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INTRODUCTION

Warburg, me and Hexokinase 2: Multiple discoveries of key molecular events underlying one of cancers' most common phenotypes, the "Warburg Effect", i.e., elevated glycolysis in the presence of oxygen

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Abstract As a new faculty member at The Johns Hopkins University, School of Medicine, the author began research on cancer in 1969 because this frequently fatal disease touched many whom he knew. He was intrigued with its viscous nature, the failure of all who studied it to find a cure, and also fascinated by the pioneering work of Otto Warburg, a biochemical legend and Nobel laureate. Warburg who died 1 year later in 1970 had shown in the 1920s that the most striking biochemical phenotype of cancers is their aberrant energy metabolism. Unlike normal tissues that derive most of their energy (ATP) by metabolizing the sugar glucose to carbon dioxide and water, a process that involves oxygen-dependent organelles called "mitochondria", Warburg showed that cancers frequently rely less on mitochondria and obtain as much as 50% of their ATP by metabolizing glucose directly to lactic acid, even in the presence of oxygen. This frequent phenotype of cancers became known as the "Warburg effect", and the author of this review strongly believed its understanding would facilitate the discovery of a cure. Following in the final footsteps of Warburg and caught in the midst of an unpleasant anti-Warburg, anti-metabolic era, the author and his students/collaborators began quietly to identify the key molecular events involved in the "Warburg effect". Here, the author describes via a series of sequential discoveries touching five decades how despite some impairment in the respiratory capacity of malignant tumors, that hexokinase 2 (HK-2), its mitochondrial receptor (VDAC), and the gene that encodes HK-2 (HK-2 gene) play the most pivotal and direct roles in the "Warburg effect". They discovered also that like a "Trojan horse" the simple lactic acid analog 3-bromopyruvate selectively enters the cells of cancerous animal tumors that exhibit the "Warburg effect" and quickly dissipates their energy (ATP) production factories (i.e., glycolysis and mitochondria) resulting in tumor destruction without harm to the animals.

Keywords Cancer · "Warburg effect" · Glycolysis · Mitochondria · Hexokinase 2 · Drug target · Cancer therapy · 3-bromopyruvate · Positron emission tomography (PET)

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Introduction

This mini-review is the introductory article in a mini-review series entitled "The Warburg Effect: Its Continued Impact on Cancer Research into the Twenty First Century". Other "corresponding authors" of articles contributed to this mini-review series are Drs. Jamie Caro, Gregg Semenza, Catherine Godinot, Paul M. Hwang, Paul C. Herrmann, Robert Gillies, Jose Cuezva, and Peng Huang. The reader is referred to each of these for a review of additional developments related to the "Warburg effect" in cancer and likely also to somewhat different points of views than the author of this introductory article and from each other.

Definition of the "Warburg effect"

Normal animal and human tissues convert most of the sugar (glucose) they consume to the compound "pyruvic acid" via a process called "glycolysis", after which the pyruvate enters intracellular factories called "mitochondria" where it

is oxidized to carbon dioxide (CO_2) and water (Devlin 2006). The free energy derived from this "oxygen dependent" process is utilized to drive the synthesis of the "high energy" compound ATP from ADP and inorganic phosphate (P_i) via an "ATP synthasome" (Ko et al. 2003; Chen et al. 2004). This large enzyme complex located in the mitochondrial inner membrane is comprised of the ATP synthase and transport systems for ADP and P_i that deliver these substrates directly to the synthase. For each molecule of blood glucose that is metabolized to carbon dioxide and water via the above noted process, sufficient free energy is generated to drive the ATP synthase to make over 30 molecules of ATP from ADP and P_i (Devlin 2006).

In sharp contrast to most normal tissues, it was shown by the German biochemist Otto Warburg (Fig. 1a) in the early part of the past century (Warburg 1930) that cancerous tumors that originate within them frequently develop a modified sugar (glucose) metabolism whereby a significant portion of the blood sugar consumed by the tumor is converted one step beyond pyruvate, i.e., to lactic acid, even when oxygen is plentiful ("Warburg effect", Fig. 1b). That is, even when the tumor mitochondria have sufficient oxygen to metabolize all the pyruvate formed from the blood glucose to carbon dioxide and water, the tumor via the process of "glycolysis", i.e., conversion of glucose to lactic acid, assumes a significant role in energy (ATP) production. As glycolysis produces only two net molecules of ATP per glucose molecule converted to lactic acid (Devlin 2006), the cancer cells remodel their glycolytic and mitochondrial machinery such that the former is up regulated (goes faster) and the latter is down regulated (goes slower). In summary, the "Warburg effect" refers to a cancerous tumor's increased utilization of the glycolytic pathway for energy (ATP) production (and likely other needs) even though plenty of oxygen is present to support mitochondrial function.

Benefits of the "Warburg effect" to cancer

The "Warburg effect" likely provides the vast majority of cancerous tumors that exhibit this phenotype with a number of benefits. One is *biosynthesis*. A rapidly dividing cancer cell needs carbon precursors that are involved in the biosynthesis of cell building blocks. The glycolytic pathway and its off-shoot the pentose phosphate pathway (hexose monophosphate shunt) are rich sources of precursors essential for the biosynthesis of nucleic acids, phospholipids, fatty acids, cholesterol, and porphyrins. Thus, maintaining a high glycolytic rate within each tumor cell of a given tumor, even in the presence of oxygen (i.e., the "Warburg effect"), assures not only the tumor's survival but its rapid growth. A second advantage of the "Warburg effect" is likely involved in both *tumor protection and invasion*. As tumor cells via glycolysis, even in the presence of oxygen, produce lactic acid and transport it out, this acid (i.e., its low pH) may both protect tumors (that are resistant to it) against attacks by the immune system while inducing negative effects (chemical warfare) on normal surrounding cells, thus preparing them for invasion. Finally, and not least important, the "Warburg effect" also assures a *longer tumor survival time if oxygen become limiting*. This is because the enzymes that catalyze the already high glycolytic rate are themselves not dependent directly on oxygen. In fact, the genes that encode them are activated by hypoxic conditions. Thus, evidence that a given tumor exhibits the "Warburg effect" is also evidence that the same tumor is likely to survive longer (not necessarily grow) when oxygen is either limiting or absent (hypoxic or anoxic conditions). In fact, in Warburg's famous book "The Metabolism of Tumors" (Warburg 1930) he states based on his own experiments that tumors can survive even in the absence of oxygen. In such a case, any glucose that enters the glycolytic pathway would likely be converted almost completely to lactic acid.

