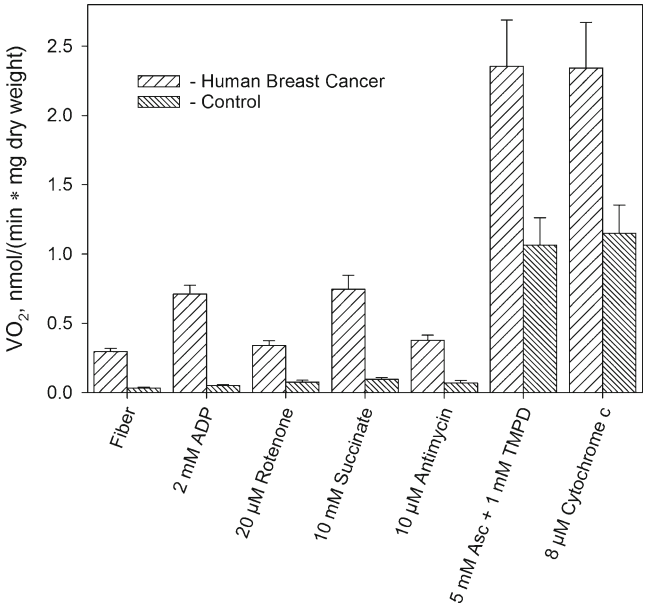

Fig. 3 Assessment of the respiratory chain functions in permeabilized human breast cancer fibers and normal adjacent tissues. The experiment was performed in medium-B with 5 mM glutamate and 2 mM malate as respiratory substrates. TMPD - N,N,N′,N′-tetramethyl-p-phenylenediamine, and Asc - ascorbic acid. The difference in the mean values of the two groups is statistically significant, $P<0.05$

ascorbate were added and this resulted in a very strong increase in the rate of O_2 consumption by human BC samples (Fig. 3). The 8 μM cytochrome c had no effect on the TMPD-ascorbate activated respiration, suggesting that the applied permeabilization procedure did not damage the intactness of the external mitochondrial membrane in human BC samples. In our experiments, TMPD strongly enhanced the COX activity but resulted sometimes in a residual CN-insensitive O_2 consumption due likely to auto-oxidation of TMPD; data not shown. The general respiration rates of respiratory chain complexes in normal breast tissue samples (control) were found to be significantly lower than that of cancer fibers.

Regulation of mitochondrial respiration in BC cells

According to previous studies, it was established that human BC cells contain creatine (Cr) and express various isoforms of creatine kinase, CK, including ubiquitous (Pratt et al. 1987). Two experimental protocols were applied to clarify the peculiarities of respiration regulation in BC cells in situ. Firstly, one protocol was designed to monitor the dependence of the respiration rate of permeabilized fibers derived from tumor and control normal tissues on the external ADP concentration in the medium with exogenously added ADP (at a final concentration of 2 mM) in the presence of respiratory substrates and inorganic phosphate (Pi). In the second approach, respiration was activated by addition of Cr and MgATP in the presence of a system consisting of pyruvate kinase (PK, 10 U/ml) and phosphoenolpyruvate (PEP, 5 mM) for trapping any extramitochondrial ADP to follow control of mitochondrial oxygen consumption by CK (Gellerich and Saks 1982).

Our experiments showed that addition of Cr (up to 10 mM, in the presence of PK-PEP system and exogenously added MgATP) to permeabilized tumor fibers had only a minor effect on the rate of O_2 consumption by cancerous tissues (Fig. 4a). Besides, studies were performed at higher than 0.1 mM concentrations of ATP, and it was found that in the presence of PEP-PK system the addition of ATP up to final concentrations of 1, 2 and 5 mM also did not induce

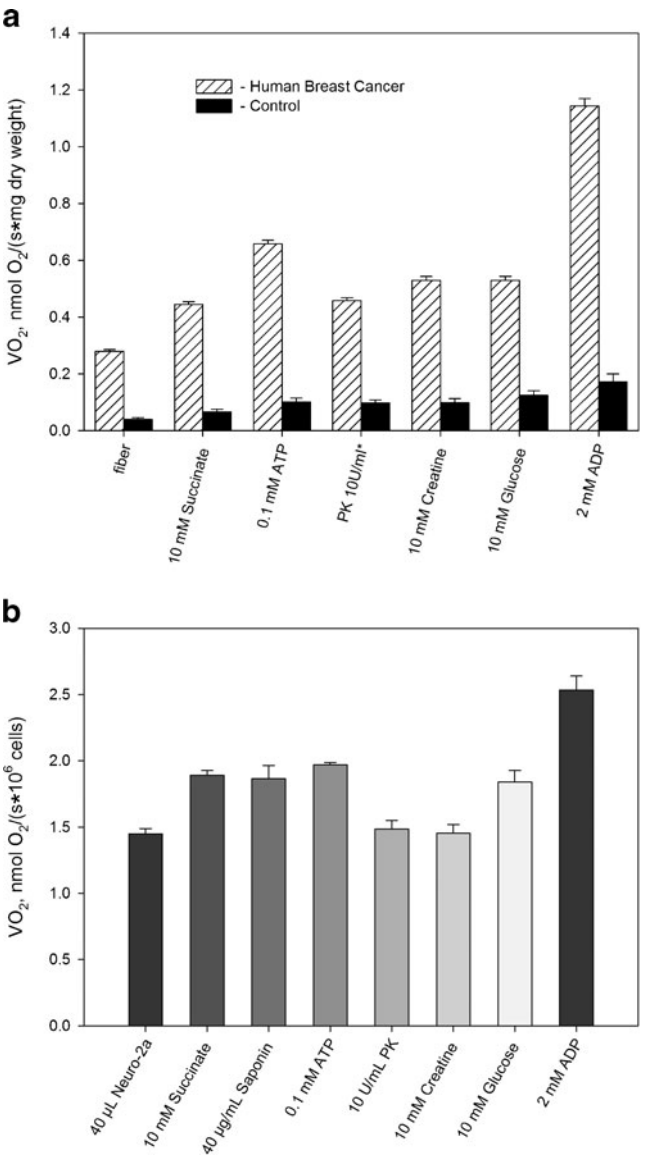

Fig. 4 Effects of exogenously added succinate, creatine, glucose and ADP on the rate of oxygen consumption by permeabilized human breast cancer fibers (**a**) and Neuro-2a cells (**b**). These respirometric investigations were carried out in medium-B supplemented with 5 mM glutamate and 2 mM malate in the presence of pyruvate kinase (PK) –phosphoenolpyruvate (5 mM) ADP trapping system. The difference in the mean values of the two groups in **a** is statistically significant, $P<0.05$

any substantial increase in respiration rates of permeabilized samples of human BC. These in situ experiments clearly indicate that mitochondria in human BC cells have, most probably, a poor ability for the production of phosphocreatine (PCr). No stimulation of respiration by Cr was also registered in nontumorous breast tissues as well as in tumor cells of another histological type – murine neuroblastoma cells of the line N2a (Fig. 4b). Thus, according to our data, MtCK plays a minor role in energy transfer in BC cells, although this enzyme was found to be expressed in human BC samples (Pratt et al. 1987). It is not clear yet whether the CK expression is low or it is not active in some tumor cells. Due to these data, we used in MCA a protocol with direct activation of respiration by ADP.

