Mitochondrion 9 (2009) 165–179

Contents lists available at ScienceDirect

Mitochondrion

journal homepage: www.elsevier.com/locate/mito

Review

Mitochondrial function and energy metabolism in cancer cells: Past overview and future perspectives

Avraham Mayevsky *

The Mina and Everard Goodman Faculty of Life-Sciences, The Leslie and Susan Gonda Multidisciplinary Brain Research Center, Bar-Ilan University, Ramat-Gan 52900, Israel

ARTICLE INFO

Article history:
Received 12 August 2008
Received in revised form 20 January 2009
Accepted 22 January 2009
Available online 30 January 2009

Keywords:
Warburg hypothesis
Mitochondrial function
NADH redox state
Tumor viability
Microcirculatory blood flow

ABSTRACT

The involvements of energy metabolism aspects of mitochondrial dysfunction in cancer development, proliferation and possible therapy, have been investigated since Otto Warburg published his hypothesis. The main published material on cancer cell energy metabolism is overviewed and a new unique in vivo experimental approach that may have significant impact in this important field is suggested. The monitoring system provides real time data, reflecting mitochondrial NADH redox state and microcirculation function. This approach of in vivo monitoring of tissue viability could be used to test the efficacy and side effects of new anticancer drugs in animal models. Also, the same technology may enable differentiation between normal and tumor tissues in experimental animals and maybe also in patients.

1. Introduction

The involvement of the mitochondrion in cancer cell metabolism, functions and therapeutic potential is well documented and accepted. These intracellular organelles have been described by cytologists since the mid 19th century (Lehninger, 1964). According to (Tzagoloff, 1982; Scheffler, 1999), the term mitochondrion was coined by Benda in 1898. However, only in the mid 20th century the role of the mitochondria in oxidative energy metabolism was established in detail (Kennedy and Lehninger, 1949). One of the major fields in cancer research, related to mitochondrial function, was started in the first quarter of the 20th century by Otto Warburg and several other investigators (Cori and Cori, 1925; Crabtree, 1929, 1928). The pioneering work of Warburg between 1923 and 1925 on the metabolism of tumors led to the hypothesis that the development of cancer may originate when cellular glycolysis increases, while mitochondrial respiration becomes impaired (Warburg,1930, 1956a,b; Weinhouse, 1956). Warburg's hypothesis, termed the "Warburg effect," explains the significance of cellular energy metabolism in the pathophysiology of cancer cells. The Warburg hypothesis was not tested under in vivo conditions while monitoring of mitochondrial function by measuring the NADH redox state for example. Very little attention has been paid, by the "Mitochondrialists," to Warburg's work, as can be seen, for example, in the review article by Scheffler (2001a), entitled "A century of mitochondrial research: achievements and perspectives" that does not cite even a single study by Warburg. The significance of energy metabolism in the "come back of the mitochondria" (Scheffler, 2001b) was not discussed. The six hallmarks of cancer cells or tumors, reviewed in 2000 by Hanahan and Weinberg, did not include the bioenergetics of cancer cells, although many publications had dealt with this issue after Warburg's death in 1970. The validity of Warburg hypothesis was not accepted by various investigators and this issue is still under investigation. For example, Weinhouse in 1956 claimed that "there is no sound experimental basis for the belief that oxidative metabolism in tumors is impaired". Burk and Schade (1956) support Warburg hypothesis and disagreed with Weinhouse and worte "Respiratory impairment in living cancer cells, first described by Otto Warburg in 1923, is an experimental fact, and not, as described by Weinhouse." Later on Weinhouse and his collaborators claimed that the high pyruvate kinase activity maybe the main factor leading to the high glycolysis in certain tumors (Gosalvez et al., 1974, 1975; Lo et al., 1968; Weinhouse, 1972). As reported by Garber in 2006, the so-called classical six hallmarks concept was recently challenged by Eyal Gottlieb, claiming that the bioenergetics pattern (aerobic glycolysis) could be the seventh element or hallmark of cancer cells, as described by Warburg 80 years ago. This idea was reviewed recently by Kroemer and Pouyssegur (2008). Very recently Frezza and Gottlieb (2008) reviewed the connection between mitochondrial function and cancer in relation to the "Warburg hypothesis." They presented two distinct scenarios in which mitochondria play a key role in tumorigenesis. Thompson's group (Chen et al., 2007; DeBerardinis et al.,2007, 2008) concluded that "Efforts to integrate modern concepts of signal transduction with cellular metabolism

* Tel.: +972 3 5318218; fax: +972 3 6354459.
E-mail address: mayevsa@mail.biu.ac.il

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doi:10.1016/j.mito.2009.01.009

are still in their infancy" and that "One area that needs to be addressed is the regulation of anaplerosis and of mitochondrial metabolism in general." The same group showed the involvement of glutamine metabolism in transformed cells (DeBerardinis et al., 2008). They found that "transformed tumor cells engage in glutamine metabolism exceeding the need for glutamine as a biosynthetic precursor". Similar studies by Rossignol et al. (2004) showed that mitochondrial structure and morphology could be modulating by change in energy substrate in cancer cells. McBride et al. (2006) reviewed the other tasks of the mitochondria in addition to the main role in being the powerhouse of all cells in the body.

It is now more than half a century since Chance and Williams published their original work on the mitochondrial metabolic state in vitro (Chance and Williams, 1955b). The discovery of pyridine nucleotides by Harden and Young (1906) a century ago was 30 years later followed by the complete description of their structure by Warburg and collaborators (1935). In 1955, the groundbreaking study of Chance and Williams (1955a) defined for the first time the metabolic states of isolated mitochondria in vitro, and correlated these states to the oxidation–reduction levels of respiratory enzymes including the NADH.

Nearly 1000 relevant publications have appeared since the initial studies of NADH fluorescence in vitro and, most importantly, in vivo (Chance et al., 1962; Chance and Williams, 1955c). Several key experiments have shown that this technique could be used for the metabolic mapping of the animal brain as well as other organs. More recently, this methodology was adapted for clinical applications (in intra-operative and intensive care units). Over the past half century, NADH redox state monitoring has been researched in vitro and in vivo, including its clinical applications that have become practical in the last few years (Deutsch et al., 2004; Mayevsky et al., 1996, 2004, 2005; Mayevsky and Rogatsky, 2007). This approach could now be used to investigate the validity of the so-called "Warburg effect" under in vivo monitoring of tissue vitality. It is possible to monitor the mitochondrial NADH in vivo, together with the microcirculatory blood flow, hemoglobin oxygenation and blood volume in various animal tumor models. This will enable the researchers to test the safety and efficacy of various anticancer drugs using in vivo tumor models. The aims of this paper are to present a very general review on cancer cell and tumor energy metabolism as an introduction to the proposal to use a new tissue monitoring approach for studying the safety and efficacy of new anticancer drugs. The same monitoring approach may and could be developed and used, in the future, as a diagnostic tool for clinical practice as well.

2. Energy metabolism in mammalian cells

All cells in the body depend on a continuous supply of ATP in order to perform their different physiological and biochemical activities. Fig. 1 describes the basic processes occurring in a typical normal cell, using glucose as a major source of energy.

The breakdown of glucose into water and CO_2 includes two steps, namely, glycolysis (the anaerobic phase) taking place in the cytoplasm, and oxidative phosphorylation (the aerobic phase) occurring in the mitochondria. Of the total yield of 38 ATP per mole of glucose, two are produced in the glycolysis process and 36 during the oxidative phosphorylation. It is important to note that oxygen availability in the mitochondrion is a critical factor for the normal ATP production in the cell. The exact level of oxygen inside the mitochondria in vivo is not well defined due to the inaccuracy of the measurement tools. It is evaluated to be around 1 mm Hg as compared to the high partial oxygen pressure existing in systemic circulation (Chance et al., 1973). Glycolysis depends on the entrance of glucose from the capillary into the cell via the glucose transporter. The end product of glycolysis, pyruvate, is transported into the mitochondria by a specific carrier protein. The pyruvate is transformed, in the matrix of the mitochondria, into acetyl coenzyme A that activates the tricarboxylic acid (TCA) cycle. In the absence of oxygen, the end product of pyruvate is lactate that may leave the cell and pass into the microcirculatory blood stream via the monocarboxylase transporter located in the plasma membrane.