Misconceptions about the "Warburg Effect"

Unfortunately, what has been attributed to Warburg (Fig. 1a) or called the "Warburg effect" in some current literature and particularly in Press releases is not completely correct. For example, it is quite common for some writers to state or implicate Warburg as showing or stating that cancerous tumors, unlike normal tissues, rely predominantly or exclusively on glycolysis for their energy (ATP) production rather than on mitochondria. Although Warburg showed that many tumors exhibit a high glycolytic rate even in the presence of oxygen, he emphasized also that the division of labor in energy production (i.e., making ATP) even in the most rapidly growing tumor cells that he tested (e.g., ascites) usually does not exceed 50% for the glycolytic contribution. Thus, in an article that appeared in Science almost 50 years ago Warburg (1956) summarized the results (oxygen and lactic acid measurements) of an experiment that he and his colleagues performed on highly malignant mouse ascites tumor cells by stating the following: "This converted to energy equivalents means that the cancer cells can obtain approximately the same amount of energy from fermentation (i.e., glycolysis to lactic acid) as from respiration (i.e., mitochondria) whereas the normal body cells obtain much more energy from respiration than from fermentation" (enclosures in parenthesis were inserted by the author).

Use of the "Warburg effect" as a method for detecting and locating cancer

As the "Warburg effect" is such a common property (phenotype) of cancer cells, it is not surprising that a method based on this phenotype has been developed for diagnosing cancers. Although the technique of PET imaging (Fig. 1c) was first described over 30 years ago by Phelps et al. 1975, its first use in the imaging of cancer was not described until 7 years later (Di Chiro et al. 1982). This method named (F-18) FDG PET imaging, i.e., ^{18}fluorine labeled 2-deoxyglucose positron emission tomography imaging) or simply "PET Imaging" is used worldwide today as a diagnostic tool to detect malignant tumors. Significantly, PET Imaging, although involving the first two steps of glycolysis, i.e., the transport of (F-18) 2-deoxyglucose (2-DG) across the tumor cell membrane via a specific transporter followed by the phosphorylation of the glucose to give (F-18) 2-DG-6-phosphate, effectively monitors the second step which is catalyzed by the enzyme hexokinase. This is due to the chemistry of 2-DG which upon forming 2-DG-6-phosphate in the hexokinase reaction is not further metabolized.

Elucidating the biochemical and gene-related basis of the Warburg Effect so that key players involved can be targeted

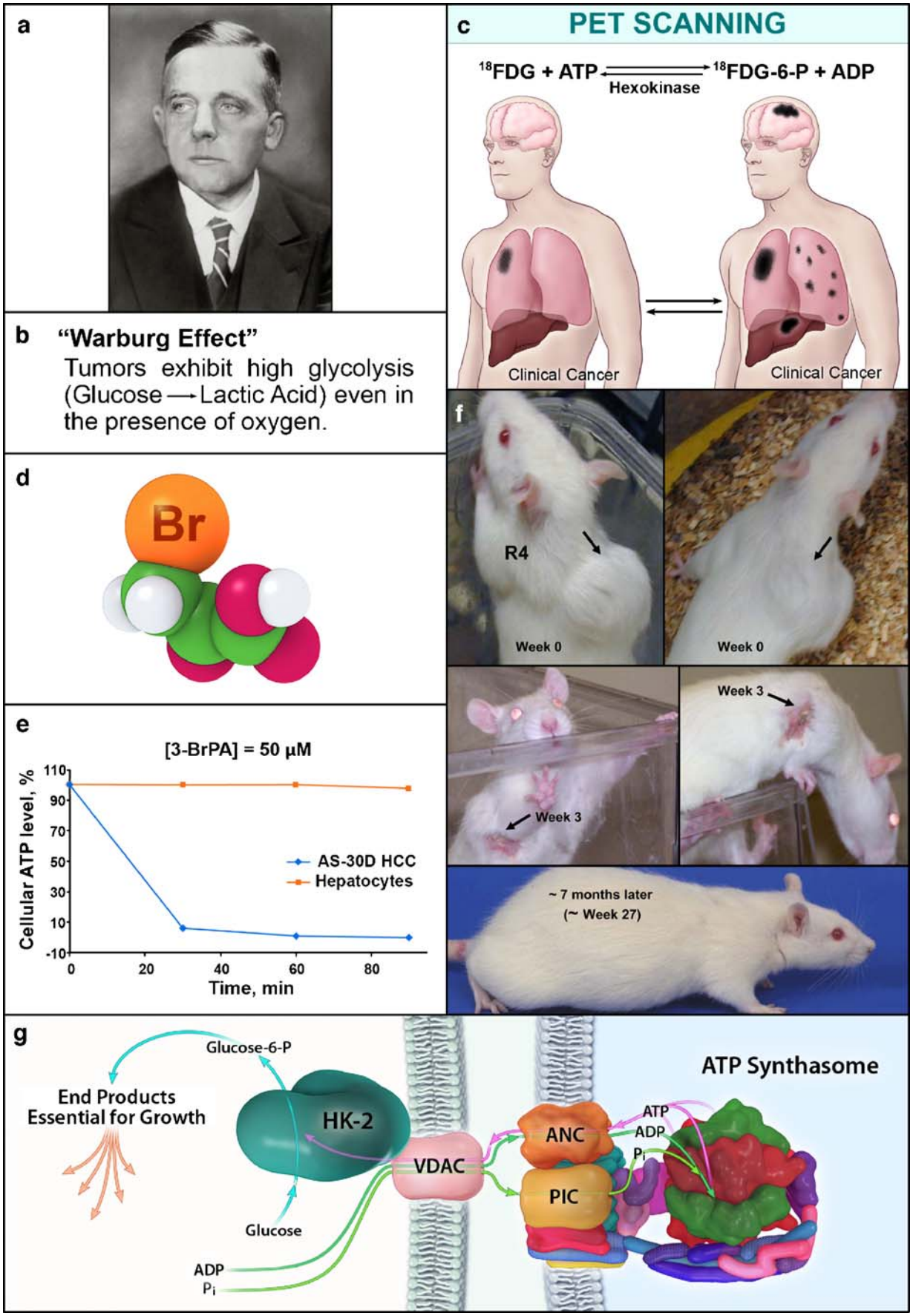
Although the development of diagnostic tools such as "PET" and other "imaging" techniques have taken physicians a giant step forward in detecting cancers in clinics throughout the world, progress in curing these detected cancers has lagged far behind. As the author emphasized in an earlier review (Pedersen 2007), unless cancer scientists resolve this very serious problem soon the ultimate cause of death for much of the worlds' current population will be cancer. In fact, if we are not fighting this disease ourselves, we likely know a family member or friend who is. Therefore, cancer is really the enemy of us all, i.e., all nations and all people, and as any military leader well knows the best way to defeat one's enemy is to understand that enemy and focus on their Achilles heel(s). For cancer, the Achilles heels are synonymous with cancer's predominant phenotypes. Here, the oldest known phenotype and certainly one of the most common, if not the most common, is the "Warburg effect", i.e., increased glycolysis (glucose metabolism to lactic acid) even in the presence of oxygen (Warburg 1930).