Previous studies have demonstrated that BC cells can over-express mitochondrially–bound HK-2) (Zancan et al. 2010). We measured the total HK activity of human BC tissues as well as special respiratory experiments were performed to estimate the functional coupling of mitochondrial HK with OXPHOS in human BC cells in situ. The total HK activity of human BC tissues was assayed as 7.42±0.86 mU/mg protein (n=8); this value exceeds considerably that for adjacent normal tissues (2.51±0.76 mU/mg protein, n=8) but is nearly equal to human skeletal muscle – 9.1 mU/mg protein (Koval et al. 1998). Surprisingly, we found that addition of glucose (in the presence of exogenous ATP, at 0.1 mM) had no effect on the respiratory activity of permeabilized BC fibers (Fig. 4a); i.e. in the used system the levels of ADP produced in HK reactions were too low in order to exert any notable respiratory stimulating action. In addition, we examined the influence of glucose administration on O_2 consumption by BC tissues at higher than 0.1 mM levels of ATP, and we did not find any marked O_2 uptake stimulation by glucose even in the presence of 5 mM ATP. These data indicate that the revealed negligible glucose effect was not associated with a low concentration (0.1 mM) of exogenously added ATP. In addition, in similar conditions (in the presence of 0.1 mM ATP) the addition of glucose to permeabilized N2a cells, in contrast to skinned human BC fibers, led to a substantial increase in respiratory rates (Fig. 4b); this indicates that the used experimental protocol for oxygraphic detection of mitochondrially-bound HK-2 in human samples was adequate, and that activity of the glycolytic enzyme in rapidly growing murine N2a cells is considerably higher than that in human BC cells. Taken together, our findings indicate on a minor role of mitochondrially-bound HK(s) in regulation of OXPHOS in human BC cells. Moreover, they suggest that OXPHOS, but not glycolysis could be a main producer of ATP in human BC cells in situ. This is in a good agreement with the recent data obtained in other laboratories; namely, Bonuccelli et al. (2010) have reported that human BC cells xenografts in nude mice show a transcriptional shift towards oxidative mitochondrial metabolism, relative to adjacent stromal tissue. Nevertheless, we cannot exclude entirely the possibility that in human BC cells HK reactions and glycolysis play a substantial role in generation of ATP. Besides HK-2, another isoforms of the enzyme could provide a glycolytic flux in human breast carcinomas. Indeed, Balinsky et al. (1983) have examined isozyme patterns of normal, benign, and malignant human breast tissues and they have found that cancerous tissues express predominantly HK-1, whereas only traces of HK-2 were revealed in some BC tissue samples. In cells HK-1, like to HK-2, can potentially bind to VDAC and this process is strongly-regulated (Pastorino and Hoek 2008). It is possible that human BC cells may enhance their glucose metabolism also by increased glucose transport; namely, it was reported (Rogers et al. 2003; Schmidt et al. 2010; Younes et al. 1995) that some GLUT(s) genes are over-expressed in malignant human breast tumors.

In order to identify the mechanisms of regulation of mitochondrial respiration in malignant breast tumors, an attempt was made to determine the apparent K_m value for exogenous ADP ($K_m^{app}{}_{ADP}$) in permeabilized human BC samples and adjacent nontumorous breast tissues. Original titration curves of our respiratory experiments with exogenously added ADP are shown in Fig. 5a and b. On the basis of data presented on Fig. 5c, the mean apparent K_m value for MgADP in BC samples was calculated as 114.8±13.6 μM (in a large range between 10.5±6.3 μM and 304±51 μM). These results are taken to show that breast tumors derived from different patients differ strongly in the regulation of mitochondrial respiration. There is no correlation between apparent K_m values and V_o or V_{max}. We still do not have enough data to suggest a correlation between K_m and either cancer type or stage. Why this is so and how this K_m values are linked to clinical parameters is not clear yet and needs further investigations. However, we cannot calculate the apparent K_m for ADP in normal breast tissues due to their very low respiratory response.

An analysis of the obtained results showed that mitochondria in human BC cells have an increased affinity for exogenously added ADP in comparison with certain normal tissues; namely, in our prior studies performed on permeabilized rat muscle fibers of oxidative type, cardiac and *soleus* muscles, the $K_m^{app}{}_{ADP}$ was measured as 297±35 μM and 354±46 μM, correspondingly (Table 1). The obtained K_m value for exogenously-added ADP in permeabilized BC fibers was found to be close to the values found in murine tumor cells of the line HL-1B (47±15 μM) (Anmann et al. 2006) as well as in permeabilized (in similar manner) cardiac and *soleus* muscle fibers (40–98 μM) after their specific pre-treatment with trypsin at low concentrations (Kuznetsov et al. 1996), see also Table 1. Taken together our results suggest that in human BC tissues, the intracellular rate of adenine nucleotides diffusion and/or the permeability of the mitochondrial outer membrane (MOM)

