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14. ABSTRACT
Most malignant cells are highly glycolytic and produce high levels of reactive oxygen species (ROS) compared to normal cells. Mitochondrial glycerophosphate dehydrogenase (mGPDH) participates in delivering reducing equivalents from this molecule into the electron transport chain, thus sustaining of glycolysis. Here we investigate the role of mGPDH in maintaining an increased rate of glycolysis and evaluate glycerophosphate-dependent ROS generation in prostate cancer cell lines (LNCaP, DU145, PC3, and CL1). Immunoblot, Real Time RT-PCR, polarographic, and spectrophotometric analysis revealed that mGPDH abundance and activity was significantly elevated in prostate cancer cell lines when compared to normal prostate epithelial cell line PNT1A. Overall, these data demonstrate that mGPDH is involved in maintaining a high rate of glycolysis and is an important site of electron leakage leading to ROS production in prostate cancer cells.

15. subject terms - reactive oxygen species, mitochondrial glycerophosphate dehydrogenase, glycerophosphate shuttle, prostate cancer, oxidative stress

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Introduction:

Prostate cancer is the most commonly diagnosed malignant disease and the second leading cause of cancer-related mortality in men in most Western countries [1]. A number of risk factors such as age, cigarette smoking, obesity, high fat diet and environmental toxins have been associated with both prostate cancer and increased ROS generation [2]. However, mechanisms by which they increase the risk of this disease are not clear. Prostate cancer etiology is in part related to oxidative stress. ROS has been proposed as a mediator in several human pathologies [3], ageing [4], necrotic and apoptotic processes [5, 6] as well as cancer in general [7]. In addition, ROS plays an important role in regulating both the growth and survival of prostate cancer [8]. Oxidative stress is associated with an increased generation of ROS derived from oxygen and oxidants, and/or impaired cellular antioxidant mechanisms. Mitochondria are the main site of ROS production, converting up to 4% of intracellular molecular oxygen to ROS as byproducts of oxidative phosphorylation [9]. It has been previously demonstrated that ROS formed during this process of energy production result from the inefficient flow of electrons through Complexes I and III of the respiratory chain [9]. However, more recent studies have shown that mitochondrial glycerophosphate dehydrogenase also participates in the production of significant amounts of ROS when glycerophosphate is supplied as a respiratory substrate [10, 11].

Mitochondrial FAD-dependent glycerophosphate dehydrogenase (mGPDH, EC 1.1.99.5) is encoded in the nucleus and is located on the outer surface of the inner mitochondrial membrane. This enzyme acts in concert with the cytoplasmic NAD-linked glycerophosphate dehydrogenase (cGPDH, EC.1.1.1.8) to form the glycerophosphate shuttle [12]. This shuttle interconverts glycerol-3-phosphate and dihydroxyacetone phosphate, transferring reducing equivalents into the

electron transport chain, in order to reoxidize cytosolic NADH generated from glycolysis. The activity of mGPDH is low in most mammalian tissues resulting in a low glycerophosphate shuttle activity [11]. However, brown adipose tissue contains mGPDH and cGPDH in equimolar proportions resulting in high shuttle activity [13]. The glycerophosphate shuttle is also active in tissues that metabolize glucose rapidly, such as skeletal muscle [14], brain [15], and insect flight muscle [16]. In addition, high mGPDH activity has also been reported in rat spermatozoa [17], pancreatic beta cells [18], heart muscle [19], placenta [20], testis [21], and the rat liver after hormonal induction [22]. The glycerophosphate shuttle also contributes to thermogenic pathways [23], the regulation of lipid synthesis [24], the energy dissipating system due to bypassing one phosphorylation site at Complex I [25] and the control of glycolysis by shuttling electrons from NADH into the mitochondria [13]. mGPDH appears to play a crucial role in fetal development as Brown et al. reported an ~ 50% reduction in viability of mGPDH knock out mice when compared to wild-type littermates [26]. However, the multiple roles of mGPDH in cellular metabolism have not been fully elucidated.

Body:

The hypothesis of this project is that increased activity of mitochondrial glycerophosphate dehydrogenase (mGPDH) causes increased production of ROS which plays an important role in the prostate cancer phenotype. Most of the results mentioned here are published in the article "High activity of mitochondrial glycerophosphate dehydrogenase and glycerophosphate-dependent ROS production in prostate cancer lines, *Biochem Biophys Res Commun* **333,** 1139-45(2005)" by S.K. Roy Chowdhury, A. Gemin, G. Singh. Please see the publication (attached in Appendix) for Figures 1-5. and Tables 1 and 2. Additional results are described in the second manuscript "Increased biogenesis of mGPDH and antioxidant enzymes in prostate cancer cells/cancer" by S.K. Roy Chowdhury, S. Raha, M. Tarnopolsky and G. Singh.

This is the first study to conduct a systematic evaluation of mGPDH using two complementary functional methods, namely polarography and spectrophotometry, in normal prostate epithelial cells (PNT1A) and prostate cancer cell lines. Like mGPDH, succinate dehydrogenase is also flavoprotein-dependent and transfers electrons directly to coenzyme Q of the electron transport chain [10]. Therefore the activity of succinate dehydrogenase was measured in each sample and compared directly to that of mGPDH activity. Oxygen consumption measurements were done in intact permeabilized cells to ensure mitochondrial and intracellular integrity as well as interorganelle structural communication [27]. Spectrophotometric measurements, on the other hand, characterize activities of isolated complexes in disintegrated membranes. Both methods provide complementary data about mitochondrial functions.

***Polarographic analysis*:** Representative measurements of oxygen consumption of DU145 and PNT1A cells are depicted in Fig. 1A and 1B, respectively. Following the addition of glycerophosphate (GP*), oxygen consumption was significantly increased in DU145 cells (Fig. 1A) when compared to PNT1A cells (Fig. 1B). Moreover, this observation appears to be a characteristic of all prostate cancer cell lines evaluated as LNCaP, DU145, PC3, and CL1 cells consumed 2.5-6.0 fold more oxygen after the addition of glycerophosphate when compared to PNT1A cells (Table 1). In contrast, oxygen consumption following the addition of succinate in prostate cancer cells and normal prostate cells showed no significant difference (Table 1).