In this brief mini-review, the author describes a series of sequential discoveries related to the "Warburg effect" that were made during the past five decades in his laboratory (or in collaborations with his laboratory). These sequential discoveries led to the conclusion that hexokinase 2 (HK-2), the protein (VDAC) that binds HK-2 to the outer mitochondrial membrane, and the gene (HK-2 gene) that encodes it, play the most pivotal and direct roles in the "Warburg effect" and likely essential roles also in cancer growth, survival, and metastasis. The author also describes briefly one of the most recent discoveries in his laboratory. That is, how a simple analog of lactic acid, by taking advantage of the "Warburg effect", enters and destroys cancer cells while sparing normal cells (Ko et al. 2001, 2004), and in live animals eradicates advanced (PET-positive) cancers without harm to the animals (Ko et al. 2004).

Preparatory work and tumor models, "the beginning"

Having carried out postdoctoral work with the late Albert L. Lehninger who gave his students considerable freedom, the author learned prior to joining the faculty at Johns Hopkins School of Medicine (Baltimore, MD) in 1969 how to single-handedly prepare intact mitochondria, monitor their basic properties (respiration and ATP synthesis), purify, assay and characterize mitochondrial enzymes, and with the help of an indefatigable postdoctoral colleague Carl Schnaitman, also how to carefully sub-fractionate mitochondria into their four compartments: outer membrane, inner membrane, intra-cristal space (soluble space between these membranes) and matrix (soluble space enclosed by the inner membrane) (Schnaitman and Greenawalt 1968; Chan et al. 1970). These basic tools, all of which are essential even today for those who wish to study mitochondria in depth, permitted the author to enter the cancer field with a focus on elucidating those key molecular events involved most directly in the "Warburg effect" and therefore in the growth and progression of numerous cancers.

The author's entry into the cancer field was made possible by Albert Lehninger for reasons noted above and by the generosity of Harold P. Morris an established cancer scientist who had spent several decades at the nearby National Cancer Institute (NCI) developing many different liver cancer (hepatoma) lines that grew in the hind limbs of rats (Morris 1965). Three of these hepatoma lines (3924A, 7800, and 9618A) kindly provided to the author varied in growth rate with hepatoma 3924A being the most rapidly growing, 9618A the slowest, and hepatoma 7800 intermediate. Together with two other cancer cell lines, a rat hepatoma line (AS-30D) developed at the M.D. Anderson Hospital and Tumor Institute by Smith et al. (1970), and the readily available Ehrlich ascites mouse line, the author had in hand the experimental systems that he and his students/collaborators needed to arrive at a molecular understanding of the "Warburg effect". With the exception of the AS-30D hepatoma line that the author's laboratory would show exhibits a pronounced "Warburg effect", all other tumor lines noted above had been shown already to exhibit this


a
b
"Warburg Effect"
Tumors exhibit high glycolysis (Glucose ⟶ Lactic Acid) even in the presence of oxygen.
c
PET SCANNING
18FDG + ATP ⟶ 18FDG-6-P + ADP
Hexokinase
Clinical Cancer
Clinical Cancer
d
Br
e
[3-BrPA] = 50 μM
Cellular ATP level, %
110
90
70
50
30
10
-10
0
20
40
60
80
Time, min
AS-30D HCC
Hepatocytes
f
R4
Week 0
Week 0
Week 3
Week 3
~ 7 months later
(~ Week 27)
g
Glucose-6-P
End Products
Essential for Growth
HK-2
VDAC
ANC
PIC
ATP
ADP
Pi
ATP Synthasome
Glucose
ADP
Pi

◀ **Fig. 1** **a** Photograph of Otto Warburg. Otto Warburg was born in Freiburg, Germany on October 8, 1883 and died on August 1, 1970. He was awarded doctoral degrees in chemistry and medicine and became widely known for his experimental work on cellular respiration and cancer summarized in his book entitled "The Metabolism of Tumors" (London Constable, 1930). He was awarded the Nobel Prize in 1931 for his work on cellular respiration and it relationship to cancer (Copyright © The Nobel Foundation. The Noble Foundation has granted Springer a one-time non exclusive permission to use the photograph in the Journal of Bioenergetics and Biomembranes. The photo should not be reproduced or redistributed without the permission of the Nobel Foundation). **b** Abbreviated Definition of the "Warburg effect": Many tumors, both animal and human, exhibit a high rate of conversion of glucose to lactic acid (glycolysis) even when sufficient oxygen is available for mitochondrial function. **c** Biochemistry on which Positron Emission Tomography (PET) for cancer is based. This imaging technique is based on the use of radiolabelled 2-deoxyglucose that is taken up by cancer cells and phosphorylated by the enzyme hexokinase (frequently HK2) to yield 2-deoxyglucose-6-phosphate (2DG) that is not further metabolized. As many types of cancer cells are known to utilize more glucose than their normal cells of origin, tumors containing such cells are frequently "PET positive". **d** The Chemical Agent 3-Bromopyruvate Acid (3-BrPA). This simple agent is an analog of lactic acid differing in only one atom. However, because of its chemistry 3-BrPA is highly reactive. Moreover, as it is a structural analog of lactic acid it is believed to takes advantage of the "Warburg Effect". That is, 3-BrPA is believed to selectively enter cancer cells via the enhanced number of lactic acid transporters that are present and once inside to use its alkylating properties to block energy production, i.e., ATP production by both glycolysis and mitochondria. **e** Capacity of 3-BrPA to kill liver cancer cells (AS-30D hepatocellular carcinoma cells) that exhibit the "Warburg effect" relative to its capacity to kill normal liver cells, i.e., hepatocytes, and **f**. Capacity of 3-BrPA to eradicate in animals (rats) large cancers (hepatocellular carcinomas) derived from the AS-30D cells. The *in vitro* experiments presented in **e** shows that 3-BrPA at the levels used by Dr. Young Ko selectively kills the cancer cells (hepatocellular carcinoma cells) while leaving the normal liver cells alone, while the *in vivo* experiment in **f** shows that when 3-BrPA is injected at the site of a large hepatocellular carcinoma growing in the upper back of the rat "R4" the tumor disappears within 3 weeks. (Permission granted from Elsevier to reproduce Figures 4 and 5 (**e** and **f** here) from the following article: Ko YH, Smith BL, Wang Y, Pomper MG, Rini DA, Torbenson MS, Hullihen J, Pedersen PL Advanced cancers: eradication in all cases using 3-bromopyruvate therapy to deplete ATP. Biochem Biophys Res Commun. 2004 Nov 5;324 (1):269–75. **g** Current view showing how HK-2 bound to VDAC on the outer membrane has preferred access to ATP synthesized on the inner membrane by the ATP synthasome, a complex between the ATP synthase and carriers (transporters) for ADP and phosphate (P_i). The structural integrity of this entire network is essential for the survival of those cancer cells that exhibit the "Warburg" effect. Thus, HK-2 while preventing apoptosis by binding to VDAC, also supports cancer cell growth by receiving preferred access to ATP newly synthesized by the ATP synthasome