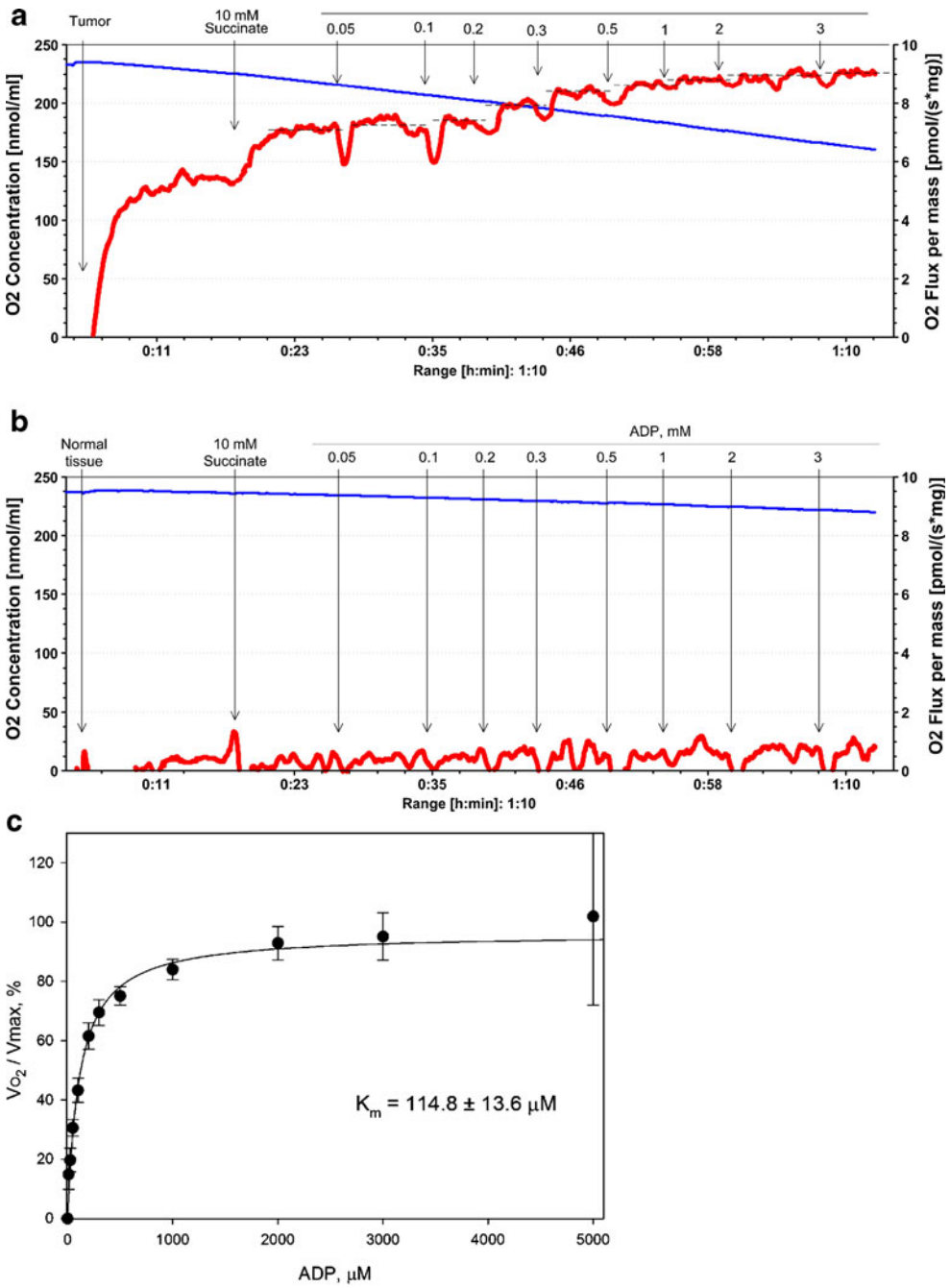

Fig. 5 Kinetics of respiration regulation by added ADP. Typical respiration traces of permeabilized human breast cancer fibers (**a**) and normal breast tissues (**b**) recorded using a two-channel high-resolution respirometer in medium-B (see "Materials and methods") supplemented with 5 mM glutamate, 2 mM malate and 10 mM succinate as respiratory substrates. Respiration kinetics, as normalized respiration rates versus ADP concentrations in permeabilized human breast cancer fibers (**c**). The value of apparent K_m for exogenous ADP was measured as 114.8± 13.6 μM

to the nucleotides is significantly higher than in normal tissues of oxidative type. These strong differences in metabolic regulation of respiration could be casually linked with a decreased expression of some cytoskeletal proteins (particularly, βII–tubulin). Indeed, previous studies have shown that in normal oxidative muscle tissues this cytoskeletal protein can bind to VDAC and thereby strongly limit the permeability of MOM towards ADP/ATP (Rostovtseva et al. 2008), but decreased levels of βII–tubulin expression were monitored in the above mentioned HL-1 tumor cells (they have substantially lower K_m value for ADP than normal cells of the same histological type) (Guzun et al. 2011) as well as in human BC cells, both in vitro (Hiser et al. 2006) and in vivo (Leandro-Garcia et al. 2010).

Metabolic control analysis of respiration regulation in malignant human breast tumors

In the presented work, we applied MCA for the quantitative characterization of the regulation of energy transfer in human BC in situ. According to our knowledge, the MCA has not been used as yet for analysis of the regulation of OXPHOS in

human cancers in vivo. In MCA, we activated the mitochondrial respiration in skinned BC fibers by exogenous ADP (at a final concentration of 2 mM); in this case, ATP produced in mitochondria is transported out of the mitochondria through MOM channels (Timohhina et al. 2009). The use of ADP for activation of respiration was largely motivated by very low activity of MTCK in BC samples (see Fig. 4a). After addition of ADP, corresponding respiratory experiments with specific inhibitors of respiratory chain complexes and ATP synthasome were carried out, and FCC(s) were then calculated.

Figure 6a shows representative traces of oxygen consumption by skinned BC fibers during their incubation with increasing concentrations of 3-NP, an inhibitor of Complex II, when respiration was activated by ADP. 3-NP at a concentration of 0.2 mM caused a strong (approximately 50 %) decrease in the rate of ADP activated respiration in human BC cells without any marked effects toward adjacent normal tissue (Fig. 6b). Similar titration curves were obtained for other respiratory chain and ATP synthasome complexes. They were plotted as relative O_2 consumption rates (VO_2 J/Jo) versus a concentration of inhibitors. From these plots (see Figs. 7 and 8) corresponding FCC(s) were calculated and they are shown on Fig. 9. The FCC(s) for BC fibers were determined by the graphical method of Fell (1997), detailed as described in our prior work (Tepp et al. 2010). Additionally, obtained results were compared with the computer estimated coefficients. In this case, non-linear regression analysis was used by fitting experimental data to the mathematical model developed by Gellerich et al. (1990); in detail, see in Materials and method. The computed FCC(s) is found to be comparable with estimations done using the graph method; for example, for skinned BC samples, in the case of ADP stimulated respiration, the following FCC(s) for mitochondrial Complex III after titrations experiments with antimycin-A were calculated: computed, 0.52±0.15; and graph, 0.54±0.11. In this case of inhibition of Complex III with antimycin-A parameters of the mathematical model are as following: N=1, K_d=0.97±0.33 nM; E_0=25.7±4.4 nM; and C_0=0.52±0.15. Our results suggest that in human BC cells the key sites of the regulation of respiration are Complex IV (FCC=0.74), ATP synthase (FCC=0.61), and inorganic phosphate carrier (FCC=0.60), since FCC(s) for other complexes were found to be substantially lower and they have approximately equal values (Fig. 9). Moreover, it was found that the FCC(s) for ATP synthase and P_i carrier in permeabilized BC samples exceed considerably (by almost 10-fold) those measured for normal tissues of oxidative type - rat cardiomyocytes (see Fig. 9); for these cells, the main regulatory complexes are MtCK and ANT, which emphasize again the MtCK and the PCr/Cr as a main energy flux passway in cardiac muscle cells (Tepp et al. 2010).