Spectrophotometric measurements:

GCCR activity: The enzymatic activity of mGPDH was evaluated in cell lysates by monitoring the rate of reduced cytochrome *c* formation at 550 nm when glycerphoshate was used as a substrate, termed glycerophosphate cytochrome *c* reductase (GCCR) activity. Using this assay, prostate cancer cells showed 2-6 fold higher GCCR activity when compared to normal PNT1A cells (Table 2). To evaluate the specific inhibition of mGPDH, cell lysates were incubated with sodium oleate for 5 min prior to the evaluation of GCCR activity. Sodium oleate diminished GCCR activity by 50-67% in both prostate cancer cell lines and normal prostate epithelial cell lysates (Fig. 2A).

SCCR activity: The enzymatic activity of succinate dehydrogenase was evaluated in cell lysates by monitoring the rate of reduced cytochrome *c* formation at 550 nm when succinate was used as a substrate, termed succinate cytochrome *c* reductase (SCCR) activity. Using this assay, prostate cancer cells showed no significant difference in SCCR activity when compared to

PNT1A cells (Table 2). Unlike GCCR activity, SCCR activity was not altered significantly in any cell line upon the addition of sodium oleate (Fig. 2B).

Lactate dehydrogenase activity: LDH catalyzes the last step in glycolysis. LDH activity was therefore used as a quantitative marker of glycolytic activity in intact cells by monitoring the formation of NAD as pyruvate is converted to lactate. Using this method, LDH activity was 1.68-4.44 fold higher in prostate cancer cells when compared to normal prostate epithelial cells (Table 2). These results confirmed that prostate cancer cells are more glycolytic than normal prostate cells.

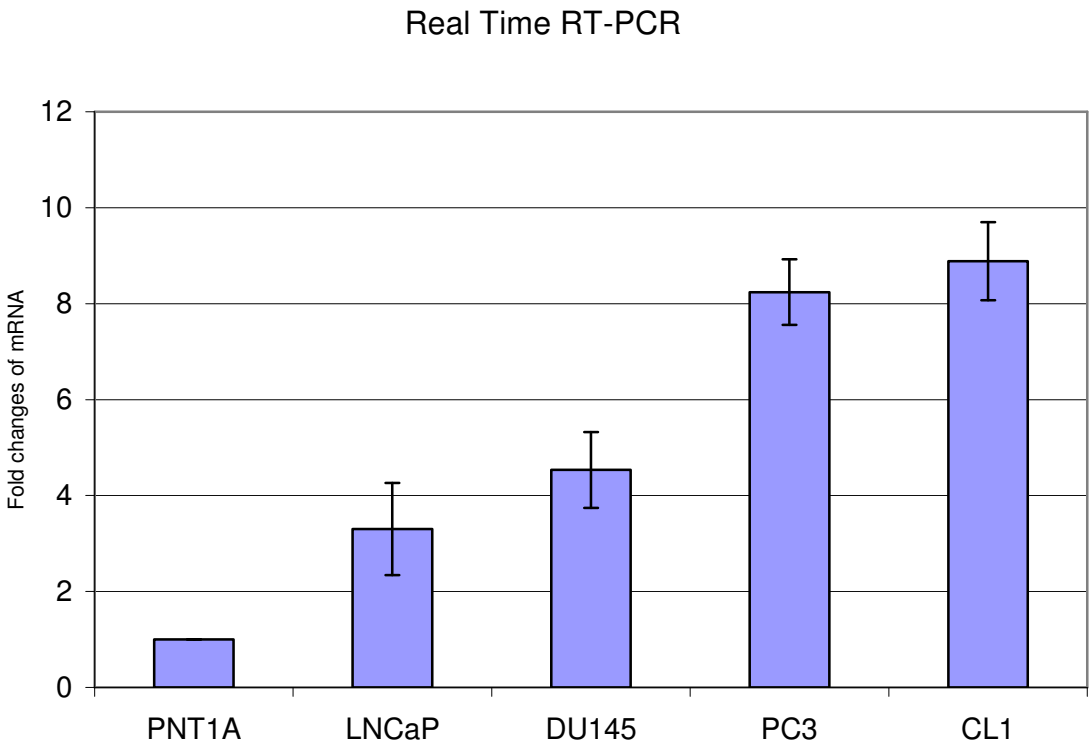
Cytoplasmic glycerophosphate dehydrogenase (cGPDH) activity: In order to determine that varying levels of cGPDH activity was not responsible for the observed differences in mGPDH activity, cGPDH activity was assessed. Spectrophotometric analysis revealed that the cGPDH activity of all cell lines used in this investigation were not significantly different from each other and therefore cannot account for the observed differences in mGPDH activity (Table 2).

Western blot analysis: To assess the amount of mGPDH in normal prostate cells and prostate cancer cells, immunoblot analysis was performed on whole cell lysates utilizing an mGPDH specific antibody. Mitochondrial glycerophosphate dehydrogenase protein levels were elevated in prostate cancer cell lines when compared to the normal prostate cell line, PNT1A (Fig. 3). Moreover, mGPDH appears to be less abundant in the androgen sensitive LNCaP cell line when compared to DU145, PC3 or CL1 cells that are all androgen insensitive.

Real Time RT-PCR

We have further studied the mRNA expression level of mGPDH using more sophisticated and reliable Real Time PCR technique from "Applied Biosystem" rather than Northern blot analysis. mRNA level of mGPDH in prostate cancer cells are 3.30-8.9-fold higher than in normal prostate epithelial cells (See Fig. I).

Fig. I



Total RNA was isolated from different prostate cancer cell lines using Qiagene minikit. A total of 100ng RNA was used to detect mRNA levels of mGPDH in different prostate cancer cells compared to normal prostate cells, PNT1A.

This observation would indicate that there is a positive correlation between mGPDH protein level, mRNA expression and activity, at least in the cell lines evaluated in this study.

It is of interest to note that there may also be a correlation between androgen sensitivity and mGPDH expression as androgen-sensitive LNCaP cells had decreased enzymatic activity, protein level and mRNA expression level of mGPDH when compared to androgen-insensitive DU145, PC3, and CL1 cells.