"effect". Although there had been some controversy related to whether the slower growing Morris hepatomas, e.g., 7800 and 9618A, exhibit a "Warburg effect", studies at the NCI by Burk et al. (1967) reported a low but significant effect. Consistent with their work and the much earlier work of Warburg (1930) is a recent genomic study (Altenberg and Greulich 2004) that shows that many of the isozymes of the glycolytic pathway are over-expressed in 24 cancer classes representing more than 70% of human cancer cases worldwide. In general, as shown by Weber and Lea (1966), Lo et al. (1968) and others near the middle of the last century, the degree of the "Warburg effect" is related to tumor growth rate with the most rapidly growing, i.e., the most viscous tumors, exhibiting the greatest "effect".

Taking advantage of the above indicated training, prior knowledge and available experimental systems, the author describes below in sequential order the discoveries made in his laboratory that played a major role in the elucidation at the mitochondrial, molecular, and gene level of those pivotal factors involved most directly in the "Warburg effect", i.e., elevated glycolysis in the presence of oxygen. An attempt is made throughout this article to acknowledge the many students (pre-doctoral, postdoctoral, and others), and some collaborators, that were involved in this exciting endeavor. Certainly, without their hard work and enthusiasm for the project it would not have been possible and ongoing even today. Also, the author's technician Joanne Hullihen is acknowledged for her participation since 1969 in many of these studies.

Discovery that cancerous tumors that exhibit the most pronounced "Warburg Effect" have functional mitochondria with the capacity to make ATP but these are reduced in content per cell resulting in a lower net oxygen consumption (respiratory) capacity

The initial studies in the author's laboratory related to the "Warburg effect" were carried out in 1969 and published as two papers in "Cancer Research" 1 year later (Schreiber et al. 1970; Pedersen et al. 1970), the year of Warburg's death. These studies were conducted with James Schrieber, a medical student, T.L. Chan, a postdoctoral fellow, John Greenawalt and Walter Balcavage, both faculty colleagues, and in collaboration with Harold Morris who had moved from the NCI to assume a faculty position in the Department of Biochemistry at Howard University, College of Medicine. Significantly, these studies employed the rapidly growing Morris hepatoma 3924A that exhibits a pronounced "Warburg effect", i.e., high glycolysis in the presence of oxygen, and also employed the slower growing Morris hepatomas 7800 and 9618A.

The initial studies conducted on purified mitochondria isolated from the slower growing hepatomas 9618A and 7800 revealed little or no difference in their capacity to respire (consume oxygen) relative to normal liver mitochondria. This included substrates the electrons of which enter the first complex of the electron transport chain (i.e., Complex I, NADH dehydrogenase) and proceed to its end (i.e., Complex IV, cytochrome oxidase) where they reduce molecular oxygen to produce water. This was the expected findings as the 9618A and 7800 hepatomas grow slowly

(9618A) or relatively slowly (7800), and although exhibiting a "Warburg effect", it is very low.

Studies on freshly isolated mitochondria from Morris hepatoma 3924A that does exhibit a pronounced "Warburg effect" were shown in the presence of succinate (electrons of which enter at the level of Complex III of the electron transport chain) to have the capacity to respire (consume oxygen) at a rate even greater than control mitochondria. Moreover, this rate was stimulated by addition of ADP, indicating that ATP synthesis via the ATP synthase is functional. Finally, measurements of the activity of cytochrome oxidase (Complex IV), i.e., the terminal enzyme of energy metabolism that interacts directly with oxygen, showed that its activity is at least as high as control liver mitochondria. Finally, direct measurements of the capacity of hepatoma 3924A mitochondria to make ATP from ADP and P_i showed that this critical function, although somewhat lower than that of liver mitochondria, is nevertheless operative. Thus, these initial studies showed that mitochondria of hepatoma 3924A that exhibit the "Warburg effect" are not markedly impaired in their capacity to respire (consume oxygen) and transport electrons from Complex III of the electron transport chain to molecular oxygen. Also, these mitochondria are not impaired in their capacity to make ATP at the level of the ATP synthase. Nevertheless, cytochrome oxidase measurements on tumor homogenates showed that on a whole cell basis the activity of this enzyme is significantly diminished implicating a marked reduction in mitochondria in hepatoma 3924A. In addition, examination of the literature available at that time (Reviewed in Table 1, Pedersen 1978) repeatedly showed that the yield of mitochondria from a variety of tumors is also markedly reduced. Therefore, these initial studies conducted in 1969 in the author's laboratory (Schreiber et al. 1970; Pedersen et al. 1970) taken together with data of others were certainly consistent with Warburg's view/experiments (Warburg 1930) that the total respiratory capacity of cancers that exhibit a pronounced "Warburg effect" may be compromised. Further support for this view, was obtained over a decade later in the laboratory of Papa and colleagues (Emboli et al. 1977) in Italy where they showed that the capacity of mitochondria isolated from tumors that exhibit the "Warburg effect" also exhibit a markedly reduced capacity to utilize pyruvate as substrate. Thus, mitochondria isolated from hepatoma 3924A and Ehrlich ascites cells had a 50% or more reduction in their capacity to transport electrons from pyruvate to molecular oxygen than did control rat liver mitochondria.