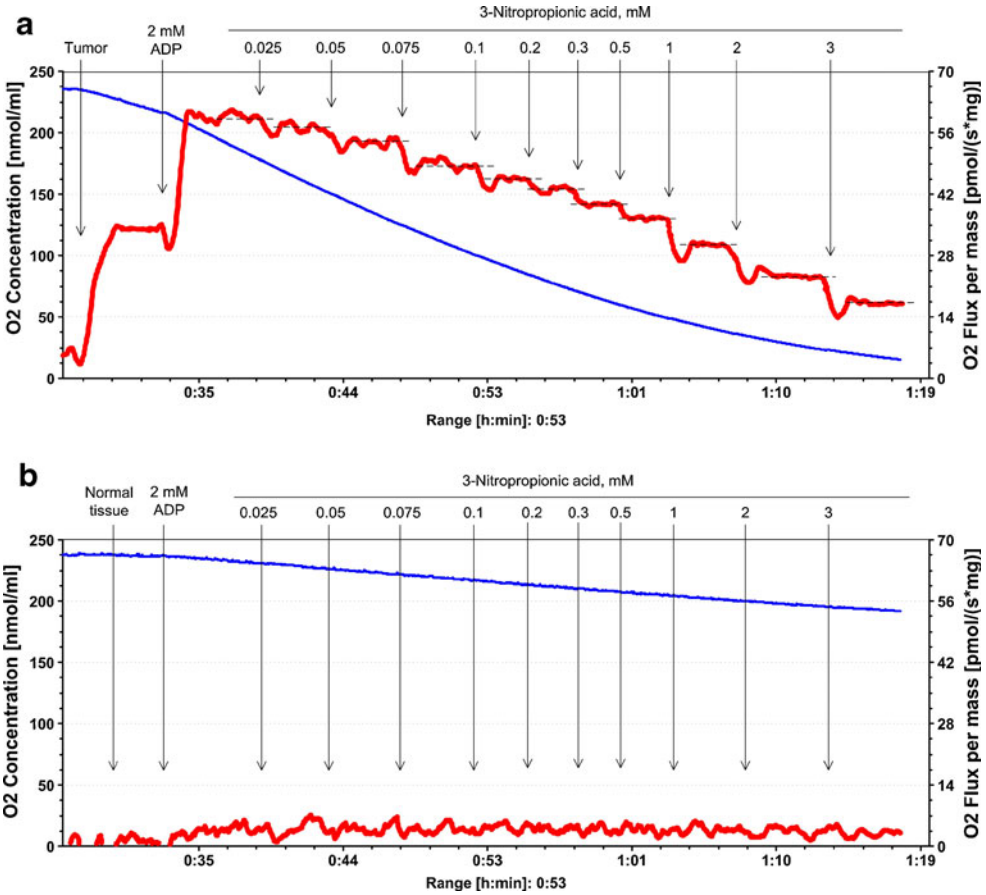

Fig. 6 Representative tracings of change in the rate of oxygen consumption by permeabilized human breast cancer fibers (**a**) and normal breast tissue samples (**b**) after their incubation with increasing concentrations of 3-nitropropionic acid (3-NP) in the presence of 10 mM succinate, and direct activation of mitochondrial respiration by 2 mM ADP. Steady state rates are marked by *dashed lines*. On these figures, total concentrations of 3-NP are shown at every steady state

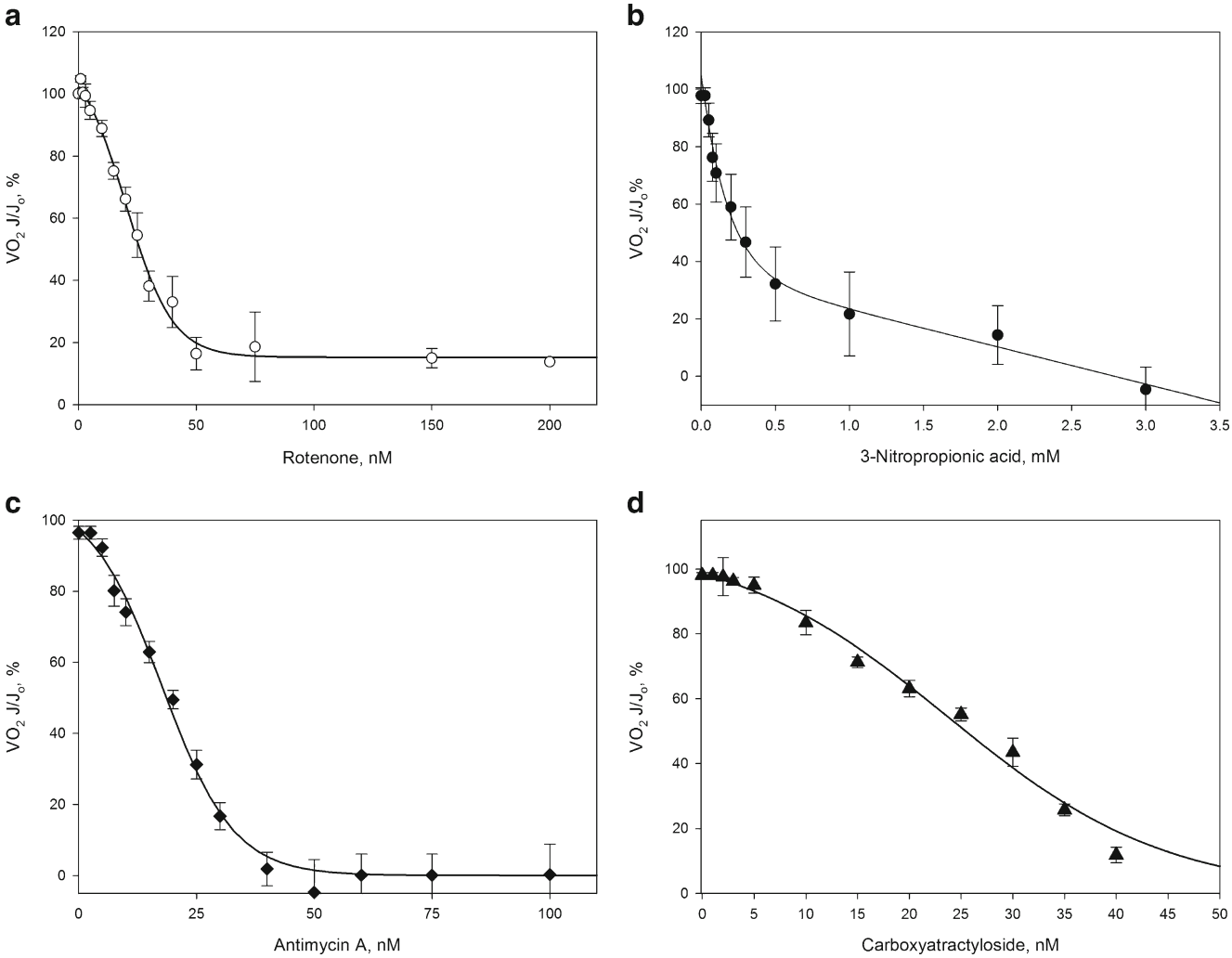

Fig. 7 Titration curves of inhibition of the mitochondrial respiratory chain complexes in permeabilized human breast cancer fibers with: **a** rotenone (Complex I) **b** 3-nitropropionic acid (Complex II) **c** antimycin-A (Complex III), and **d** carboxyatractyloside (ANT). On the graphs, every data point was calculated as the mean of 10–15 independent experiments, *bars* are SEM. From these data, the corresponding flux control coefficients were calculated