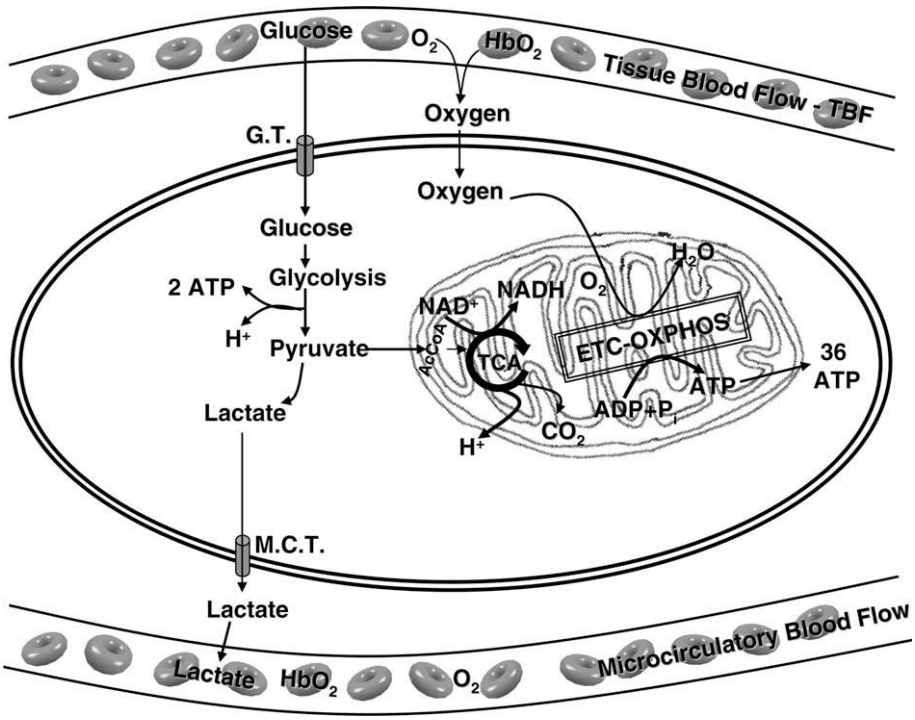

Fig. 1. Schematic representation of cellular energy metabolism and its relationship to microcirculatory blood flow and hemoglobin oxygenation.

In the mitochondria, the TCA cycle generates NADH which enters the electron transport chain (ETC) leading to the oxidative phosphorylation that generates ATP. The CO_2 released from the TCA cycle exit the cell as HCO_3^-, via the anion exchanger, into the blood stream. The continuous supply of oxygen to the mitochondria depends on two microcirculatory parameters: tissue blood flow (TBF) in the diffusible small vessels (small arterioles and capillaries), and the level of hemoglobin oxygenation or saturation (HbO_2) in the small vessels. Any change in the oxygen consumption by the mitochondria will be compensated either by downloading the extra oxygen needed from oxygenated hemoglobin or via an increase in the blood flow. Under a restriction of oxygen supply (hypoxia or ischemia), mitochondrial function will be inhibited and ATP production will decrease, while glycolysis will become stimulated. In most normal tissues and organs, this stimulation is not sufficient to provide the amount of ATP needed for the normal physiological and biochemical activities.

The pioneering work of Chance and collaborators, started in the early 1950th, ushered in a new era of studying mitochondrial function, in health and disease, using spectroscopic techniques (Chance, 1954, 1952; Chance and Legallias, 1951). The monitoring of NADH redox state in isolated mitochondria in vitro (Chance and Baltscheffsky, 1958), and later under in vivo conditions (Chance et al., 1962), established the foundations for the understanding of cellular bioenergetics, under various pathophysiological conditions in experimental animals and patients (Mayevsky and Chance, 2007; Mayevsky and Rogatsky, 2007).

2.1. Mitochondrial function and human diseases

In addition to the mitochondrial role in cellular bioenergetics, the pivotal role of mitochondrial dysfunction in various human diseases has become increasingly clear. For example, the involvement of the mitochondria in tumor cell pathogenesis was initially described by Warburg 80 years ago (Warburg, 1930), and later followed by many studies (Warburg, 1956a; Weinhouse, 1956). Since then, a large body of investigations has shown the involvement of the mitochondria in many human diseases. In this section the reader will find a list of human diseases in which mitochondrial dysfunction plays a central role in its pathogenesis and pathology. The aging process has been profoundly researched in relation to the mitochondrial function (Linford et al., 2006; Navarro and Boveris, 2007). Many studies documented in detail the relationship between apoptosis and mitochondrial function in various neurodegenerative diseases, such as Parkinson's or Alzheimer's disease (Kermer et al., 2004; Lin and Beal, 2006; Tatton and Olanow, 1999).

The role of the mitochondria in the nervous system pathologies has been elaborated by numerous investigators. Sims and Anderson (2002) showed the contribution of the mitochondria to tissue damage developing under stroke. Also, the potential role of the mitochondria in CNS injury has been established, such as in pediatric brain injury (Robertson et al., 2006), newborn rat injury (Robertson et al., 2007) or spinal cord injury (Sullivan et al., 2007), to name just a few examples (Mizuno et al., 1995; Preston et al., 2001). The implication of the mitochondrial dysfunction in cardiovascular diseases was recently reviewed (Ballinger, 2005). The relationship between mitochondrial activity and prolonged endotoxemia in the liver, skeletal muscle and kidney, has been reported (Porta et al., 2006; Rotig, 2003) as well as its role in sepsis (Crouser, 2004; Fink, 2002). The involvement of the mitochondria in apoptosis was investigated also under the influence of anesthetics (Olney et al., 2004). The effect of Cocaine on mitochondrial respiration was reported a few years ago (Boess et al., 2000). In the past few years, the connection between diabetes, obesity and mitochondrial activity has been researched (Berdanier, 2001; Duchen, 2004). Gottlieb et al. (2002) found "that the regulation of mitochondrial outer membrane permeability contributes to respiratory control". Despite the collection of vast knowledge on the mitochondrial function and human health, the accumulated information did not translate into practical clinical tools, such as new drugs or medical devices. The critical need for a breakthrough in cancer research, diagnosis and therapy stimulates us to consider the application of our novel multiparametric monitoring approach, including mitochondrial function in vivo.

2.2. Cancer cells, tumors and mitochondrial activities

In this section a general and short overview on the connection between cancer cells and cellular energy metabolism is given.

The interrelation between the two components of cellular energy metabolism, namely, glycolysis and mitochondrial oxidative phosphorylation, was first studied by Louis Pasteur in 1857 (Krebs, 1972). He found that when oxygen is provided to cells metabolizing anaerobically, the O_2 consumption increases (the Pasteur Effect) and the utilization of glucose and production of lactic acid decline. The "aerobic glycolysis" in cancer cells, described by Warburg in the 1920th, was contrary to the normal Pasteur Effect. The Crabtree effect described about the same time (Crabtree, 1929, 1928) was tested in the mid 60th by Bickis and Henderson (1966) and Bickis et al. (1966) in sliced of tissues in vitro. They tried to estimate the tumor malignancy from metabolic measurements as well as the sensitivity of tumors to anticancer agents. Although the mitochondrial energy metabolism in cancer cells was the main subject of Warburg's study 80 years ago, the investigation of his hypothesis, by real time monitoring of mitochondrial function in vivo, was relatively neglected during the last 30 years. This issue was discussed in details by Gatenby and Gillies (2004). They suggested new avenues of investigation related to critical issues of the" relationship in timing between angiogenesis switch and the glycolytic switch". The involvement of the mitochondria and angiogenesis in hematologic malignancies were discussed by various investigators (Biswas et al., 1997; Fontenay et al., 2006; Mesters et al., 2001; Moehler et al., 2004). During these past few decades, the dominant approach in the research of the mitochondria involvement in cancer and tumor development, focused on signal transduction and other non-energetic aspects, such as apoptosis and reactive oxygen species (ROS) generation. These various investigation subjects were based on Warburg's assumption that cancer could be the result of mitochondrial defects. Brandon's group concluded that "mitochondrial dysfunction does appear to be a factor in cancer etiology, an insight that may suggest new approaches for diagnosis and treatment" (Brandon et al., 2006).The actuality of Warburg relevance to renal cancer metabolism was discussed by Simonnet et al. (2002) and Godinot et al. (2007) Fig. 2 presents six main subjects that are relevant to the mitochondrial function in cancer cells and tumors. It is important to note that this classification was designed to show the complex relationship between mitochondrial activity and cancer in a simplified scheme. Each item presented in the figure has been deeply investigated, with a large number of experimental and clinical studies published during the last few decades. The multiple interconnections between each of the presented items made it almost impossible to draw lines between them. A short description of the six subjects is as follows.