Glycerophosphate-dependent H_2O_2 production by prostate cancer cells: Hydrogen peroxide production was evaluated in both prostate cancer cell lines and PNT1A cells. Prostate cancer cells produced 2-3 fold more H_2O_2 when compared to PNT1A cells under basal conditions (Fig. 4). LNCaP, DU145, PC3, and CL1 cell lines generated 2.7-4.1 fold higher H_2O_2 than PNT1A cells, when glycerol-3-phosphate was used as a substrate (Fig. 4). The complex IV inhibitor, KCN, dramatically increased H_2O_2 production rates. Glycerophosphate-induced H_2O_2 production in the presence of KCN was elevated 5-7 fold in prostate cancer cells when compared to PNT1A cells. Moreover, glycerophosphate-induced H_2O_2 production in the presence of KCN exceeded H_2O_2 production when both succinate and KCN were administered in combination (Fig. 4).

***H_2O_2 production following administration of the GCCR inhibitor sodium oleate*:** GP-dependent generation of H_2O_2 in DU145 was decreased 57% by sodium oleate, (Fig. 5). In contrast, GP-dependent H_2O_2 generation in PNT1A cells was unaffected by the addition of sodium oleate.

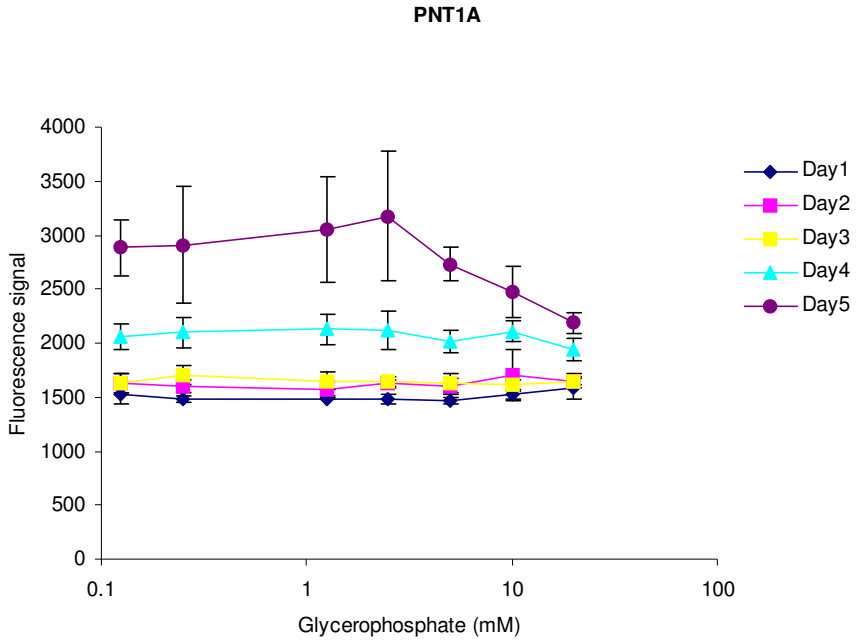
Our data are the first to suggest that mGPDH acts as an ROS generator in prostate cancer cells (Fig. 4). Moreover, glycerophosphate-dependent ROS production could be decreased with the addition of sodium oleate in DU145 cells, however this inhibitor had no effect on ROS production in PNT1A cells. These findings suggest a direct involvement of mGPDH in ROS generation in prostate cancer lines, but not in normal cells. Sodium oleate does not act as an antioxidant as there was no effect on ROS production under any condition tested in PNT1A cells (Fig. 5). It is not yet clear why sodium oleate has no observable effect on succinate dehydrogenase activity (Fig. 2), but is capable of decreasing succinate-dependent ROS production (Fig. 5).

Fluorometric analysis revealed that glycerophosphate-dependent ROS production exceeded that of succinate-dependent ROS production (Fig. 4). This observation may be attributable to the absence of a CoQ-binding protein in mGPDH. This CoQ-binding protein evidently has a natural protection of ubisemiquinone formed during CoQ reduction by succinate dehydrogenase [28].

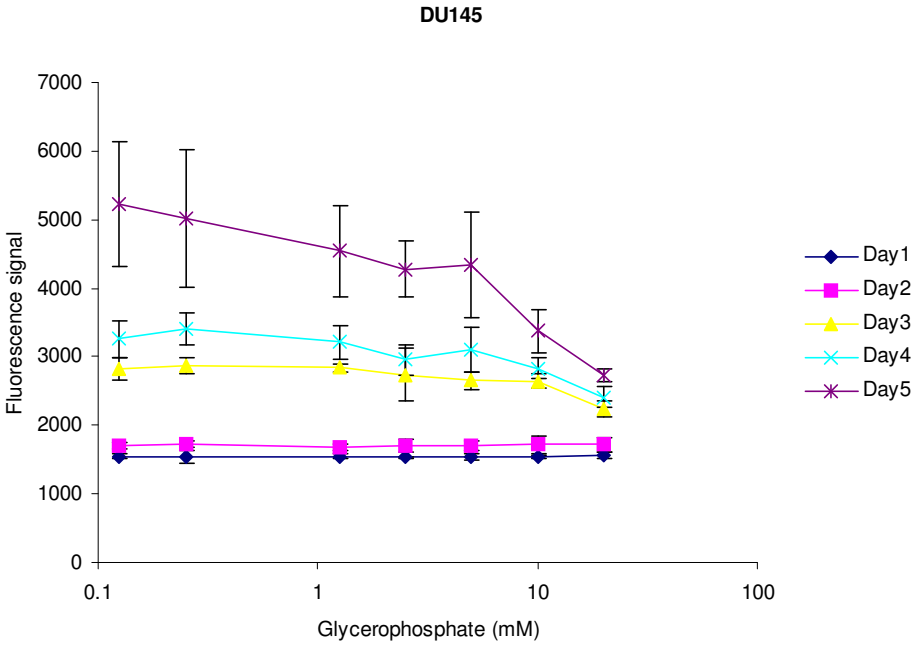
Evaluation of cell proliferation with glycerophosphate in prostate cancer cells. We further studied the effect of glycerophosphate on the cell proliferation of prostate cancer cell lines, DU145, LNCaP, and CL1 including normal prostate epithelial cells, PNT1A. All cell lines are stopped to proliferate at the concentration of 5 mM glycerophosphate after 3 days of cultivation (Fig. II A-D). It could be either due to the certain amount of cells in a limited area of tissue culture plates or an excessive production of H_2O_2 was harmful for further cell proliferation.