The combination of these initial studies in the author's laboratory (Schreiber et al. 1970; Pedersen et al. 1970), a number of other studies revealing a reduced content of mitochondria in tumors exhibiting the "Warburg effect" (Reviewed in Table 1, Pedersen 1978), and the studies of Emboli et al. (1977) demonstrating a reduced capacity of such tumor mitochondria to oxidize pyruvate, the end product of glycolysis in aerobic cells, certainly provided support for Warburg's view that some dysfunction in mitochondria and/or their turnover may contribute to the "effect" bearing his name.

However, with the above said, it will be noted below that additional experiments by the author and his students/collaborators that commenced in 1975 demonstrated that the "Warburg effect" (phenotype) per se, i.e., increased glycolysis even in the presence of oxygen, results most directly from the involvement of the following three critical factors: (1) hexokinase 2 (HK-2) that is over-expressed, (2) the voltage dependent anion channel (VDAC) that binds HK-2 to the mitochondria, and (3) the gene that encodes HK-2 (HK-2 gene) that is both amplified and up-regulated. Justification for a pivotal role of each of these three factors in the "Warburg effect" is based on past experiments conducted in the author's laboratory as summarized below.

Discovery that an over-expressed mitochondrial bound form of hexokinase plays the pivotal biochemical role in the "Warburg effect"

The discovery in the author's laboratory in 1976/1977 that a form of hexokinase plays the pivotal biochemical role in the "Warburg effect" had a "round about" origin. Ernesto Bustamante, a pre-doctoral student, was experimenting with different culture media to support the growth of the rat hepatoma ascites cell line (AS-30D) that the author had obtained from the M.D. Anderson Hospital and Tumor Institute. In fact, Bustamante had adapted the cell line to grow in tissue culture and named the cultured cells the "H-91" cell line. In the course of these studies, he found that when glucose is the sugar in the medium the H-91 cells produced high amounts of lactic acid in the presence of oxygen ("Warburg effect") as expected, but when the sugar was replaced with galactose the cells still grew but much less lactic acid was produced. Transport studies ruled out a difference in the rates of entry into the hepatoma cells of galactose vs glucose. Confused, Bustamante and the author examined a metabolic chart. As expected, it showed that when glucose enters hepatocytes it is converted via the enzyme hexokinase (HK-4/glucokinase) immediately to glucose-6-phosphate that is further metabolized via glycolysis to pyruvic acid, most of which then enters mitochondria with only a small amount being converted to lactic acid. In sharp contrast, however, we learned from the same chart that when galactose enters such cells it bypasses the hexokinase step in its conversion to glucose-6-P via a pathway known as the "LeLoir pathway", named after a Brazilian Scientist Luis LeLoir who received for its discovery the Noble prize in 1970, the same year of Warburg's death.

Bustamante and Pedersen (1977) concluded that the simplest interpretation of the above noted experimental results was that an isozyme of hexokinase, distinct from that found in normal liver, plays the key role in facilitating the high glycolytic activity of the H-91 liver cancer cell line and therefore the key (direct) biochemical role in the "Warburg effect". In the same study, evidence was provided that showed hexokinase is bound to the outer mitochondrial membrane and is directly coupled to ATP synthesis on the inner membrane. This provides high levels of glucose-6-phophate that "jump start" the glycolytic pathway ultimately leading to high levels of lactic acid in the presence of oxygen, i.e., the "Warburg effect". Finally, these studies showed also that the form of hexokinase bound to the outer membrane, unlike the unbound form, was not inhibited by its product glucose-6-phosphate.

The pivotal role of the mitochondrial bound hexokinase in the "Warburg effect", i.e., increased glycolysis even in the presence of oxygen, was brought home in much greater detail 4 years later in a second report by Bustamante et al. (1981). These studies were conducted first with Ehrlich ascites tumor cells that are known to exhibit a pronounced "Warburg effect". Significantly, the high aerobic glycolytic rate catalyzed by the Ehrlich tumor cytoplasm was reduced markedly when the hexokinase-containing mitochondria were removed and almost completely restored upon adding purified Ehrlich ascites mitochondria to the tumor cytosol free of mitochondria. Perhaps even more impressive was the finding that upon addition of the hexokinase-containing tumor mitochondria to the liver cytosol, that exhibits little glycolysis, the glycolytic rate was elevated to the level of the tumor cytoplasm. In the same study it was shown that hexokinase is bound to mitochondria isolated from all highly glycolytic tumor cells (or tumors) examined including the H-91, Ehrlich, L1210, Novikoff, and Morris 3924A.

Finally, a confirmatory study was conducted shortly thereafter by David Parry a new postdoctoral fellow whose work focused on Novikoff hepatoma ascites cells carried in rats (Parry and Pedersen 1983). This study involving a careful sub-fraction procedure confirmed that the hexokinase found in these cancer cells is bound also to the outer mitochondrial membrane. In addition, this study revealed that there is no detectable glucokinase (HK-4), the predominant high Km (for glucose) hexokinase isozyme found in liver, the hepatoma's tissue of origin. Thus, in the transformation of a liver hepatocyte to become a highly glycolytic hepatoma cell that exhibits the "Warburg effect", the expression of the high Km (for glucose) hexokinase (HK-4) is silenced while the low Km (for glucose) hexokinase, later identified by the author's laboratory as hexokinase-2 (HK-2), is overexpressed.

In summary, these original biochemical based studies that commenced with a variety of cancer cell types in 1969 and were completed by 1983 left little doubt that a form of hexokinase bound to the outer mitochondrial membrane plays the pivotal biochemical role in the "Warburg effect", i.e., high glycolysis in the presence of oxygen. Although studies by Rose and Warms (1967) had shown earlier in some very careful studies that a form of hexokinase, later identified as hexokinase 2 (HK-2), is bound to mitochondria of sarcoma ascites tumor cells, their studies were focused on the kinetic properties of the enzyme and not on the elucidation of the "Warburg effect" per se, the objective of the author's laboratory.