2.2.1. Mitochondrial respiration

As mentioned earlier, the metabolic activity of cancer cells has been characterized by various groups after the groundbreaking work of Otto Warburg, the Nobel laureate of 1931. There is still no detailed information on the microenvironment activities, especially on the mitochondrial function and cellular energy metabo-

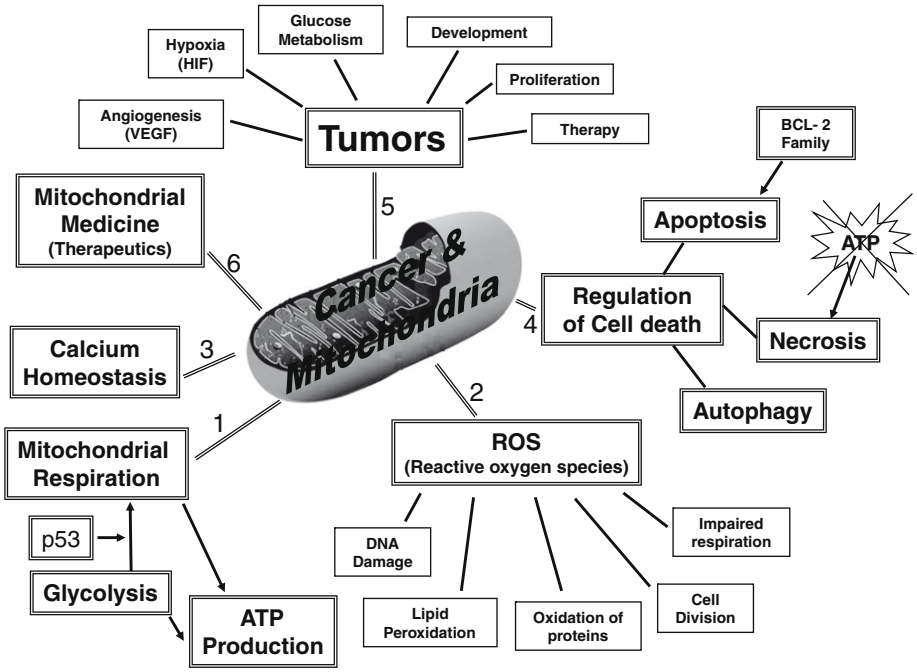

Fig. 2. Schematic representation of the central role of the mitochondrion in the various processes involved in the pathology of cancer cells and tumors. Six issues marked as 1–6 are discussed in details in the text.

lism at the tumor tissue level in vivo. The change in cellular glucose metabolism was the first biochemical hallmark in cancer cells, as was recently discussed in detail by Shaw, 2006. He concluded that "80 years after Warburg's initial observations; we now know many of the molecular details of how glucose metabolism is altered by specific signaling pathways during tumor genesis. The onus is now on us to therapeutically exploit this phenotype." Ramanathan et al. (2005) concluded that "the cancer gene and Warburg concepts are fully consonant but, presumably, with cancer genes as the cause and metabolic alterations as an effect". Other investigators claim that the Warburg hypothesis was not proven to account for all the experimental results obtained since his time (Guppy et al., 2002). Zu and Guppy (2004), after reviewing the data published in 40 years, asserted 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 hypoxic environment". Fang et al. (2008) discussed the adaptation to hypoxia and acidosis in carcinogenesis and tumor progression. The relationship between the bioenergetics of cancer cells and tumors has been reviewed by Moreno-Sanchez et al. (2007) and investigated in animal experiments as well as in human studies (Alirol and Martinou, 2006; Cuezva et al., 2002; Gottlieb et al., 2002; Smallbone et al., 2007; Wu et al., 2007; Xu et al., 2005). The involvement of the tumor suppressor gene, p53, in the bioenergetics of cancer cells and tumors, has been described by many investigators Matoba et al. (2006) and Ma et al. (2007) described the role of p53 in the regulation of mitochondrial respiration and its balance with glycolysis. They showed that the stimulation of mitochondrial activity by p53 affects the synthesis of cytochrome c oxidase 2 while glycolysis is inhibited. They concluded that "a primary defect in aerobic respiration is sufficient to alter glycolysis through both p53 dependent and independent pathways, providing a genetic model for the Warburg effect in cancer cells" (Assaily and Benchimol, 2006; Green, 2006; Kruse and Gu, 2006; Ma et al., 2007; Matoba et al., 2006). Assaily and Benchimol (2006) claimed that the new player – p53 is very important in future studies of cellular metabolism. Due to the close relation between cancer cells activities and their energetic state, it was proposed to use this relation for therapeutic purposes (Chen et al., 2007). In their review, Brandon et al. (2006) summarize the connection between mitochondrial mutations and the oxidative phosphorylation process suggesting its open new directions to anticancer drug development. Ristow et al. (2000) describe the role of Frataxin, a mitochondrial protein, that could stimulate mitochondrial oxidative phosphorylation. In his review Ristow (2006) summarized the interaction between tumor growth and mitochondrial metabolism suggesting new approaches to prevention and treatment of malignant tumors. Recently, Haridas et al. (2007) described the role of Avicins, pro-apoptotic and anti-inflammatory material, on energy metabolism in cancer cells and concluded that it could be used to treat cancers. Robey and Hay (2005, 2006) described the role of mitochondrial hexokinases in keeping mitochondrial homeostasis and coupling cellular metabolism and survival. King et al. (2006) described the connection between the activity of two enzymes, succinate dehydrogenase and fumarate hydratase and mitochondrial dysfunction in cancer cells. Pedersen (2007a,b) reviewed in details the possible role of the mitochondria as a target for new therapeutic approaches.

2.2.2. Reactive oxygen species-ROS

The mitochondria are the main intracellular source of ROS production, generated by the respiratory chain. As shown in Fig. 2, an excess of ROS production can lead to cellular damage, affecting various patophysiological events. These include DNA damage, lipid peroxidation, protein oxidation, cell division and impaired respiration, as discussed in detail by Ott et al. (2007). ROS are also involved in the regulation of cell death by releasing cytochrome c and other pro-apoptotic proteins.

2.2.3. Calcium homeostasis

The significant role of calcium ions in mitochondrial function in normal cells is a well-established fact (Duchen, 2000a,b; Rizzuto et al., 2000). In cancer cells, damage to the mitochondria involves the regulation of calcium signaling (Preston et al., 2001) taking part in the apoptotic process.

2.2.4. Regulation of cell death

This subject has been investigated in detail during the last few decades and has been related to the activities of normal cells as well as cancer cells, proceeding through diverse mechanisms. Cell death regulation can occur in three different interrelated mechanisms (Fig. 2). When energy is depleted and ATP synthesis is significantly decreased, the process of necrosis will develop. Such a process may be part of diverse pathophysiological conditions that develop in normal and cancer cells. The apoptotic process has been also researched in great depth in normal cells and even more intensively in cancer cells. Many factors appearing in Fig. 2 are linked to apoptosis and to autophagy, and they were described in numerous publications (Bonnet et al., 2007; Dohi et al., 2004; Gogvadze et al., 2008a,b; Gogvadze and Zhivotovsky, 2007; Gonzalvez and Gottlieb, 2007; Muravchick and Levy, 2006; Schultz and Harrington, Jr., 2003; Schwarz et al., 2007).The regulation of cell death and especially of apoptosis, related to mitochondrial function, includes pro-apoptotic and anti-apoptotic peptides or proteins, such as proteins of the BCL-2 family (Certo et al., 2006; Green, 2006; Lessene et al., 2008).

2.2.5. Tumors in experimental animals and patients

Various aspects of tumor development, proliferation and many other factors, related to mitochondrial dysfunction, are shown in Fig. 2. In many fast-growing tumors, regions of hypoxia are formed and they can be detected by measuring partial oxygen pressure (Gullino et al., 1967a,b; Vaupel, 2004). A vast number of publications describe the involvement of the hypoxia inducible factor (HIF) in the process of malignant transformation in cancer cells (Brahimi-Horn et al., 2007; Kim et al., 2007; Lopez-Lazaro, 2006; Lu et al., 2002). The involvement of hypoxia in tumor progression and therapy was described in an editorial publication several years ago (Coleman et al., 2002). As a response to hypoxia, angiogenesis is regulated, via the HIF system, in order to maintain the balance between oxygen supply and demand (Airley and Mobasheri, 2007; Pugh and Ratcliffe, 2003). According to Guppy et al. (2005), hypoxic tumors are very aggressive and have a bad prognosis due to their potential to metastasize. Hypoxia and HIF in solid tumors were used as a metabolic target to enhance cytotoxic chemotherapy (Cairns et al., 2007) and tumor phenotype can be regulated by different types of HIF (Gordan and Simon, 2007). Hyperbaric oxygenation was used as a therapeutic tool against malignancy (Daruwalla and Christophi, 2006). Another very important aspect of tumor progression is the angiogenesis process that is related to the hypoxic state of the tumors as well as other factors (Blanc-Brude et al., 2003; Choi et al., 2003; Raghunand et al., 2003).