Fig. II

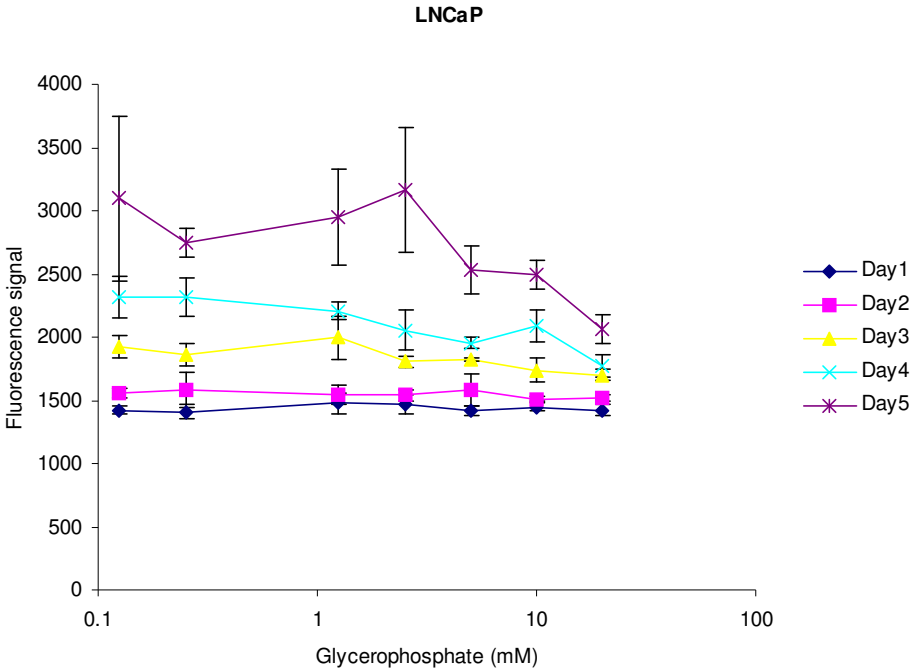
(A)


PNT1A
Fluorescence signal
4000
3500
3000
2500
2000
1500
1000
500
0
0.1
1
10
100
Glycerophosphate (mM)
Day1
Day2
Day3
Day4
Day5

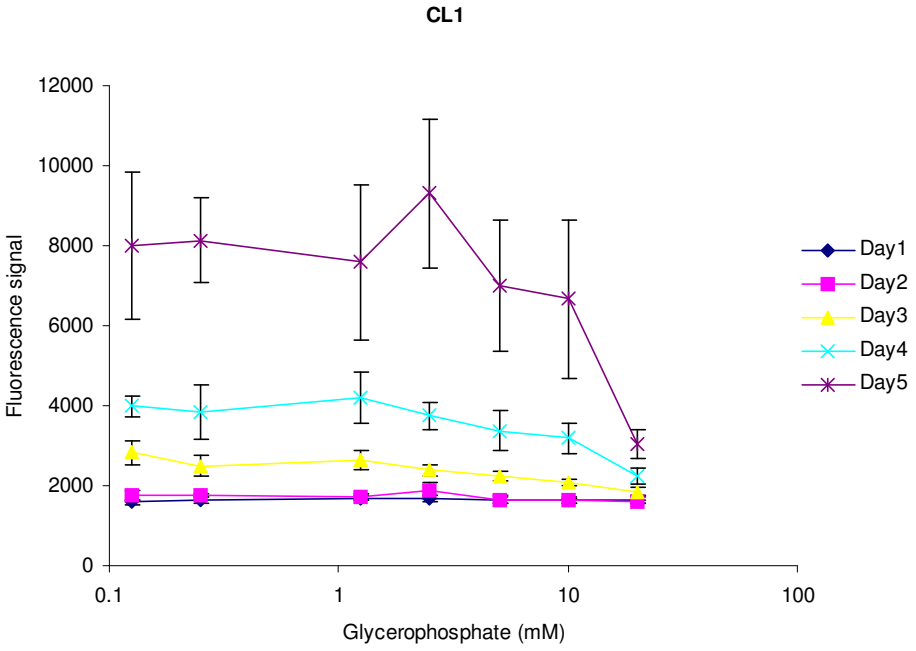
(B).


DU145
Fluorescence signal
7000
6000
5000
4000
3000
2000
1000
0
0.1
1
10
100
Glycerophosphate (mM)
Day1
Day2
Day3
Day4
Day5

(C).


LNCaP
Fluorescence signal
4000
3500
3000
2500
2000
1500
1000
500
0
0.1
1
10
100
Glycerophosphate (mM)
Day1
Day2
Day3
Day4
Day5

(D).


CL1
Fluorescence signal
12000
10000
8000
6000
4000
2000
0
0.1
1
10
100
Glycerophosphate (mM)
Day1
Day2
Day3
Day4
Day5

Comparison of in vitro anchorage-independent growth of cells between mGPDH-downregulated (antisense-transfected) and control (vector-transfected) cells: We are actively trying to establish mGPDH-downregulated (antisense-transfected) and control (vector-transfected) cells. The cDNA of mGPDH (from ATCC) was inserted in the antisense direction into the multiple cloning site of the mammalian expression vector pcDNA3.1 (obtained from Invitrogen) to form the mGPDH antisense vector. Cells were transfected with prepared plasmid using Lipofectamine Plus (Invitrogen). However, we were not able to transfect cells successfully.

In vivo determination of tumor formation in immune-incompetent mice: As we did not have vector-transfected or antisense-transfected prostate cancer cells, we did not further investigate *in vivo* tumor formation in immune-incompetent mice.

Other major results: Other results derived from this fellowship were described in the manuscript S.K. Roy Chowdhury, S. Raha, M. Tarnopolsky and G. Singh, Increased biogenesis of mGPDH and antioxidant enzymes in prostate cancer cells/cancer.