Our in situ experiments do not suggest that the activity of mitochondrial ATP synthase may be substantially decreased in human breast cancers. This is different from the data obtained in other laboratories; in fact, in several studies performed on biopsy material derived from untreated patients with primary BC, a specific and significant suppression of the expression of the β–catalytic subunit of the mitochondrial H^+-ATP synthase, was revealed (Isidoro et al. 2004, 2005). The reason of this discrepancy is not clear and needs further studies.

Discussion

To our knowledge, this is the first clinical study of human cancer bioenergetics with the use of permeabilized cell technique and MCA. Our results obtained with the application of the methods mentioned above show that human BC is a non-hypoxic oxidative tumor in which mitochondrial respiration is significantly increased but is very sensitive to use of its inhibitors. From the results of this work we conclude also that MCA in combination with permeabilized cell technique may be very useful method for diagnostics and possibly for choice of therapeutic strategy in cancer treatment (see also in (Moreno-Sanchez et al. 2010)).

To date a glycolytic phenotype was found in a high percentage of human breast cancers, and it was suggested that inhibition of glycolysis might evolve as a future option for therapy of these malignancies (Pedersen 2007b; Pelicano et al. 2006; Schmidt et al. 2010). There are also some indications in the literature that HK-2 is a key glycolytic enzyme that plays a role in the regulation of the mitochondria-initiated apoptosis in cancer cells. Namely, Chen et al. (2009) showed that in

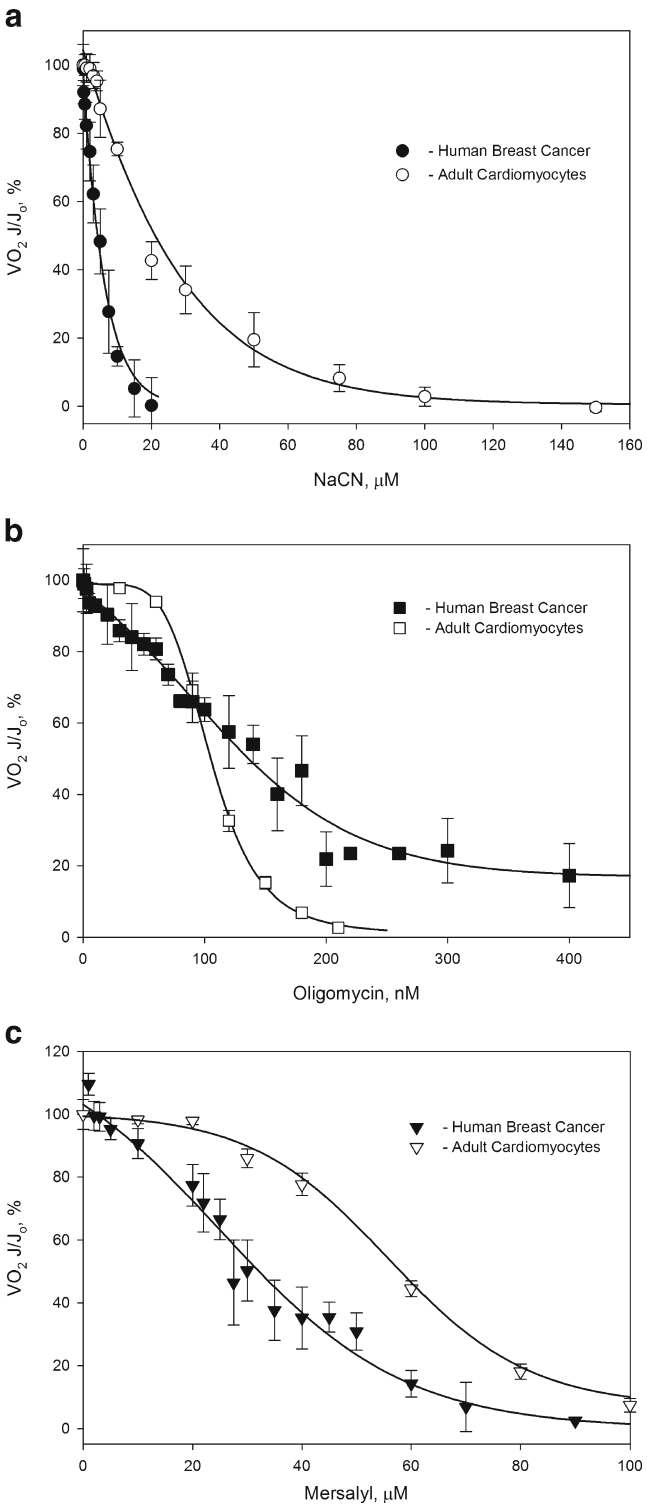

Fig. 8 Comparative titration curves of inhibition of certain energy transfer complexes (IV (**a**), ATP synthase (**b**), and inorganic phosphate carrier (**c**)) in permeabilized human breast cancer fibers and adult rat cardiomyocytes. Ranges of inhibitor concentrations used for complete inhibition of respiration are similar both for cancer fibers and isolated cardiomyocytes. *Bars* are SEM