2.2.6. Mitochondrial medicine

Since the mitochondria are involved in a wide range of diseases, a new therapeutic approach was developed 30 years ago, aimed to develop drugs targeting the mitochondria. This approach was recently reviewed by Armstrong (2006, 2007) and others (Garber, 2005; Goldin et al., 2007; Preston et al., 2001; Schulz et al., 2006; Varmus, 2006). A new group of anticancer agents affecting the mitochondria, termed the "Mitocans," have been recently developed (Neuzil et al., 2007a,b). A new novel anticancer agent that was isolated from Jasmonate was described by the group of Flescher (Flescher, 2007; Goldin et al., 2007; Heyfets and Flescher, 2007; Rotem et al., 2005) to be active at the mitochondrial activity level. Other investigators had proposed also to use the mitochondria or the glycolysis as targets for anticancer agents development (Gatenby and Gillies, 2007; Gillies and Gatenby, 2007; Nguyen and Hussain, 2007; Smeitink et al., 2006; Wallace, 2005; Weissig et al., 2004).Very recently Ganapathy et al. (2009) pointed out the significance of nutrient transporters, such as glucose transporter, as a target for drug development and cancer therapy.

3. In vivo monitoring of tissue energy metabolism

Although a variety of methods have been tested over the years, recently several real time invasive and noninvasive techniques have been developed to determine tissue energy metabolism. This section briefly describes the available techniques for monitoring tissue viability described by various investigators (Fig. 3). These techniques could be adapt and use in cancer cells as well as in tumors.

3.1. Microcirculatory blood flowmetry (A)

Real time monitoring of tissue blood flow (TBF) can be achieved using laser Doppler flowmetry (LDF) (Dirnagl et al., 1989; Haberl et al., 1989; Stern et al., 1977). This method has been calibrated against the hydrogen clearance probe and carbon 14 iodoantipyrine autoradiography, the two well-established methods for quantitative TBF monitoring. The LDF measures relative changes, which correlate with the relative TBF alterations measured by the other two quantitative approaches (Wadhwani and Rapoport, 1990). The principle of LDF is to measure the Doppler shift, namely, the frequency change undergone by the light reflected from moving red blood cells. The results are presented as percentages of a full scale (0–100%), providing relative flow values. Although the changes in the total back-scattered light are an indirect indicator of the blood volume in the tested tissue volume, it cannot be calibrated because of the nature of the instrument. In our laboratory, we used LDF to measure TBF (tissue blood flow) in the brain, kidney, and liver (Barbiro et al., 1998; Mayevsky et al., 1999b, 2001). This method has been successfully employed in many animal models, including rodents, cats and baboons under stroke.

3.2. Hemoglobin oxygenation (B)

The level of oxygenated hemoglobin can be monitored at the microcirculatory level using the absorption spectrum of hemoglobin, which is different in its oxygenated or deoxygenated state. The tissue surface is illuminated with 585 nm light which is an isobestic point, and with 577 nm light, which is a non-isosbestic point, in which the oxy-hemoglobin absorb more light than the deoxy-hemoglobin form. By subtracting the 585 nm reflectance from the 577 nm reflectance, a parameter correlated to blood oxygenation is obtained. A detector collects the light reflected from the tissue and converts it into oxy-hemoglobin levels (Frank et al., 1989; Rampil et al., 1992).

3.3. Mitochondrial NADH redox state fluorometry (C)

NADH monitoring from the organ surface (brain, kidney, liver, testis, etc.) is performed by the fluorometric technique based on the original work by Chance and Williams (Chance and Williams, 1955b; Mayevsky, 1984). The excitation light (366 nm) is passed from the fluorometer to the tissue through a bundle of quartz optical fibers. The emitted light (450 nm), together with the reflected light at the excitation wavelength, is transferred to the fluorometer through another bundle of fibers. The measured changes in fluorescence and reflectance signals are calculated as percent values relative to the calibrated signals under normoxic conditions. This type of calibration is not absolute, but it provides reliable and reproducible results for different animals and different laboratories (Mayevsky, 1984; Mayevsky and Chance, 1982). The combination of NADH fluorescence and LDF techniques has been routinely utilized in our laboratory for the past 10 years (Mayevsky et al., 2001, 2002, 1999a; Sonn and Mayevsky, 2000).

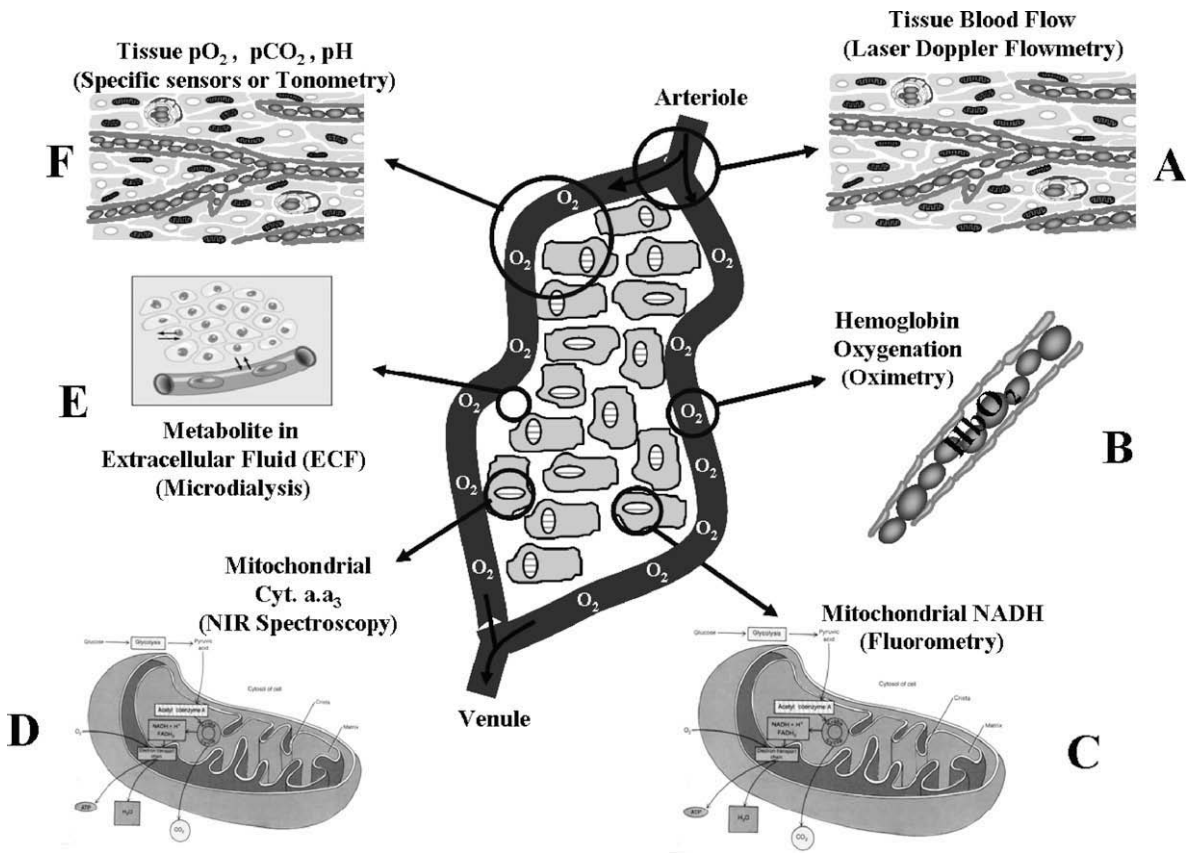

Fig. 3. Schematic presentation of the six parameters that could be monitored for the evaluation of tissue energy metabolism (see text for details).