Key research accomplishments:

1. The methodology for conducting the prepared experiments was validated and some of this was published in the manuscript.
2. The data has been presented at the AACR (American Association for Cancer Research) and subsequently the manuscript has been published in the scientific journal, BBRC (Biochemical and Biophysical Research Communications).
3. The second manuscript is in preparation and almost ready to publish.

Reportable outcomes:

The following publication and manuscript describe the reportable outcome that has resulted from this training grant.

S.K. Roy Chowdhury, A. Gemin, G. Singh, High activity of mitochondrial glycerophosphate dehydrogenase and glycerophosphate-dependent ROS production in prostate cancer lines, *Biochem Biophys Res Commun* **333,** 1139-45(2005).

S.K. Roy Chowdhury, S. Raha, M. Tarnopolsky and G. Singh, Increased biogenesis of mGPDH and antioxidant enzymes in prostate cancer cells/cancer in preparation.

Conclusion:

The data presented here suggest a coordinated higher activity and abundance of mGPDH and increased rate of glycerophosphate-dependent ROS production in prostate cancer cells in comparison to normal prostate epithelial cells. The elevated activity of mGPDH and GP-dependent ROS production may be a characteristic of neoplastic cells. The elevated expression

and activity of mGPDH in prostate cancer cells indicates that the glycerophosphate shuttle is activated along with the malate-aspartate shuttle to maintain highly glycolytic environment in cancer cells. This is the first report to demonstrate that mGPDH is an important source of ROS in prostate cancer cells that could be used as a strategy to target their destruction.

References

1. S. H. Landis, T. Murray, S. Bolden, P. A. Wingo, *CA Cancer J Clin* **49,** 8 (1999).

2. S. K. Pathak, R. A. Sharma, W. P. Steward, J. K. Mellon, T. R. Griffiths and A. J. Gescher, *Eur J Cancer* **41**, 61 (2005).

3. B. Halliwell, *FASEB J* **1,** 358 (1987).

4. G. Barja, *J Bioenerg Biomembr* **31,** 347 (1999).

5. G. Kroemer, B. Dallaporta, M. Resche-Rigon, *Annu Rev Physiol* **60**, 619 (1998).

6. P. L. Pedersen, *J Bioenerg Biomembr* **31,** 291 (1999).

7. A. L. Jackson, L. A. Loeb, *Mutat Res* **477,** 7 (2001).

8. S. S. Brar et al., *Am J Physiol Cell Physiol* **285,** 353 (2003).

9. G. Lenaz et al., *Ann N Y Acad Sci* **959,** 199 (2002).

10. Z. Drahota, S. K. Chowdhury, D. Floryk, T. Mracek, J. Wilhelm, H. Rauchova, G. Lenaz, J. Houstek, *J Bioenerg Biomembr* **34,** 105 (2002).

11. P. Jesina, D. Kholova, R. Bolehovska, Z. Cervinkova, Z. Drahota, J. Houstek, *Physiol Res* **53,** 305 (2004).

12. M. Klingenberg, *FEBS Lett* **6,** 145 (1970).

13. J. Houstek, B. Cannon, O. Lindberg, *Eur J Biochem* 54, 11 (1975).

14. E. S. Cole, C. A. Lepp, P. D. Holohan, T. P. Fondy, *J Biol Chem* **253,** 7952 (1978).

15. I. R. Cottingham, C. I. Ragan, *Biochem J* **192,** 19 (1980).

16. R. S. Sohal, *Mech Ageing Dev* **60,** 189 (1991).

17. J.B. Schenkman, D.A. Richert, W.W. Westerfeld, *Endocrinology* **76,** 1055 (1965).

18. H. Ishihara, M. Nakazaki, Y. Kanegae, K. Inukai, T. Asano, H. Katagiri, Y. Yazaki, M. Kikuchi, J. Miyazaki, I. Saito, Y. Oka, *Diabetes* **45,** 1238 (1996).

19. T. D. Scholz, S. L. Koppenhafer, C. J. TenEyck, B. C. Schutte, *J Mol Cell Cardiol* **29,** 1605 (1997).

20. J. Swierczynski, P. Scislowski, Z. Aleksandrowicz, *Biochim Biophys Acta* 429, 46 (1976).

21. J. M. Weitzel, N. B. Shiryaeva, R. Middendorff, M. Balvers, C. Radtke, R. Ivell, H. J. Seitz, *Biol Reprod* 68, 699 (2003).

22. S. Muller, H. J. Seitz, *Proc Natl Acad Sci U S A* **91,** 10581 (1994).

23. W. N. Aldridge and B. W. Street, *Biochem J* **107**, 315 (1968).

24. R.R. Chaffee, J.R. Allen, Y. Cassuto, R.E. Smith. *Am J Physiol* **207,** 1211 (1964).

25. V. Bobyleva, L. Pazienza, U. Muscatello, N. Kneer, H. Lardy, *Arch Biochem Biophys* **380,** 367 (2000).

26. L. J. Brown, R. A. Koza, C. Everett, M. L. Reitman, L. Marshall, L. A. Fahien, L. P. Kozak, M. J. MacDonald, *J Biol Chem* 277, 32892 (2002).

27. T. G. Frey, C. A. Mannella, *Trends Biochem Sci* **25,** 319 (2000).

28. H. Rauchova, R. Fato, Z. Drahota, G. Lenaz, *Arch Biochem Biophys* **344,** 235 (1997).

Appendices:

The manuscript in preparation: S.K. Roy Chowdhury, S. Raha, M. Tarnopolsky and G. Singh, Increased biogenesis of mGPDH and antioxidant enzymes in prostate cancer cells/cancer in preparation.

The published journal article: S.K. Roy Chowdhury, A. Gemin, G. Singh: High activity of mitochondrial glycerophosphate dehydrogenase and glycerophosphate-dependent ROS production in prostate cancer lines, Biochem Biophys Res Commun 333, 1139-45 (2005).