tumor cells 3-bromopyruvate, a potent inhibitor of HK, can cause a covalent modification of HK-2 protein, and directly triggered its dissociation from mitochondria, leading to a specific release of apoptosis-inducing factor from the mitochondria to cytosol and eventual cell death. Alterations in the expression of OXPHOS proteins, which are encoded not only by nuclear but also mitochondrial DNA (mtDNA), have also been observed. Hence, in human tumors, including BC, severe irreversible mutations in mtDNA, arising during multistep oncogenesis, could also mediate a decrease in the bioenergetic capacity of mitochondria and shift the cellular production of ATP towards glycolysis. It was reported (Desouki et al. 2005) a near absence of mtDNA-encoded COX subunit II expression in more than 40 % of breast cancers that could cause a severe injury of OXPHOS. Mutations of cytochrome b genes were also revealed in the mitochondrial genome of human BC cells (Tan et al. 2002) and they could result in a defective function of the respiratory chain of mitochondria. Other laboratories have measured mtDNA levels in various malignancies and report a decrease in mtDNA content in human breast tumors (Tseng et al. 2006); this depletion of mtDNA could be also supported by a decrease in OXPHOS levels. In several works, repression of expression of β–subunit of ATP synthase was described (Isidoro et al. 2004, 2005). In many cells, the OXPHOS system is closely related to the phosphotransfer systems, including creatine kinase isotypes. Prior studies have shown that some human malignant tumors, including BC, express CKBB (brain type creatine kinase) (predominant isoform), and only traces of MtCK, and MM isoform (muscle type creatine kinase) (Balinsky et al. 1983, 1984; DeLuca et al. 1981; Kanemitsu et al. 1984; Pratt et al. 1987; Roguljic et al. 1989; Thompson et al. 1980). Protection of cells from anoxia was reported after addition of creatine (Cr) to hippocampal brain slices, which already express CK at high levels (Holtzman et al. 1998). However, in sarcoma the Cr/CK system decreases drastically (Bera et al. 2008; Patra et al. 2008). In the cancerous HL-1 cells of cardiac phenotype, both beta 2 tubulin and MtCK are absent (Guzun et al. 2011). Thus, the absence of MtCK co-expressed with beta 2 tubulin may be characteristic for many types of cancer.

Very remarkably, in recent years new data have been rapidly accumulating showing that while all tumor cell types are characterized by enhanced glycolytic flux, not all have a diminished mitochondrial respiration: certain types of cancer cells show increased respiration and up-regulation of OXPHOS components (Jose et al. 2011; Moreno-Sanchez et al. 2007). Some researchers believe that there is no evidence that cancer cells are inherently glycolytic, but that some tumors might indeed be glycolytic in vivo as a result of their adaptation to hypoxic environment (Zu and Guppy 2004). That is, BC cells could maintain a significant level of OXPHOS capacity to rapidly switch from glycolysis to OXPHOS during carcinogenesis. Indeed, Bonuccelli et al. (2010) have reported that human BC epithelial cells in vivo display a transcriptional shift towards mitochondrial

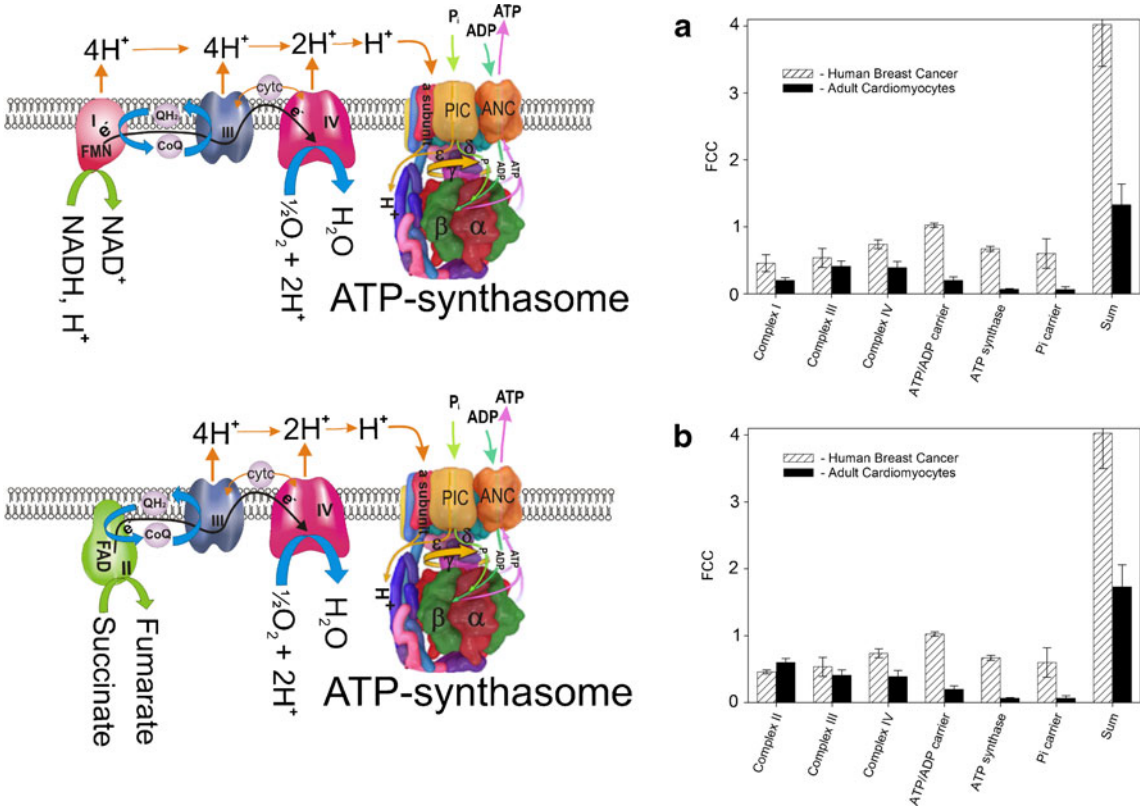

Fig. 9 Flux control coefficients (FCC) of permeabilized human breast cancer fibers and the model of regulation of mitochondrial respiration. Two ways of electron transfer were examined: **a** NADH and **b** succinate dependent electron transfers; here, ATP-synthasome is a large mitochondrial complex consisting of ATP synthase, adenine nucleotide carrier (ANC) and inorganic phosphate carrier (PIC). Bars are presented as average values ± SEM. [See legend to Fig. 10 for acknowledgment for the artwork of the "ATP synthasome"]

OXPHOS, relative to adjacent tumor stromal cells and can use lactate and ketone bodies as a metabolic fuel. Moreover, an analysis of the literature data showed that the high glycolytic rates of breast cancers might be in some part attributed to the presence in the tumor stroma of nonmalignant cells, namely, activated tumor-associated macrophages which are characterized by an enhanced glycolysis (Chen et al. 2011; Garedew et al. 2010). De Groof et al. (2009) have shown that increased OXPHOS activity precedes rise in a glycolytic rate in H-RasV12/E1A transformed fibroblasts that develop a Warburg phenotype. Whitaker-Menezes et al. (2011a) detected increased mitochondrial respiratory chain activities in epithelial tumor cells of human breast cancer that was associated with overexpression of markers of aerobic glycolysis in tumor-associated fibroblasts. The authors validated their observations with data from >2.000 breast cancer patients, which showed the transcriptional upregulation of mitochondrial OXPHOS in human breast tumors and concluded that upregulation of OXPHOS in epithelial tumor cells is a common feature of human breast cancers. Nieman et al. (2011) showed that adipocytes provide the fatty acids to ovarian cancer cells for rapid tumor growth suggesting adipocytes act as an energy source for the cancer cells for increased mitochondrial β-oxidation.