3.4. Near-infrared spectroscopy (D)

Near-infrared spectroscopy (NIRS) is a noninvasive optical technique that evaluates the relationship between oxygen availability and consumption by continuous in vivo measurement of changes in oxy-hemoglobin (HbO_2), deoxy-hemoglobin (Hb), and total blood volume in intact living tissue. In some circumstances, it can be used to assess regional oxygen consumption and the oxidation–reduction state of cytochrome aa3 (Cyt aa3), although the artifacts of the hemoglobin signal in the Cyt aa3 signal remain problematic. Since tissue oxygenation is indicated by the state of cytochrome oxidase, the terminal enzyme of the respiratory chain, dynamic measurements of oxygen utilization and perfusion can be achieved by NIRS (Jobsis, 1977; Thorniley et al., 1997). The method utilizes the ability of near-infrared light (700–1000 nm) to pass through biologic materials, such as skin, bone and muscle, with much less scattering than at shorter wavelengths. Differences in the absorption spectra of the iron (heme) or copper centers of tissue chromophores under oxygenation or oxidation, permit determining relative variations in their concentration. Hemoglobin, myoglobin and Cyt aa3 are the only major tissue chromophores known to have significantly oxygen-dependent absorption spectra. These are the same compounds that vary with tissue oxygen availability; therefore NIRS is well suited to evaluate tissue oxygenation.

3.5. Microdialysis (E)

The technique of tissue microdialysis allows continuous on-line monitoring of changes in tissue energy-related metabolites, e.g. glucose, lactate, pyruvate, adenosine and xanthine (Johnston and Gupta, 2002). It involves the insertion of a catheter (0.62 mm in diameter) lined with a polyamide dialysis membrane into brain parenchyma which is perfused with a physiological solution (e.g. Ringer's lactate) at ultra-low flow rates (0.1–2.0 μl/min) using a precision pump. Small molecules diffuse from the extracellular space into the perfusion fluid, which is then collected into vials that are changed every 10–60 min, allowing for up to 70% equilibration across the dialysis membrane (Hutchinson et al., 2000b; Hutchinson, 2005). Cerebral microdialysis has been applied to patients in many different clinical situations, including TBI, SAH, epilepsy, ischemic stroke, tumors and during neurosurgery (Bhatia and Gupta, 2007; Hillered et al., 2006; Hutchinson et al., 2000a; Hutchinson, 2005; Peerdeman et al., 2000; Sarrafzadeh et al., 2002; Verweij et al., 2007). Cerebral microdialysis has great potential for exploring the pathophysiology of acute brain injury, drugs pharmacokinetics within the central nervous system and responses to therapeutic interventions.

3.6. Tissue pO_2, pCO_2 and pH (F)

Measurements of tissue pO_2, pCO_2 and pH are used as a monitoring modality in the neurosciences, critical care units (Doppenberg et al., 1998; Maas et al., 1993; Zauner et al., 1997) and as a marker of tissue oxygenation in research protocols (Lacombe et al., 1992; Van Hulst et al., 2002; Van et al., 1992). The technique involves the insertion of a micro-sensor into the brain parenchyma, either through a bolt inserted into the skull or directly through the craniotomy site and tunneled under the skin. Two commercially available micro-sensors allow direct, continuous measurement of brain tissue gases. One of these sensors measures brain tissue oxygen tension using polarographic Clarke-type electrodes. These are flow-dependent oxygen electrodes covered with a highly permeable membrane (Beppu et al., 2002; Gonzalez et al., 2003; Sjoberg

et al., 1999). The other measures PaO_2, $PaCO_2$ and pH using fiber-optic technology.

4. Future directions

Our laboratory has developed and applied a multiparametric monitoring approach based on tissue-light interactions. The tissue is illuminated by light at various wavelengths (from different light sources) and the following parameters are continuously monitored from the tissue surface; tissue blood flow, tissue reflectance (indicating changes in tissue blood volume), microcirculatory blood oxygenation and mitochondrial NADH.

The principles of the monitoring techniques were described in detail in the previous section. Our approach is unique in the fact that all the four parameters are measured in almost the same tissue volume. This enables us to correlate between the various parameters and comprehend the oxygen balance of the tissue. There is no interference between the various monitored parameters, since the principles of each technique are different.

4.1. Optical spectroscopy of tissue energy metabolism in vivo

Monitoring of tissue physiological parameters, in real time, is a continuous challenge to scientists performing experimental animal studies as well as in clinical practice. Since the regulation of tissue physiology is dependent on many factors, the monitoring of a single physiological parameter, such as mitochondrial function, provides a very small fraction of the information needed for data interpretation and understanding of the pathological state. The diagnostic value of a single parameter monitoring device is limited, preventing its implementation in daily experimental and clinical practice. In order to overcome this limitation, we have developed a multiparametric monitoring system that can provide real time information on changes in various physiological and biochemical parameters, taking place in the brain (Friedli et al., 1982; Mayevsky, 1983; Mayevsky et al., 1985), heart (Osbakken and Mayevsky, 1996), kidney (Luger-Hamer et al., 2008; Mayevsky et al., 2003) and liver (Barbiro et al., 1998) exposed to various pathophysiological states.

In order to identify the vitality of any tissue in the body, it is necessary to monitor parameters representing the O_2 balance in the tissue. The O_2 supply is evaluated by monitoring the microcirculatory blood flow using laser Doppler flowmetry as well as the level of hemoglobin oxygenation in the microvasculature. The evaluation of oxygen demand is not performed directly in the present work, but the oxygen balance is evaluated by monitoring the mitochondrial function using the NADH fluorometry/reflectometry approach. All these three monitored parameters could be measured in all tissues or organs in the body, and therefore could be used to evaluate the vitality of all tissues using the same principles. By utilizing the multiparametric monitoring approach, it is possible to show the interrelation between various physiological and biochemical processes under various pathophysiological conditions. The coupling or uncoupling between microcirculatory blood supply and the intracellular mitochondrial function can be studied by monitoring the various parameters simultaneously in the same tissue volume. For example, when the brain is deprived of oxygen, by anoxia (0% oxygen), hypoxia or ischemia, the oxygen availability will decrease and the mitochondrial function will be inhibited (NADH will increase). As a result, the ATP turnover will decrease, the Na^+–K^+–ATPase activity will decline and the ionic homeostasis will be disrupted (Mayevsky et al., 1999b, 2004; Meilin et al., 1999). On the other hand, if the brain is activated by a cortical spreading depression (SD) wave (Mayevsky, 1992; Mayevsky and Weiss, 1991; Sonn and Mayevsky, 2000), the energy demand will increase and the mitochondrial function will be stimulated (NADH will become oxidized), leading to a significant cerebral blood flow (CBF) elevation. The various aspects of NADH monitoring in vivo were described in many details in our recent published review (Mayevsky and Rogatsky, 2007).

4.2. The multiparametric monitoring system

The multiparametric monitoring system is based on the combination of several independent techniques (Fig. 4). The simultaneous monitoring of mitochondrial NADH and HbO_2 from the brain surface is accomplished using the Time Sharing Fluorometer Reflectometer (TSFR) (Rampil et al., 1992). The tissue blood flow (TBF) is monitored by laser Doppler flowmetry (Fig. 6). All of the optical fibers are joined into one bundle of fibers that is placed on the tissue surface of the animal used. As seen in Fig. 4, the filter wheel of the TSFR (A) contains eight appropriate filters, as seen in table B in the figure, enabling the measurements of three parameters, namely, the tissue reflectance, NADH fluorescence and blood oxygenation, as described previously (Rampil et al., 1992). In part C the absorption spectra of oxygenated and deoxygenated hemoglobin is shown together with the two wavelength selected in our device.

4.2.1. Animal model preparation and experimental procedure

It is important to note that in the following sections the brain will used as a typical organ to illustrate the use of the multiparametric monitoring systems. Practically it is possible to use the same monitoring system in other organs such as the heart, kidney, liver or the small intestine (Mayevsky and Rogatsky, 2007).

All experimental protocols were approved by the institutional animal care committee under the instruction of the National Institutes of Health. The experimental procedures were detailed previously (Mayevsky et al., 2005; Mayevsky and Chance, 1982). In order to demonstrate the performance of the Multiparametric monitoring system we used the brain of male rats .The animals were anesthetized by Equithesin (E-th = Chloral hydrate 42.51 mg; Magnesium sulfate 21.25 mg; Alcohol 11.5%; Propylene glycol 44.34% and Pentobarbital 9.72 mg) IP injection of 0.3 ml/100 g body weight. The animals were kept anesthetized during the operation and during the entire monitoring period, by IP injections of E-th 0.1 ml every 30 min. Body heat was measured by a rectal probe (YSI) and was regulated to be in the range of 36–37 °C using a heating blanket. The rats were anesthetized and secured in a head holder. After a midline incision of the skin, an appropriate hole was drilled in the parietal bone of the right hemisphere. The dura mater remained intact and an appropriate light guide holder was placed in the hole. Two stainless steel screws in the right parietal bone were used, with dental acrylic cement, to fixate the probes, which were positioned by a micromanipulator on the cortex. The two common carotid arteries were isolated just before brain surgery, and ligatures of 4–0 silk threads were placed around them.