Increased biogenesis of mGPDH and antioxidant enzymes in prostate cancer cells/cancer

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Abstract

Cancer cells generate energy via glycolysis and produce more reactive oxygen species (ROS) in the process. The involvement of mitochondrial glycerophosphate dehydrogenase (mGPDH) in production of ROS has been established in prostate cancer cell lines (LNCaP, DU145, PC3, and CL1). We have further found that the mRNA level of mGPDH in prostate cancer cells is 3.3-8.9-fold higher compared to the normal prostate epithelial cell line, PNT1A. This is consistent with the enzymatic activity and protein level of mGPDH. However, cytochrome *c* oxidase (COX) activity is 2.9-3.2-fold downregulated in androgen-independent prostate cancer cell lines. The level of antioxidant enzymes, catalase, MnSOD and CuZnSOD is upregulated in prostate cancer cell lines, thus far examined. Furthermore, we obseved that the activity of mGPDH is significantly higher in liver tissues from all mice with cancer compared to liver tissues from control mice. These data suggest that the upregulation of mGPDH due to highly glycolytic environment is responsible in ROS generation and implicated in cancer progression.

Key Words: Mitochondrial glycerophosphate dehydrogenase; Reactive oxygen species; Cytochrome *c* oxidase; Antioxidant enzymes; Liver tissues; Glycerophosphate shuttle; Prostate cancer

Intrduction

The primary characteristic metabolic phenotype of cancer cells is the high rate of glycolysis [1]. This high glycolysis is considered to be due to defective mitochondrial energy provision or to its lower capacity in competing for common intermediates [2]. It has been shown that the activity of mGPDH is upregulated in the highly glycolytic environment of prostate cancer cell lines. It was reported that an increase in glycolysis also activated mGPDH activity through a Ca^{2+}-dependent mechanism in insulin-secreting cells [3]. mGPDH is located on the outer side of the inner mitochondrial membrane and constitutes the rate-limiting part of the glycerophosphate shuttle. The glycerophosphate shuttle enables the transfer of electrons from NADH generated from glycolysis to coenzyme Q in the electron transport chain. Histochemical studies for pathways of insulin demonstrated the importance of mGPDH in hydrogen shuttle system, which reoxidize the glycolysis-derived NADH in insulin secretion [4]. The other consistent feature of cancer cells is that they produce more reactive oxygen species (ROS) compared to normal cells. ROS play an important role in regulating growth and survival of cancer. High level of ROS results in oxidative stress, a deleterious process that can be an important mediator of damage to cell structures, including lipids membranes, and DNA. In contrast beneficial effects of ROS occur at low/moderate concentrations and involve physiological roles in cellular response to a noxia, and in the function of a number of cellular signaling pathways. The mitogenic responses are presumably interacting with nuclear regulatory factors (AP-I, NFKB APE), regulatory kinases (Src kinase, protein kinase C, mitogen activated protein kinase), receptor tyrosine kinases, protein-tyrosine phosphatases and angiogenic factors [5]. The delicate intracellular interplay between oxidizing and reducing equivalents allows ROS to function as second messengers in the control of cell proliferation and differentiation. A number of sources have

been identified for the origin of ROS generation, however the role of mGPDH in ROS generation is substantially altered in cancer cells. Mitochondria are a major source of cellular free radicals and are packed with various redox carriers that can potentially leak single electrons to oxygen and convert it into superoxide anion. The superoxide anion can be converted to number of molecular species of ROS. Recently, it has been shown that mGPDH is involved in the production of ROS in rat liver mitochondria [6], brown adipose tissue mitochondria [7], different prostate cancer cell lines [8], as well as in mitochondria from human placenta [9]. mGPDH (unlike succinate dehydrogenase in mammalian mitochondria) has the capacity to generate large amounts of superoxide, much of it is produced on the cytosolic side of the inner membrane, which is the known location of the active site of this enzyme [10]. Although cytochrome *c* oxidase (COX) itself is not a source of ROS, inhibition of COX may facilitate ROS production from other complexes [11]. Mutations of COX have been shown to inhibit OXPHOS and stimulate increaseds ROS production, resulting in increased tumor growth [12].

In order to clarify the role of mGPDH in ROS production and its role in the progression of tumor formation, we have investigated the expression of mGPDH and the cellular content of antioxidant enzymes such as catalase, MnSOD, and CuZnSOD, in a high ROS environment characteristic of tumor formation. We have also correlated the activity of COX in different prostate cancer cell lines to study its role in ROS production. In order to correlate the function of mGPDH to pathology, we have further compared the enzymatic activity of mGPDH in the liver tissue of aged C57 black mice with and without cancer.

Materials and methods

Cell cultures and rat liver tissues. The Normal immortalized prostate epithelial cell line, PNT1A was a generous gift of Prof. Norman J. Maitland (University of York, United Kingdom) [13-15]. This cell line was maintained in RPMI 1640 (Life Technologies, Inc., Ontario, Canada) supplemented with 10% fetal bovine serum (Life Technologies, Inc.), 1% penicillin-streptomycin (Life Technologies, Inc.), and 1.0 mM glutamine. The androgen-sensitive human prostate cancer cell, LNCaP and androgen insensitive DU145 and PC3 cell lines were obtained from the American Type Culture Collection (Manassas, VA). The cell lines were maintained in RPMI 1640 supplemented with 10 mM HEPES, 1.0 mM sodium pyruvate, 10% fetal bovine serum, and 1% penicillin-streptomycin. The CL1 androgen-independent cells derived from the LNCaP line, were a generous gift from Dr. A. S. Belldegrun (University of California Los Angeles Medical School, Los Angeles, CA), was maintained in RPMI 1640 supplemented with 10% charcoal-stripped fetal bovine serum and 1% penicillin-streptomycin. Cell lines were cultured at 37°C in a humidified atmosphere with 5% CO_2.

Using a total of 13 C57 black mice, we divided the group into 3 categories. Those that were unaffected by cancer, those having detectable tumors in any tissue other than liver and those having some form of hepatocellular carcinoma (see Table 1). In all cases liver tissues obtained were extracted and homogenized in STE medium (0.25 M sucrose, 10 mM Tris-HCl, 1 mM EDTA, pH 7.4) at 4^0C in a glass-glass homogenizer (50-80 mg liver/1 ml of STE buffer).