Discoveries showing that the receptor for hexokinase in the outer mitochondrial membrane of cancer cells that also contributes to the "Warburg effect" is the protein named "VDAC" (Voltage Dependent Anion Channel) and that the Isoform of Hexokinase is HK-2

As indicated above, the earlier studies of Bustamante and Pedersen (1977) showed that a form of hexokinase bound to the outer mitochondrial membrane of the cancer lines studied is essential for the "Warburg effect", i.e., high rate of conversion of glucose to lactic acid in the presence of oxygen (i.e., high aerobic glycolysis). Moreover, in these same studies we had shown also that when bound to the mitochondria the enzyme is not inhibited by glucose-6-phosphate, the product of the hexokinase reaction. However, upon freezing and thawing the mitochondria to release the hexokinase from its outer membrane binding site the enzyme became markedly inhibited by glucose-6-phosphate. This implicated the importance of a second protein, i.e., an integral membrane "receptor" protein, as being critical for the "Warburg effect" as its prevention of product inhibition of hexokinase has the net effect of stimulating glycolysis. For this reason, our efforts then focused on identifying the mitochondrial receptor for hexokinase.

A new postdoctoral fellow, Richard Nakashima, took on this challenging task in the author's laboratory in 1985 and isolated a protein from the outer mitochondrial membrane of AS-30D hepatoma cells that bound the carboxyl labeling agent dicyclohexylcarbodiimide (DCCD). Nakashima showed also that DCCD prevented the solubilized hepatoma hexokinase from binding to rat liver mitochondria. This project was completed (Nakashima et al. 1986) via a collaboration with Marco Colombini and Patrick Mangan at the nearby University of Maryland in which the DCCD-binding protein that Nakashima had isolated and purified from AS-30D hepatoma cells was identified as VDAC (voltage dependent anion channel), a protein that Colombini had purified earlier from rat liver mitochondria (Colombini 1979). Finally, in a subsequent study in the author's laboratory by Nakashima, a second postdoctoral Fellow, Marco Paggi, and predoctoral student Laura Scott, the form of hexokinase bound to the

outer mitochondrial membrane was identified biochemically as hexokinase 2 (HK-2) (Nakashima et al. 1988).

In confirmation of the above studies, Linden et al. 1982 working independently in Stockholm with Zajdela hepatoma cells showed that 75% of the total cellular hexokinase is bound to the mitochondria via a protein they referred to at the time as a "pore protein". Significantly, they showed also that addition of glucose to such cells respiring only on endogenous mitochondrial substrates markedly inhibited their respiration (Nelson et al. 1984), thus further confirming a pivotal role for mitochondrial bound HK in the "Warburg effect", i.e., high glycolysis even in the presence of oxygen.

Discovery that HK-2 binding to VDAC in the outer mitochondrial membrane facilitates the "Warburg Effect" by allowing preferred access to ATP synthesized on the inner membrane

As emphasized above the "Warburg effect", i.e., increased glycolysis in the presence of oxygen, is facilitated in many cancers not only by the high amounts of HK-2 but also by the binding of this enzyme to VDAC in the outer mitochondrial membrane. This prevents the enzyme from being severely inhibited by its product glucose-6-P. In still other work carried out in the author's laboratory Krishan Arora, a postdoctoral fellow, showed that HK-2 bound to the outer mitochondrial membrane also synthesizes glucose-6-P faster when the ATP is provided by the ATP synthase located in the inner mitochondrial membrane than when the ATP is provided in the medium (representative of the cytosolic compartment; Arora and Pedersen 1988). In other words, HK-2 bound to VDAC in the outer mitochondrial membrane has preferred access to ATP synthesized in the inner membrane and thus the rate at which glucose-6-P is synthesized by this "design" is higher than that which would result if the newly synthesized ATP diffused into the cytosol and then to the active site of HK-2 bound to the outer membrane.

Reflections on the completed biochemical studies described above in relation to both the "Warburg Effect" and to PET imaging now used worldwide to diagnose human cancer

The biochemical studies that were commenced in the author's laboratory in 1969 and completed almost two decades later in 1988 had demonstrated without the use of any genetic or molecular biological tools that the underlying biochemical basis of the "Warburg effect", i.e., increased glycolysis even in the presence of oxygen, resides at the level of the mitochondria and involves two key proteins, HK-2 and VDAC. Although it might be argued that glycolytic enzymes other than HK-2 are involved in the "Warburg effect", and likely they do contribute, it should be re-emphasized that in our early studies that removal of tumor mitochondria containing VDAC-bound HK-2 from the tumor cytosol markedly suppressed glycolysis (Bustamante et al. 1981), an event that could be reversed almost completely by re-addition of the same. This speaks highly for HK-2 as the pivotal enzyme involved in the "Warburg effect" and, together with its binding partner VDAC, one of two major protein players. This conclusion is further reinforced by the finding that addition of tumor mitochondria (containing HK-2 bound to VDAC) to liver cytosol enhances its very low glycolytic rate to levels approaching those of the tumor cytoplasm (Bustamante et al. 1981). It is reinforced also by numerous reports over the past three decades that mitochondria isolated from a number of different cancer types, including human cancers, contain HK-2 (Reviewed in Pedersen et al. 2002 and Mathupala et al. 2006).

As it relates to PET imaging now used to both diagnose/detect many human cancers throughout the world and monitor their treatment (Reviewed in Phelps 2000), it should be noted that early PET monitored an elevation of a product of the hexokinase reaction using [F18] 2-deoxyglucose that was synthesized by Indo et al. (1978), 1 year after the author's laboratory (Bustamante and Pedersen 1977) reported that a mitochondrial bound form of hexokinase is a major player in the high glycolytic activity of cancers, i.e., the "Warburg effect". Four years later [F18] 2-deoxyglucose was reported for use in cancer patients (Di Chiro et al. 1982) following an extensive study at the National Cancer Institute. Although reference to the earlier work in the author's laboratory, i.e., Bustamante and Pedersen (1977) and Bustamante et al. (1981) is not found in the later report of Di Chiro et al. (1982), it is comforting to know that the pioneering efforts of Bustamante and the author (1977, 1981) also supported by the National Cancer Institute, likely helped, or perhaps led to a clinical application that has proved invaluable throughout the world.