4.2.1.1. The rats were exposed to the following perturbations. Anoxia: The animals were exposed to oxygen deficient atmosphere by spontaneous breathing of 100% N_2, for 25–30 s.

Ischemia: Reversible occlusion (~1 min) of the two common carotid arteries (by constricting them with threads) led to temporary brain ischemia in the rat.

Spreading depression (SD): is a phenomenon initiated by a rapid depolarization of neuronal tissue in the gray mater of the cerebral cortex with a massive redistribution of ions between the intracellular and extracellular spaces, a decrease in the DC steady potential and a depression of the spontaneous electrical activity (EEG or ECoG). No deleterious effects on neuronal tissue function was re-

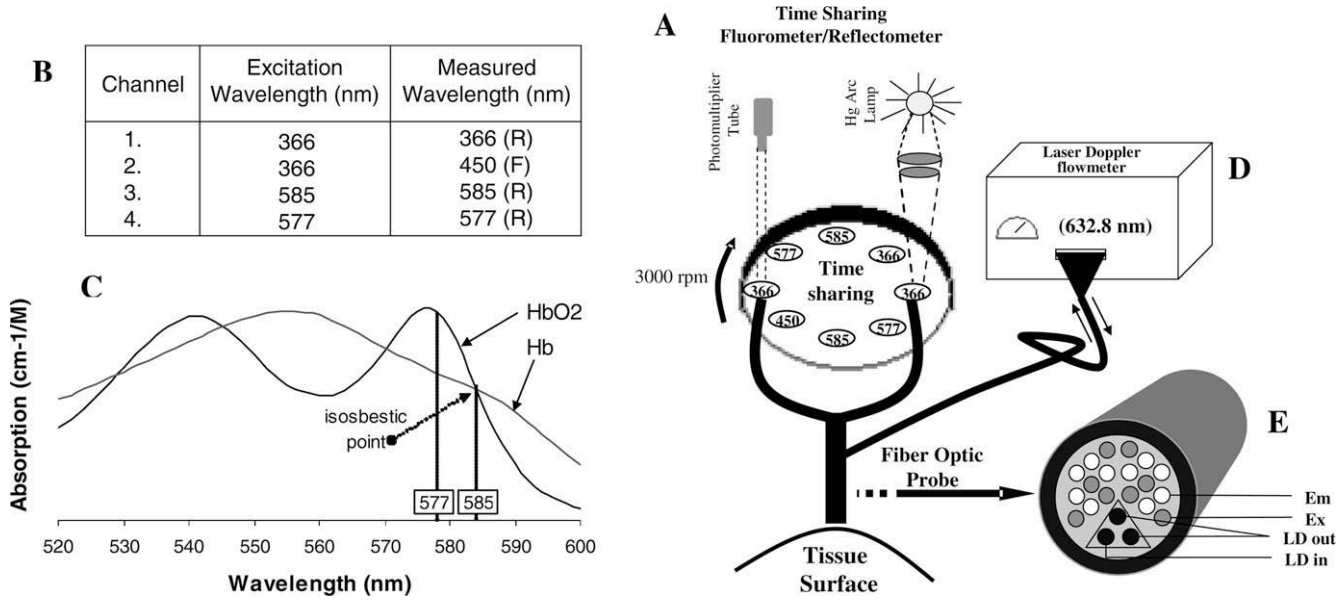

Fig. 4. (A) Schematic representation of the Time Sharing Fluorometer Reflectometer (TSFR) combined with the laser Doppler flowmeter (D) for blood flow monitoring. The time sharing system includes a wheel that rotates at a speed of 3000 rpm with eight filters: four for the measurements of mitochondrial NADH (366 nm and 450 nm) and four for oxy-hemoglobin measurements (585 nm and 577 nm) as seen in (C). The source of light is a mercury lamp. The probe includes optical fibers for NADH excitation (Ex) and emission (Em), laser Doppler excitation (LD in), laser Doppler emission (LD out) as seen in part E The absorption spectrum of Oxy- and Deoxy- Hemoglobin indicating the two wavelength used (C).

ported under conditions of increased oxygen supply compensating for the extra oxygen needed by the SD process. The SD has a wave shape propagating from the point of its initiation to the entire ipsilateral cerebral hemisphere. SD was induced by a special cannula located epiduraly and cemented to the skull together with the monitoring devices. In order to induce SD the brain will be washed (through the SD cannula) with KCl solution (0.2–0.6 M) until the appropriate number of waves appear. Then, the wave of SD will be stopped by washing the brain with saline solution. More details on the effects of those perturbations on the brain and other organs were published recently (Mayevsky and Rogatsky, 2007).

4.2.2. Typical metabolic responses and interpretation

In order to demonstrate the performance of the suggested monitoring system the responses of the rat brain to three typical perturbations are presented in Fig. 5.

The results of a typical anoxic episode are presented in Fig. 5A. As seen, inhalation of N_2 caused a decrease in CBF and HbO_2. Concomitantly, NADH increased due to the lack of oxygen. The reflectance trace shows a two step decrease due to the increase in tissue blood volume. Upon recovery, when the rat breathed air, a massive hyperemia in CBF (as compared to the basal level) was observed and HbO_2 remained high. When CBF returned to its basal level, the reflectance and NADH recovered. Fig. 5B presents the responses of the rat brain to ischemia induced by bilateral occlusion of the carotid arteries. As observed, the occlusion of both arteries caused a decrease in CBF and HbO_2 while mitochondrial NADH increased. When the occlusion was removed, CBF rapidly increased, reaching hyperemic levels of and HbO_2 increased. Consequently, NADH was oxidized and returned to its basal level. When SD was induced (Fig. 5C), three waves where developed. The first wave was characterized by a biphasic response of HbO_2 starting by HbO_2 decrease followed by a large increase of CBF and an increase in HbO_2. As for the NADH, here also a two steps reaction was recorded. NADH was oxidized rapidly by following with a further oxidation at the stage of recovery from the first wave, no one of the parameters fully recovered. CBF drop, HbO_2 decreased and NADH was almost at base line levels. The second and third waves, showed an increase in CBF which was associated with HbO_2 oxygenation. As a result, NADH was oxidized. These type of perturbations were used in many protocols and studies published by our group in the past (Mayevsky and Rogatsky, 2007). In order to illustrate the variability of the responses between animals, the responses of the brain to anoxia, in a group of rats, are shown in Fig. 6 (Meirovich et al., Unpublished results). As seen, a comparison between the responses of normoxic rats ($n = 35$) to partial ischemic animals ($n = 14$) is presented. Ischemia was induced by occlusion of the two common carotid arteries 24 h before the monitoring. As can be seen the responses in the NADH levels and the microcirculatory oxygenation were very similar in the two groups. The main difference in the responses was recorded in the CBF and the reflectance signals. In the partial ischemic brain the change in the reflectance, represent the change in blood volume, was very small as compared to the normal brain in which the blood volume increased due to the lack of oxygen. The CBF response to anoxia presents a different pattern in the two groups. In the normoxic brain, there is a very small drop in CBF during the short anoxia followed by a very large hyperemia during the post anoxic period. In the ischemic group, a clear drop in CBF was recorded before the smaller hyperemic change recorded after the anoxic episode.

It is well accepted that microcirculatory blood flow, cellular energy metabolism and mitochondrial function are potentials targets for anticancer drugs. Each one of those factors is an indicator of tissue vitality or viability and therefore could be used in the testing of new anticancer drugs safety and efficacy. The relationship between mitochondrial metabolic state in vitro, as described by Chance and Williams (1955a,b,c), and the metabolic states of tissues in vivo is shown in Fig. 7. As seen, there are four discrete metabolic states of mitochondria in vitro depending on the availability of oxygen, substrate and ADP as well as the activity of the respiratory chain. In the resting state, state 4, the synthesis of ATP is very low due to the limitation in ADP availability. By addition of ADP, to the suspension of mitochondria, synthesis of ATP will be stimulated leading to the new active state, state 3. In this state the substrate is a