Spectrophotometric measurement. Activity of glycerophosphate cytochrome *c* reductase (GCCR) and succinate cytochrome *c* reductase (GCCR) was measured in prostate cancer cell lines and rat liver tissues, as described previously [16]. Briefly, the reaction mixture contained 10 mM potassium phosphate (pH 7.4), 2 mM EDTA, 0.01% bovine serum albumin (fatty acid free),

0.2 mM ATP, 1 mM KCN, 5 □M rotenone, and 20 mM glycerophosphate or 20 mM succinate (Sigma-Aldrich, Ontario, Canada). Sonicated cell lysates (0.2 mg protein) or liver homogenates (0.3 mg protein) were incubated in the reaction mixture for 3 min, after which time 40 □M oxidized cytochrome *c* was added. Changes in absorbance were monitored for 5 min at 550 nm and 30^0C. For calculation of enzyme activities, the extinction coefficient 19.6cm^2 mol^{-1} was used. Citrate synthase activity was evaluated in a medium containing 150 mM Tris-HCl, pH 8.2, 8 mg of lauryl maltoside/mg of protein, 0.1 mM dithionitrobenzoic acid and 0.15 mg of liver homogenate. The reaction was initiated with the addition of 300 □M acetyl CoA and changes in absorbance at 412 nm were measured for 1 min. This rate was subtracted from the rate obtained after the subsequent addition of 0.5 mM oxalacetic acid. Absorbance values were measured for a total of 5 min. Cytochrome *c* oxidase was measured at 30^0C by following the rate of oxidation of reduced cytochrome *c* at 550 nm. COX activity measurements in cultured cells were performed using 40 □M reduced cytochrome *c*, 20 mM phosphate buffer, 0.1 mg of protein from freshly cultured cells and 8 mg of lauryl maltoside/mg protein (0.16% LM). All measurements for enzymatic activity were performed using a Beckman DU-640 spectrophotometer (Beckman Instruments, Fullerton, CA).

Quantitative RT-PCR of mGPDH mRNA in prostate cancer cell lines: Total RNA was prepared from PNT1A, DU145, PC3, LNCaP and CL1 cell lines using RNeasy Mini Kit (Qiagen Inc, Ontario, Canada) according to the manufacturer's protocol. Real-time quantitative RT-PCR (TaqMan PCR) for *mGPDH* was performed using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) and predeveloped TaqMan assay reagents of human *mGPDH* and *GAPDH* control probe were purchased from Applied Biosystems. Briefly, reactions consisted of 12.5 □l 2X TaqMan Universal PCR MasterMix, 0.625 □l Multiscribe and

RNAse Inhibitor Mix, 1.25 □l fluorescently-labeled primer in a total volume of 25 □l with 100 ng RNA. Standard curves for each primer were performed using 0, 12.5, 25, 50, and 100 ng concentrations of RNA, to determine whether the amplification efficiencies of each primer were comparable. All reactions of the samples were performed in quadruplicate. The PCR reaction was carried out according to the manufacturer's protocol. The thermal cycler conditions were 48^0C for 30 min, 95^0C for 10 min, then 40 cycles of 15 sec at 95^0C, and 60^0C for 1 min. Data was analyzed using the Sequence Detection Software, which calculated the threshold cycle (C^T) values. The expression of mGPDH was normalized to GAPDH, and the relative change in gene expression was calculated according to the $-2^{\Delta\Delta CT}$ [17].

Western blot analysis. Whole cellular lysates from PNT1, DU145, PC3, LNCaP and CL1 were prepared in the NP40 lysis buffer containing 1% NP40, 150 mM NaCl, 50 mM Tris base (pH 7.4), and 1mM EDTA. Briefly, cells were washed with ice cold PBS, pelleted through centrifugation, and resuspended in ice-cold NP40 lysis buffer supplemented with 1x protease inhibitor (Sigma). Cells were kept rocking in a cold room of 4^0C for 45 minutes and then spun at 3000 rpm for 5 minutes and supernatant collected. Protein concentrations were determined according to Bradford as described below. 5 μg of protein was added to SDS sample buffer, boiled for 10 minutes, and loaded onto a 10% SDS-PAGE gel. Following electrophoresis, separated proteins were transferred onto a nitrocellulose membrane and blocked with 5% skimmed milk in TBST for 1 hour. Membranes were then incubated overnight at 4^oC with rabbit anti-human catalase (1:2000, AbCam, Cambridge, MA), rabbit anti-human MnSOD (1:2000, Upstate, Chicago, IL) and sheep anti-human Cu-ZnSOD polyclonal antibodies (1:5000, Upstate, Chicago, IL) with 5% skimmed milk in TBST for overnight at 4^0C. After three washes in TBST, the membranes were incubated in horseradish peroxidase-linked goat anti-rabbit IgG antibody

(1:5000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and the membrane of Cu-ZnSOD was incubated HRP-conjugated antisheep antibody (1:10000, Invitrogen Canada Inc., Ontario, Canada) for 1 hour, and proteins were detected using the enhanced chemiluminescence Western blotting detection kit (Amersham Biosciences).

Protein determination: The protein content of rat liver tissues and cellular lysates was measured according to the method of Bradford [18] using bovine serum albumin as a standard.

Statistical analysis. Data are presented as means ± SD. Statistical significance was determined through the use of a Student's t-test with two-tailed distribution and one-way single factor ANOVA test. Significance was considered at $P<0.05$.

Results

Determination of enzyme activities (Glycerophosphate cytochrome c reductase (GCCR) ; succinate cytochrome c reductase (SCCR) and citrate synthase (CS)

In our previous publication we demonstrated that the enzymatic activity, protein level of mitochondrial glycerophosphate dehydrogenase and also oxygen consumption with glycerophosphate was significantly higher in prostate cancer cell lines (DU145, PC3, LNCaP, and CL1) compared to normal prostate cell line, PNT1A. We have further examined the activity of mitochondrial glycerophosphate dehydrogenase in relation to the other respiratory chain enzyme, succinate dehydrogenase and to the citrate synthase, a matrix-soluble enzyme. Both mitochondrial glycerophosphate dehydrogenase and succinate dehydrogenase are membrane integrated subunit complexes and these two complexes transport electrons to the CoQ pool. The enzymatic activity of mGPDH and succinate dehydrogenase was evaluated by monitoring the rate of reduced cytochrome *c* formation at 550 nm when glycerophosphate or succinate was used as a substrate, termed as glycerophosphate or succinate cytochrome *c* reductase (GCCR or SCCR), respectively. Prostate cancer cells showed 1.8- to 6.6- fold higher GCCR activity related to SCCR and 2.1- to 7.4- fold increased GCCR activity related to citrate synthase when compared to normal PNT1A cells (Table 2). Although COX itself is not a source of ROS, inhibition or dysfunction of COX may facilitate ROS production from complexes I or III. COX inhibition by KCN and/or mutation in its subunits can block at COX site and thus increase ROS generation. COX activity in prostate cancer cells is 2.9- to 3.2- fold decreased in androgen-independent prostate cancer lines compared to normal prostate cells (Table 2). However, COX activity in androgen-dependent LNCaP cells was not changed significantly compared to normal

prostate cells. COX activity related to CS was found very similar to alone COX activity in prostate cancer cell lines (Table 2).