This and other data allowed to propose a new hypothesis of two-compartment tumor metabolism, according to which the catabolism in stromal host cells "fuels" the anabolic growth and increased mitochondrial OXPHOS in cancer cells via energy (substrate) transfer (Martinez-Outschoorn et al. 2012). Our data are in line with these new data and developments, fully supporting this new concept of two-compartment tumor metabolism (Martinez-Outschoorn et al. 2012; Witkiewicz et al. 2012). This concept described in (Martinez-Outschoorn et al. 2012) (its modified version is shown schematically on Fig. 10a) accommodates both the initial Warburg observation of elevated lactate production by tumors (Warburg et al. 1924; Warburg 1956a, b) and rapidly increasing observations of upregulation of OXPHOS in tumor cells (Bonuccelli et al. 2010; de Groof et al. 2009; Jose et al. 2011; Martinez-Outschoorn et al. 2012; Moreno-Sanchez et al. 2007; Nieman et al. 2011; Whitaker-Menezes et al. 2011a), and our study. This concept is based on an assumption that it is crucial to include the supporting microenvironment when studying cancer cell metabolism and that simply examining primary cancer cells alone may not be adequate (Martinez-Outschoorn et al. 2012). The two-compartment mechanism is not in contradiction with the initial Warburg's hypothesis but describes in more details

the tumor metabolism. The increase of aerobic lactate production via Warburg - Pedersen pathway (Pedersen 2007a) due to HK-2 binding to mitochondria may occur in stromal host cells which secrete L-lactate via MCT4 transporters (Whitaker-Menezes et al. 2011b). These cells, due to activated lipolysis and mitophagy, may produce fatty acids, which fuel increased OXPHOS and anabolism in tumor cells. This mechanism may be called glycolytic-oxidative Warburg mechanism (it has been called also the "reversed Warburg effect" (Witkiewicz et al. 2012)). This may explain high anticancer activity of 3-bromopyruvate, entering the cells via the monocarboxylate transporters, which expression is increased (see refs. (Hussien and Brooks 2011; Pedersen 2012; Whitaker-Menezes et al. 2011a, b) and the whole recent volume of Journal of Bioenergetics and Biomembranes, volume 44, number 1, 2012). This new hypothesis is consistent with the observation that the use of mitochondria-targeted drugs and inhibitors of glycolysis may be the most effective anticancer therapy (Cheng et al. 2012).

The use of MCA applied in this work for the first time in permeabilized human BC cells in situ gave important information about the character of changes of cellular energy metabolism and mechanisms of its control. Until now studies in the field were carried out mostly in vitro on many human BC cell lines, metabolic profile of which may differ cardinally from parent cells. The use of such cell lines gives very different information of the bioenergetics of human breast cancers. For example, MCF-7, one of the most popular human BC cell lines in corresponding bioenergetic studies, has too high proliferative index that exceeds manifold that for original malignant cells (DeBerardinis et al. 2007; Deberardinis et al. 2008). Our data indicate that the regulation of mitochondrial respiration in human BC cells differs from that in normal oxidative type tissues and that in this type of tumor cells not glycolysis, but OXPHOS could play a key role in the generation of ATP. For applying the Metabolic Control Analysis, inhibitor titration studies were carried out on Complex I, II, III, IV, ATP synthase, ATP/ADP carrier and Pi carrier. All the coefficients were found to be with extremely high values. The reason for these high control coefficients is clearly not diffusion restrictions, because the concentration range for inhibitors did not differ from those for isolated cardiomyocytes. We found that in human BC cells the key sites of the regulation of respiration

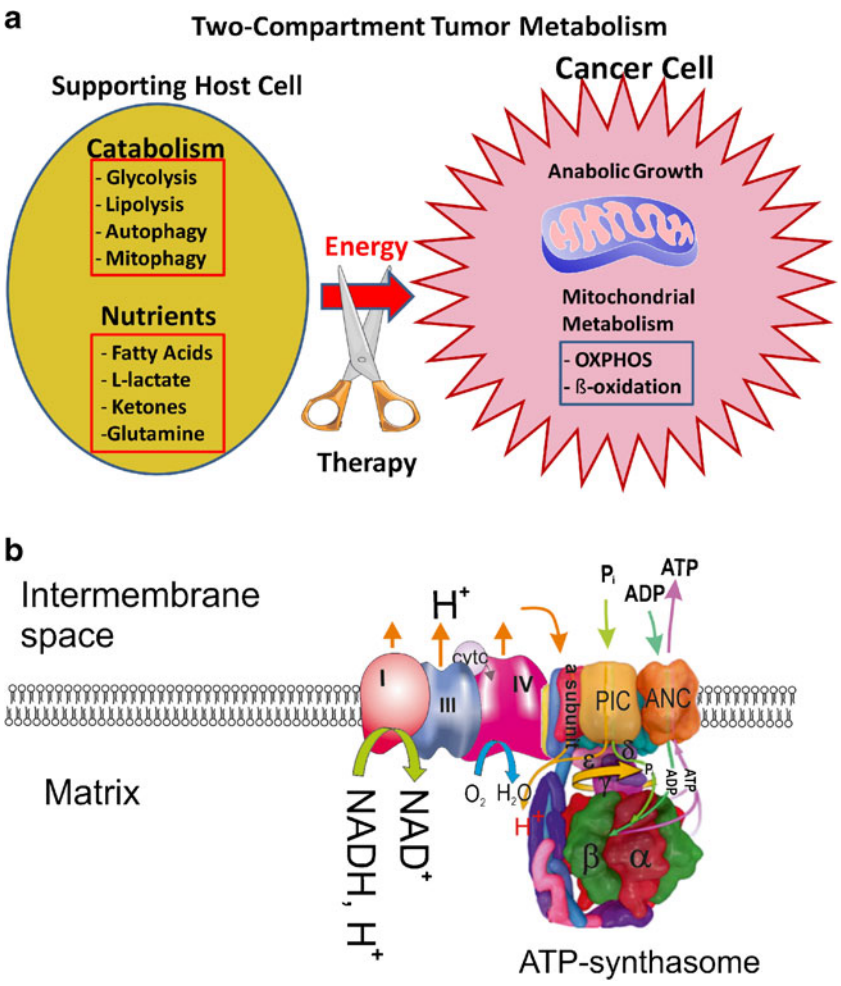

Fig. 10 **a** Two-compartment tumor metabolism, modified from (Martinez-Outschoorn et al. 2012). Targeted new therapies can metabolically uncouple anabolic cancer cells from catabolic host cells. **b** Hypothetical formation of supercomplexes in cancer cells. Respiratory complexes I, III and IV, Cyt-c and ATP synthasome are not randomly distributed within the inner mitochondrial membrane, but assemble into a big supramolecular structure. Art work of the ATP synthasome in Figs. 9 and 10a was reproduced with kind permission from P.L. Pedersen in part from Fig. 1 of reference (Pedersen 2008) and Fig. 2 of reference (Pedersen 2007c) and is the result of the combined efforts of Drs. Young H. Ko and David J. Blum