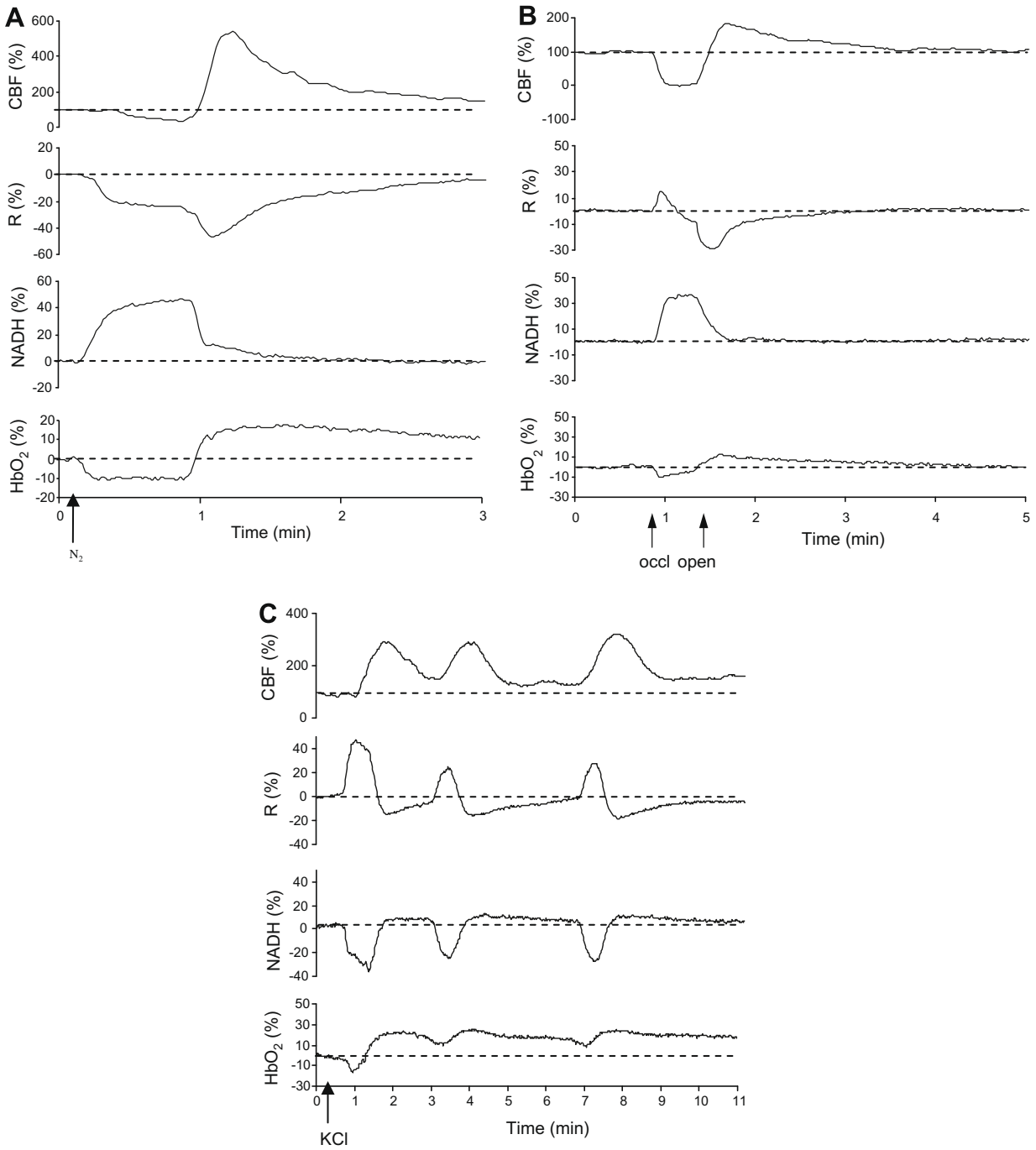

Fig. 5. Typical responses of the rat brain to anoxia (A), Ischemia (B) and cortical spreading depression (C). CBF-cerebral blood flow, R-reflected light at the excitation wavelength (366 nm), NADH-corrected fluorescence of NADH, HbO_2 – Hb oxygenation in the microcirculation.

unlimited factor, oxygen consumption is increased and the limiting factor in ATP synthesis is the activity of the respiratory chain. Elimination of oxygen or substrate will create state 5 or 2, respectively. The three parameters shown in the right side of the figure, namely, NADH redox state, tissue blood flow and HbO_2 are those monitored by the multiparametric monitoring system. The changes in those three parameters under various perturbations are shown on a continuous scale ranging between minimal and maximal levels, since it is impossible to determine the values in absolute units. In our previous reviews we concluded that a tissue under in vivo normoxic conditions is located between states 3 and 4, probably 3.5, of the in vitro concept (Mayevsky and Rogatsky, 2007; Meirovithz et al., 2007). When oxygen is not available (state 5) NADH will reached its maximum level under in vitro and in vivo conditions. This condition will be developed under severe dysfunction of the tissue in vivo i.e. death and blood flow as well as hemoglobin oxygenation will reach its minimal levels. The changes in the three parameters under limitation in microcirculatory blood flow, such as under ischemia, will be proportional to the level of ischemia. Under ischemia, in all tissues and organs, NADH is elevated while blood flow and hemoglobin oxygenation will decrease. When tissue energy metabolism is activated (mimicking the addition of ADP in vitro), the mitochondria will be shifted toward state 3 and blood flow will increase. In parallel, hemoglobin oxygenation may in-

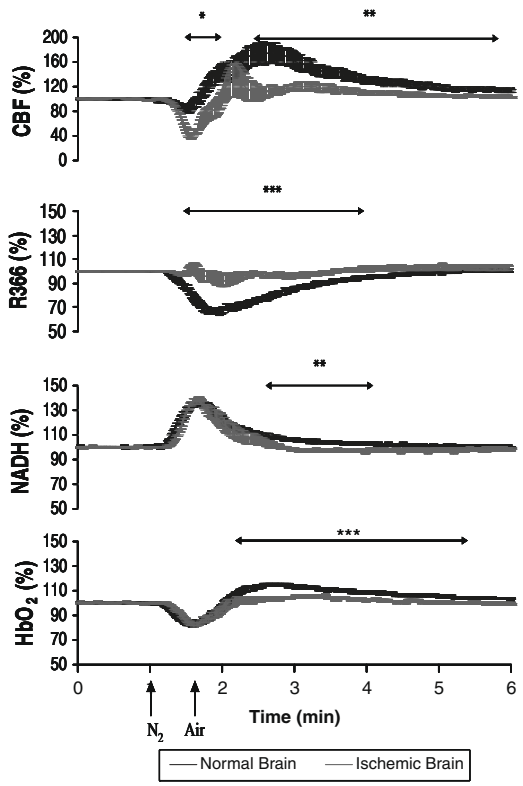

Fig. 6. The effects of anoxia on the monitored parameters in the brain of normoxic ($n = 35$) and ischemic brains ($n = 14$). The abbreviations are as in Fig. 5. (*) $P < 0.05$, (**) $P < 0.01$ and (***) $P < 0.001$.

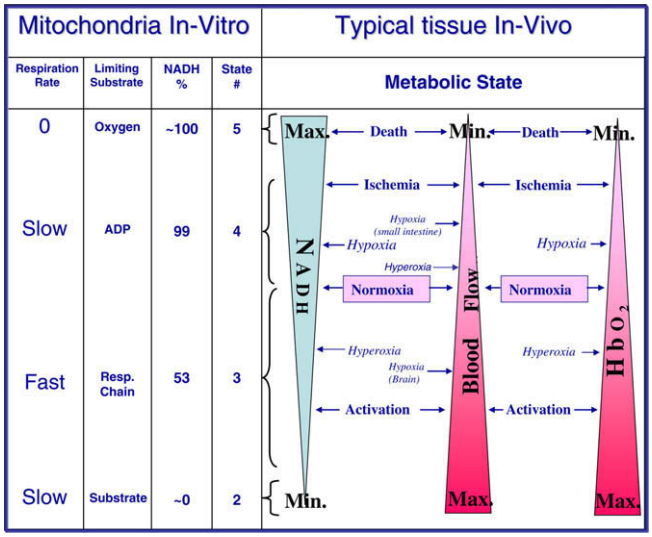

Fig. 7. Comparison between mitochondrial metabolic states in vitro and the typical tissue metabolic states in vivo evaluated by NADH redox state, tissue blood flow and hemoglobin oxygenation as could be measured by the suggested monitoring system.

crease if the oxygen balance is positive namely that extra oxygen consumed by the mitochondria will be compensated by the elevation in microcirculatory blood flow. Under hypoxic conditions the changes in the three parameters, in various organs, will be different from those measured under ischemic conditions although in those two conditions oxygen is the limiting factor. In the brain, hypoxia will lead to increase in cerebral blood flow and NADH is elevated to higher levels depending on the oxygen supply. On the other hand in the small intestine the blood flow will decrease and NADH will increase as well.