Discoveries of structure/function relationships within hexokinases

Following the discoveries above that clearly showed a direct involvement of HK-2 and VDAC in the "Warburg effect" that is characteristic of the vast majority of cancers, the focus in the author's laboratory turned briefly to better understanding hexokinases as enzymes. Although a three-dimensional structure of the yeast hexokinase had been obtained earlier (Bennett and Steitz 1980) that revealed putative substrate binding sites, these had not be verified by site directed mutagenesis in a mammalian hexokinase. Therefore, Krishan Arora a postdoctoral fellow and two visiting scientists Charles Filburn and Maurizio Fanciulli

tackled this problem in the author's laboratory. Following the cloning and sequencing of a gene for hexokinase from a mouse hepatoma cell line, they proceeded to over-express both wild type and mutant forms of the enzyme (Arora et al. 1990; 1991; 1993), the latter involving site directed mutations based on the known crystal structure of the homologous yeast enzyme (Bennett and Steitz 1980). These studies confirmed the identification of amino acid residues at the catalytic site and showed also that both the catalytic and inhibitory sites for glucose-6-phosphate reside within the C-terminal half. Although it seems likely that HK-2 binds via its N-terminal half to the mitochondrial VDAC protein in highly glycolytic cancer cells, this remains to be demonstrated using tumor mitochondria.

Discoveries that the gene encoding HK-2 is amplified in cancer cells exhibiting the "Warburg Effect", its message is over-expressed, its promoter promiscuous in terms of activators (including HIF-1 and mutated p53), and that it is subject to epigenetic control

Although work in the author's laboratory described above demonstrated via biochemical methods alone that HK-2 and VDAC play pivotal roles in the "Warburg effect" characteristic of numerous cancers, it seemed important in the last decade of the past century, to move this problem to the gene level. This was done successfully and extends into the current century with the bottom line that the HK-2 gene, as expected, plays a major "behind the scenes" role in the "Warburg" effect. Published work related to this topic involved a number of dedicated students (pre- and postdoctoral) together with some collaborators. Collectively, these include Saroj Mathupala, Annette Rempel, Curt Heese, Ashish Goel, and Min Gyu Lee, Constance Griffin, and Anita Hawkins, the latter two participating as collaborators. Much of their work that has emphasized the importance of the HK-2 gene in the over-expression of HK-2 in cancer cells has been reviewed recently (Mathupala et al. 2006) and will not be dealt with in detail here. Suffice it to say, that in cancer cells that exhibit the "Warburg Effect" the HK-2 gene is amplified (Rempel et al. 1996), its message (m-RNA) is stable (high levels), and its promoter promiscuous in its acceptance of activators including insulin, glucagon, mutated p53, cAMP, and hypoxic conditions (Mathupala et al. 1995; 1997; 2001). In addition, the HK-2 gene via its promoter is subject also to epigenetic control, i.e., methylation and demethylation (Goel et al. 2003). Most of the promoter activity involved in activating the HK-2 gene lies in the proximal region (Lee and Pedersen 2003). This project is still under intensive investigation in the author's laboratory as the influence of environmental factors via epigenetic mechanisms that possibly promote or induce cancer is now coming to center stage (Herceg 2007; Ahmed 2007).

Discovery that "PET" positive cancers in animals can be eradicated by taking advantage of the "Warburg Effect" via the use of the small molecule 3-Bromopyruvate

As indicated earlier in this review, cancer is the enemy of all nations and all people, and to defeat one's enemy one must identify their Achilles heels and then devise a clever way to target one or more of these. Clearly, we know today from both the pioneering work conducted in the author's laboratory and more recent work conducted in other laboratories that the "Warburg effect" and all that it entails is central to the growth and survival of numerous cancers. Therefore, the "Warburg effect" and all that it entails also becomes one of cancers' most vulnerable phenotypes. With this in mind, the author and his colleagues/collaborators set out to seriously tackle this problem near the end of the past century commencing with an antisense RNA approach. In fact, in studies reported briefly by Mathupala and Pedersen (1999) very encouraging results were obtained using an antisense RNA to HK-2. However, upon Dr. Mathupala's good fortune in obtaining a well deserved independent faculty position at another institution, this approach was replaced by a "small molecule" approach described briefly below.

Dr. Young Ko, Department of Biological Chemistry, Dr. J.F. Geschwind, Department of Radiology and Radiological Sciences, and the author (all at Johns Hopkins School of Medicine) set out with the long term goal of finding a new agent that would arrest the growth of tumors (VX2 of dermatoid origin) in a rabbit model for liver cancer (Pauser et al. 1996). The first objective of the project was to establish whether the VX2 tumor exhibited a "Warburg effect", i.e., high glycolysis in the presence of oxygen. Assuming this would be the case, the second objective was to discover an agent that selectively targeted this common phenotype in isolated VX2 tumor chunks. Finally, assuming that such an agent would be found, the third objective was to assess its capacity to arrest the growth of VX2 tumors implanted in the livers of rabbits.

Dr. Ko who performed all the chemical/biochemical studies showed within a very short time that the VX2 tumor does exhibit a pronounced "Warburg effect", and discovered also that a small molecule named 3-bromopyruvate (3BrPA; Fig. 1d) that she had used earlier as a pre-doctoral student to inhibit a plant enzyme (Ko and McFadden 1990) was also a potent inhibitor of the glycolytic activity catalyzed by VX2 tumor chunks. In addition, Dr. Ko discovered that (3BrPA) was not only an inhibitor of the high glycolytic activity of VX2 tumor chunks but it inhibited also ATP synthesis in isolated mitochondria derived from these chunks (Ko et al. 2001). Thus, in one tiny molecule she (we) had in hand an

agent that would inhibit both ATP production factories, glycolysis and mitochondria, of VX2 tumor cells. To cap off this initial project, Dr. Ko went on to show that 3-BrPA quickly kills every cell growing within a culture plate comprised of AS-30D hepatocellular carcinoma cells, the same cells that Ernesto Bustamante had found on the same lab bench over two decades earlier to exhibit a marked "Warburg effect" dependent on mitochondrial bound hexokinase (Bustamante and Pedersen 1977).