are Complex IV, ATP synthase, and ANT. Application of MCA suggested also that the ANT complex, major physiological role of that is to export the mitochondrially produced ATP and import of ADP (Dorner and Schultheiss 2007), may be up-regulated (FCC=1.02) in malignant breast tumors, and this assumption is supported by recent literature data. Indeed, there are some indications in the literature that certain isoforms of ANT (a component of the ATP synthasome) might play a very important role in the maintenance of energy metabolism of human BC cancer cells, providing their effective adaptation to decreased tissue O_2 tension, multiplication as well as the high resistance to apoptosis. The human genome presents four ANT isoforms (ANT1-4) (Dolce et al. 2005). Each ANT isoform possesses a specific expression depending on cell type, nature of tissue, developmental stage and status of cell proliferation. ANT1 is known to be highly expressed in differentiated tissues such as skeletal muscle, heart and brain (Stepien et al. 1992). Unlike the ANT1 and ANT3 isoforms, ANT2 is found to be strongly over-expressed in various types of human cancer cells, including BC, and in several hormone-dependent cancers compared with normal non-transformed cells (Chevrollier et al. 2005, 2011; Heddi et al. 1994; Le Bras et al. 2006). In hypoxic conditions, BC cells could maintain the integrity of their mitochondria and effective survival due to over-expression of ANT2. It was assumed by Chevrollier et al. (2011) that when OXPHOS activity is impaired, ANT2 imports glycolytically produced ATP into the mitochondria. In this case, the F_1F_o-ATPase complex hydrolyzes the ATP, pumping out a proton into the intermembrane space. The reverse operations of ANT2 and F_1F_o-ATPase under glycolytic conditions contribute to maintaining the mitochondrial membrane potential, ensuring specific intra-mitochondrial anabolic pathways as well as cell survival and proliferation. Unlike the ANT1 and ANT3 isoforms, ANT2 is not pro-apoptotic and may therefore contribute to carcinogenesis (Bauer et al. 1999; Zamora et al. 2004). Since the expression of ANT2 is closely linked to the mitochondrial bioenergetics of tumors, it should be taken into account for individualizing cancer treatments and for the development of anticancer strategies. In addition, Bianchi et al. (1995) have reported that Complex IV (COX II) of the mitochondrial respiratory chain is elevated in human BC cells.

It is most interesting that in BC cells in situ the sum of FCC (s) for ADP activated respiration exceeds significantly one normally observed in oxidative tissues and isolated mitochondria (Tepp et al. 2011a) is close to 4 (Fig. 9). Earlier, similar high value was seen for Cr-activated respiration in permeabilized heart cells with ADP recycling in ATP synthasome and mitochondrial creatine kinase reactions (Tepp et al. 2011a). In this case, direct metabolic channeling and recycling of ADP in the coupled reactions explained increased values of FCC(s) and their sum (Tepp et al. 2011a). It is well established in theoretical analysis that in an ideal linear system the sum of FCC(s) is 1 (Fell 1997; Groen et al. 1982; Heinrich 1985; Kacser and Burns 1973; Moreno-Sanchez et al. 1991, 2008, 2010; Rapoport et al. 1974; Rossignol et al. 2000; Westerhoff et al. 2009a, b), but may become higher if the system includes enzyme-enzyme interactions, direct substrate channeling and recycling within multienzyme complexes (system becomes non-linear) (Kholodenko et al. 1993, 1994). In isolated mitochondria or permeabilized cells from normal tissue the system of oxidative phosphorylation behaves usually as a quasi-linear system with the sum of FCC(s) equal or slightly higher than 1, if respiration is activated by ADP (Moreno-Sanchez et al. 1991, 2010; Rossignol et al. 2000; Tepp et al. 2011a). A high value of the sum of FCC(s) in mitochondrial respiration activated by ADP evidently shows an altered structure of the mitochondrial respiratory chain in human BC cells. Indeed, our results suggest that the mitochondrial respiratory chain and OXPHOS system contain supercomplexes with direct substrate (electron carrier) transfer (Fig. 10b). This conclusion is consistent with many earlier and confirmed in recent studies (Bianchi et al. 2004; Dudkina et al. 2008; Genova et al. 2008; Lenaz et al. 2010; Lenaz and Genova 2009; Quarato et al. 2011; Vonck and Schafer 2009). Quarato et al. (2011) studied kinetics of respiration regulation by applying MCA in a permeabilized human hepatoma-derived cell line and found high FCC(s) in the de-energized state for three protonmotive complexes I, III and IV, for which the sum of FCC(s) exceeded 1. These and our data are well explained by results of a series of studies from the Lenaz laboratory (Bianchi et al. 2004; Genova et al. 2008; Lenaz et al. 2010; Lenaz and Genova 2009). The authors showed by using flux control analysis that that respiratory complexes may kinetically behave as single supramolecular units with FCC approaching the unity for each component suggesting the existence of substrate channeling within supercomplexes (Bianchi et al. 2004; Genova et al. 2008; Lenaz et al. 2010; Lenaz and Genova 2009). In this case, the sum of FCC(s) significantly exceeds 1 (Bianchi et al. 2004, Genova et al. 2008; Lenaz and Genova 2009, 2010). Supercomplex formation and distribution of respiratory complexes between supercomplex and free form depend upon the protein/lipid ratio and phospholipid composition (Lenaz et al. 2010; Lenaz and Genova 2009). Supercomplex formation provides a kinetic advantage due to direct channeling of electron carriers or substrates (like ADP between ANT and ATP synthase), and they have even been isolated and purified by solubilization by mild detergents and visualized by electron microscopy (Dudkina et al. 2008; Lenaz et al. 2010; Lenaz and Genova 2009; Vonck and Schafer 2009). Formation of the mitochondrial respiratory chain supercomplexes may result also in fixation of cytochrome c within this complex (see Fig. 10b) and thus suppress the mitochondria-driven apoptosis. These conclusions are consistent with the results of electron microscopic investigation of mitochondria in cancer cells showing their altered morphology and condensed appearance

(Gabriel 2009). Further studies are required, however, to identify precisely the structure of mitochondrial supercomplexes in human BC cells.

In conclusion, MCA in combination with the techniques of permeabilized cell may be a very useful method for studies of cancer bioenergetics, tumor metabolism, diagnostics of cancer, and possibly for the choice of an effective therapeutic strategy.

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