The reason for the difference in the responses of the two organs was discussed in our previous publication (Barbiro-Michaely et al., 2007) Exposing the various organs to hyperoxia will increase the level of blood oxygenation and a small oxidation of NADH will be recorded while tissue blood flow may decrease slightly or may remain the same (Meirovithz et al., 2007). It is important to note that the saturation level of HbO_2 is different along the arterial system starting at close to 100% in the large arteries as measured by the pulse oximeter technology. On the other hand, in the microcirculation the saturation level may range between 70–80%, in less active tissues, and 20–40% in very active tissues such as the brain. The oxygenation level is also affected by the size of the vessels (small arterioles and capillaries) monitored by the tissue oximeter.

It is assumed that the changes in the three monitored parameters could vary in different perturbations in tumor tissue as compared to the normal tissue, thus enabling to study the efficacy of new anticancer drug in vivo as well as studying the validity of the Warburg hypothesis.

5. The use of the multiparametric monitoring system in cancer research

Mitochondrial dysfunction and angiogenesis are two major factors in cancer cell activity and tumor proliferation. Therefore, the multiparametric monitoring system could be used to study the "Warburg effect" as part of the pathophysiology of cancer cells and tumors, and to test the efficacy of new anticancer drugs (Certo et al., 2006). It is clear that in order to test the cancer cell bioenergetics, including the mitochondrial dysfunction, only an in vivo monitoring approach will provide meaningful results. Therefore, appropriate animal models should be developed and utilized for such studies.

The critical step in drug development is its testing under in vivo conditions either in experimental animal models or in clinical trials. These studies pursue three major goals:

(1) To evaluate the side effects and safety of new anticancer drug on the various organs in the normal body.
(2) To test the expected drug effects on the tumor in vivo. The mechanism of action of anticancer drug could be tested by the multiparametric monitoring system.
(3) Our monitoring system could be developed into a system that will differentiate between normal and cancerous tissue as was reviewed by Ramanujam (2000). He suggested using fluorescence spectroscopy as a tool to differentiate between neoplastic and non-neoplastic tissues. The use of various endogenous fluorophores such as NADH and flavoproteins was discussed. In the last few years Chance and his collaborators had used the low temperature scanner to monitor NADH and flavoproteins in a frozen sections of tumors (Li et al., 2007; Poptani et al., 2003; Zhang et al., 2004a,b) They rescribed the relationship between the level of mitochondrial redox state and the malignancy of tumors. The disadvantage of their technology is that the tissue is analyzed only in the frozen state therefore the time coarse effect could not be measured in the same tumor and in the same animal.

In order to evaluate the efficacy of a drug or its side effects, it is most appropriate to monitor various physiological or biochemical activities, in real time, as was investigated in many details by the research group of Vaupel (Kelleher et al., 1996; Thews et al., 2002; Vaupel et al., 2006, 2007; Vaupel, 2008; Vaupel and Mayer, 2007). They monitored oxygen partial pressure (pO_2 electrode) to-

gether with microcirculatory blood flow (laser Doppler flowmetry) as indicators of tissue oxygenation in tumors. In our previous review we demonstrated the practical approach of using our monitoring system in drug tissue interaction (Mayevsky et al., 2000). One of the absent tools in the development of anticancer drugs is the lack of experimental animal models for the evaluation of mitochondrial function in vivo. Therefore, we propose to use the multiparametric monitoring system in cancer research. The combination of various monitored parameters at the tissue level enable a better understanding of the relationship between microcirculatory physiological events and the intracellular compartment, namely, the mitochondria. The advantage of this monitoring approach is that, with a single probe connected to the monitoring system, it becomes possible to measure several interconnected parameters representing tissue vitality in various body organs. Although mitochondrial function is the most significant parameter to be monitored, the other three parameters are complementary to the mitochondrial NADH values. This monitoring system could be used in order to evaluate the safety of new drugs developed using in vivo models .This subject of drug safety was reviewed by Boelsterli (2003).

As mentioned in the previous sections, the involvement of the mitochondria in diverse pathophysiological aspects of cancer cells and tumors is very clear and well documented. Also, the angiogenesis process is a significant part of tumor development. Therefore, the monitoring of intravascular parameters (microcirculatory blood flow, blood volume and Hb oxygenation) together with intracellular mitochondrial function will have a tremendous value in testing anticancer drug efficacy and safety in intact animals in vivo.

5.1. Experimental models and protocols for testing anticancer drugs

In this section, few principles of designing experimental protocols, by the user, are presented. This approach must be developed by the user, according to his/her specific experimental setup and the preferred tissue or organ used in the laboratory.

5.1.1. Testing the safety of anticancer drugs

We are suggesting using the multiparametric monitoring system for testing the safety of anticancer drugs. As of today the toxicity testing of new drugs does not include tissue level real time monitoring of physiological and biochemical parameters. Safety could be tested in various organs of different experimental animal models. For example one could monitor simultaneously the cerebral cortex of the brain (most vital organ) together with the wall of the small intestine (less vital organ). This approach was proven to be a reliable and sensitive method in testing the effects of hypoxia, anoxia or hypercapnia (Barbiro-Michaely et al., 2007) They described the technological details of the monitoring system as well as the various tested protocols. In short, the animal is prepared according to our published procedures (Barbiro-Michaely et al., 2007; Mayevsky and Chance, 2007). It is suggested to use three groups of animals that will include at least 10 animals in each group and will have also a 4th group that will be injected in various doses of the tested drug. In order to plot a dose response curve in terms of safety. In the control group, saline will be injected IV or IP (after 1 h of base line recording) according to the injection site of the tested drug. In the second group, the carrier of the drug (if different from saline) will be injected and in the third group the tested drug will be injected. After the injection, the monitoring could continue for 4–6 h and the changes in the four monitored parameters are recorded. The behavior of the parameters in the treated animal is compared to the recording in the two control animal groups. Another possibility is to challenge the monitored organ by a various perturbations such as anoxia, hypoxia or adrenaline injection and the changes in the responses of the various groups, before and after the injection, could be analyze (Barbiro-Michaely et al., 2007; Mayevsky et al., 2000).

It is important to note that each investigator could and should design his own protocols based on his previous experience.

5.1.2. Testing the efficacy of anticancer drugs

In order to test the efficacy of anticancer drugs in vivo it will be necessary to develop the animal model as well as the experimental protocols. It is important to note that our proposed approach was not used yet in testing the effects of anticancer drugs. We have used this approach in testing the mechanism of action of other drugs such as neuroprtectors against ischemia. The main limitation of the suggested multiparametric monitoring approach is the need of direct contact between the fiber-optic probe and the monitored tissue. Therefore one could use the system in tumors located in the surface of the body. Another option is to expose the internal tumor and attached the probe to the surface of the tumor or to penetrate with a needle type probe into the tumor.

In order to test the efficacy of anticancer drugs in tumors two type of protocols could be used. The first approach is to use an acute model, namely, to prepare the tumor for monitoring and measure the effect of the anticancer drug for 6–8 h after the injection. The second and probably the more practical approach is to test the long term effects of the anticancer drug.

5.1.2.1. Acute model. In this approach the short term effects of the anticancer drug is tested assuming the drug may affect the metabolism of the tumor within 6–8 h and two groups of animals are required in this protocol. After monitoring the tumor for 1 h control period, one group of animals will be injected by the tested drug while a second group will receive saline injection. The animal will be monitored for 6–8 h and the level of the parameters during the monitoring period will be recorded. One half of the animals in each group will be exposed to one or more perturbations during the monitoring period and the responses before and after the drug injection will be compared. If the drug will decrease the vitality of the tumor, it is expected that blood flow and oxygenation will be decreased while NADH will be elevated along the time axis. During the perturbation such as anoxia (100% nitrogen breathing) the amplitude of the NADH increase will be smaller along the time axis.

5.1.2.2. Chronic model. Since most anticancer drugs will start to affect the tumor within few days, it will be necessary to monitor the tumor every day and the response to the well define perturbation will be compared. This type of approach will require large groups of animals due to the high variability in the responses. A control group of animals will be injected by saline and the responses to the perturbations will be compared to the drug injected group. For example one can use our system to tests the mitochondrial function after injection of drugs that affect the glycolysis (2-deoxy glucose) or the mitochondria and testing the responses of the tissue to various perturbations in the acute or the chronic model.

In conclusion, the use of the multiparametric monitoring system has an added value to the other approaches used to test the activity of various anticancer drugs. The main advantage in our approach is that mitochondrial function is monitored in vivo and the effect of the anticancer drug is evaluated continuously in real time. Also, the comparison between the mitochondrial function (NADH redox state) and the intravascular parameters (blood flow and oxygenation) will provide information on the effect on angiogenesis as compared to cellular energy metabolism.

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