Following publication of this initial in vitro study (Ko et al. 2001), the in vivo rabbit studies were quickly initiated with the addition to the project of Dr. Michael Torbenson of the Department of Pathology (Johns Hopkins, School of Medicine). Here, 3-BrPA formulated by Dr. Ko was injected intra-arterially by Dr. Geschwind, an interventional radiologist, into VX2 tumors that had been implanted previously into the livers of a number of rabbits and allowed to grow. The results obtained pathologically by Dr. Torbenson were quite remarkable with the cells in most tumors being destroyed by 70% or more with a single 3-BrPA injection. In contrast to the author's expectation of considerable toxicity, this was not evident at the doses used either at the whole animal level or as shown by Dr. Torbenson at the tissue level. Also, there was little damage to normal liver tissue surrounding the implanted tumor. Finally, it was found also by pathological examination that other tissues were not affected. Additional studies with other animals showed that injection of the 3-BrPA into an ear vein did not harm the animals at the doses used and in fact suppressed lung cancer that had developed from metastasis of the liver implanted VX2 tumor.

Following publication of the above rabbit study (Geschwind et al. 2002), Dr. Ko went on to lead a third study on 3-BrPA in collaboration with the laboratory of Dr. Martin Pomper, an expert on PET imaging and also a member of the Department of Radiology. This study (Ko et al. 2004) employed a second animal model, i.e., Sprague–Dawley albino rats. Control studies by Dr. Ko were performed first on both AS-30D hepatoma cells growing in tissue culture and intact hepatocytes (liver cells) obtained from a commercial source. It is important to note from the results shown in Fig. 1e that the anticancer agent used, i.e., 3-BrPA, had little or no effect on control hepatocytes but destroyed almost all the hepatoma (cancer) cells within 30 min. Regarding the AS-30D hepatoma cells that Dr. Ko injected into the peritoneal cavity or into the upper back, these grow quickly. In the peritoneal cavity they result in death of the animal within 2 weeks while in the upper back they develop into large solid tumors (2–3 cm diameter) in the same time frame. Prior to treatment with 3-BrPA, Dr. Pomper and his colleagues Dr. Yuchuan Wang and James Fox subjected the solid tumors to PET analysis following injection with ^{18}FDG. This confirmed the tumors suspected high glycolytic metabolism. Following treatment with 3-BrPA, animals containing tumor cells growing in the peritoneal cavity continued to live a normal life whereas untreated animals died within 2 weeks. In addition, following treatment with 3-BrPA the large solid tumors growing in the upper back of other animals, e.g., R4 (Fig. 1f, upper panel) completely disappeared within a month (Fig. 1f, middle panels) and never returned (Fig. 1f, bottom panel). In the case of the latter animals, PET analysis revealed no remaining glycolytic activity and the animals lived thereafter a normal life, ate well, and gained weight (Fig. 1f, lower panel). Remarkably, of the 19 tumor bearing animals involved in the study, all were cured, and all continued to live out their life without return of the tumor (Ko et al. 2004).

Concluding remarks/future directions

The above summarized studies conducted in whole or in part in the author's laboratory that resulted in the discovery that HK-2, VDAC, and the HK-2 gene play pivotal roles in the "Warburg Effect" entailed a period touching five decades. This work has contributed not only to a better understanding of the "Warburg effect" at a molecular (enzyme/protein) level but also at a gene level. In addition, work published as early as 1977 and 1981 by Bustamante and the author (Bustamante and Pedersen 1977; Bustamante et al. 1981) on the important role of hexokinase in the "Warburg effect" likely contributed to the development/use of PET imaging for cancer (Di Chiro et al. 1982) as this technique monitors the product of the hexokinase reaction. Finally, and perhaps one of the most important contributions, is the work conducted with the tiny molecule 3-bromopyruvate (3-BrPA). This work clearly shows that by targeting the "Warburg effect" (Ko et al. 2001) that cancers exhibiting this phenotype can be either cured (Ko et al. 2004) or ameliorated (Geschwind et al. 2002) in animal models.

Regarding future directions, the above "story" from the author's laboratory is likely to become even more exciting and interesting as a close neighbor of HK-2 and VDAC is the ATP synthasome. It was first isolated and characterized in projects in which Dr. Ko also played a lead role (Ko et al. 2003; Chen et al. 2004, 2006). The ATP synthasome's three major components, i.e., the ATP synthase, P_i carrier, and ADP/ATP carrier, have been studied also in the author's laboratory for a period touching five decades, and it would appear from our most recent data that this large complex may be a "partner in crime" with HK-2 and VDAC in promoting the "Warburg effect" in cancer cells (Fig. 1g).

Acknowledgement of the work of others

As it regards the work of others, it should be noted first that the author has been working continuously since 1969 in order

to understand the "Warburg effect". Others contributing to this mini-review series have been working also for some time in the field of cancer research on somewhat different aspects of the problem as it relates directly or indirectly to the "Warburg Effect". Despite this, Warburg's views have generally not been appreciated by most cancer researchers for the past 30–40 years. For the author of this mini-review, it has been a rather lonely area to have chosen as one of his projects. Now, over thirty years later, interest in the "Warburg effect" is coming back to center stage likely because of a publication from C.B. Thompson's laboratory (Vander Heiden et al. 2001) entitled "Growth Factors can Influence Cell Growth and Survival through Effects on Energy Metabolism" and a publication the following year from J.B. Hoek's laboratory (Pastorino et al. 2002) entitled "Mitochondrial Binding of Hexokinase 2 Inhibits Bax-Induced Cytochrome c Release and Apoptosis. ***Significantly, both papers emphasize the very important role of mitochondrial bound HK-2 to either cell growth and/or to suppressing apoptosis.*** Considering that the author of this mini-review had been emphasizing the importance of HK-2 to the "Warburg Effect" and cancer growth for over 20 years, the above noted papers were for him a breath of fresh air. Significantly, since this time numerous other papers describing either original work or reviewing the subject have appeared. Among these are Pastorino and Hoek (2003), Majewski et al. (2004a), Azoulay-Zohar et al. (2004), Hammerman et al. (2004), Birnbaum (2004), Majewski et al. (2004), Zaid et al. (2005), Robey and Hay (2005, 2006), Rostovtseva et al. (2005), Kelloff et al. (2005), Kim and Dang (2006), Lemasters and Holmuhamedov (2006), Wallace (2005), Ristow (2006), Galluzzi et al. (2006), Merida and Avila-Flores (2006), Modica-Napolitano et al. (2007). The author apologizes for not being able to acknowledge many other papers or reviews.

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