mRNA level of mGPDH in prostate cancer cell lines:

To evaluate the mRNA level in these prostate cancer cell lines we used the RT PCR using ABI PRISM 7700 Sequence Detection System from Applied Biosystem. Relative mRNA levels of mGPDH were found 3.3-fold (LNCaP), 4.5-fold (DU145), 8.2-fold (PC3) and 8.9-fold (CL1) higher than in control prostate cell lines, PNT1A (Fig. 1). A strong correlation was observed between mRNA and protein levels of mGPDH as well as its enzymatic activity and ROS production.

Prostate cancer cell lines exhibit increased levels of antioxidant enzymes:

The antioxidant system is important for cells in the defense against endogenous and exogenous oxidative stress. The antioxidant enzymes work coordinately to achieve an equilibrium between pro-oxidant and antioxidant systems. Alteration of antioxidant enzymes changes the cellular redox environment, thereby modulating redox-sensitive signals that influence the cell cycle. Tumor cells appear to have an altered/upregulated antioxidant system compared to normal cells. Protein levels of catalase, and MnSOD were significantly elevated where as cytoplasmic CuZnSOD was altered in prostate cancer cell lines when compared to normal prostate cell line, PNT1A (Fig. 2).

Enzymatic activity of glycerophosphate cytochrome c reductase (GCCR) activity in mouse liver with cancer:

To investigate the function of mGPDH from *in vitro* scenario to an *in vivo* model, we used C57 black mice, which developed cancer either in liver or in other organs. Similarly to the results found in prostate cancer cell lines model [Ref], mice liver from animals with cancer either in liver itself or in other organs showed higher GCCR activity when compared to animals with no tumors (Table 3, Fig. 3. A and B). The significance was verified using one-way ANOVA. Significance was considered at $P<0.05$. The mouse liver tissues used in our experiments related to the investigation of other mitochondrial enzymes and we were limited for more investigation.

Discussion

A common characteristic of primary and metastatic cancers is upregulation of glycolysis. The glycolytic phenotype of many cancer cells and tumors have been demonstrated at both the biochemical and molecular levels. In this study we have attempted to delineate the mechanism by which highly glycolytic environment participates in the production of ROS via the glycerophosphate shuttle. mGPDH is the rate limiting enzyme of this shuttle to transfer electrons from cytoplasm to the electron transport chain. Since the enzyme activity of mGPDH as well as its protein level in prostate cancer cell lines are distinctly higher than normal prostate cells (ref), we have evaluated its activity related to other respiratory chain enzyme activity (succinate dehydrogenase) and citrate synthase enzyme activity and mRNA levels (Fig. 1 and table 2). As the enzyme activity of mGPDH related to succinate dehydrogenase and CS as well as mRNA levels of mGPDH are significantly higher in prostate cancer lines compared to normal prostate cells, it can be proposed that mGPDH plays a key role in transferring electrons from the cytoplasm to the electron transport chain.

The mitochondrial electron transport chain is the major endogenous source of ROS. For several years a growing body of evidence has accumulated to indicate signaling role of ROS in the pathogenesis of cancer. mGPDH is involved in maintaining the highly glycolytic environment of prostate cancer cell lines. The present study as well as a previous one offers to support the proposal that mGPDH is involved in ROS production by showing evidence that antioxidant enzymes are upregulated in all prostate cancer cell lines (Fig. 3). The high production of ROS can be also facilitated by the down regulation of COX in cancer cells which would limit the flux of electrons down the respiratory chain, and therefore, the generation of superoxide radicals.

We have also studied the enzyme activity of mGPDH in ovarian cancer cell model, 2008 and its cis-pltain treated variation cells, C13 with higher ROS production. C13 cells have shown significantly higher activity of mGPDH (data are not shown). These reports suggest that mGPDH exerts an important site of ROS production in cancer cells.

Tumor cells have been shown to generate endogenously ROS at high levels. The consequences of elevation of intracellular redox state by ROS is correlated to the uncontrolled growth of cancer cells. To counterbalance elevated intracellular ROS levels and to escape oxidative stress, different numbers of the antoxidative systems, e.g., CuZn, Mn superoxide dismutases, and catalase are upregulated under oxidative stress. The upregulation of mGPDH is involved in maintaining the highly glycolytic environment of prostate cancer cells.

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References

[1] O. Warburg, The Metabolism of Tumors. Arnold Constable, London, (1930) 254-270.

[2] P.L. Pedersen, Mitochondrial events in the life and death of animal cells: a brief overview. J. Bioenerg. Biomembr. 31 (1999) 291-304.

[3] H. Ishihara, M. Nakazaki, Y. Kanegae, K. Inukai, T. Asano, H. Katagiri, Y. Yazaki, M. Kikuchi, J. Miyazaki, I. Saito, Y Oka, Effect of mitochondrial and/or cytosolic glycerol 3-phosphate dehydrogenase overexpression on glucose-stimulated insulin secretion from MIN6 and HIT cells, Diabetes. 45 (1996) 1238-1244.

[4] M.J. MacDonald, P.C. Kelley, Laclau M. Histochemical evidence for pathways insulin cells use to oxidize glycolysis-derived NADH.Metabolism. 51(32002) 318-321.

[5] M. Valko, D. Leibfritz, J. Moncol , M.T. Cronin , M. Mazur, J. Telser, Free radicals and antioxidants in normal physiological functions and human disease, Int J Biochem Cell Biol. 39 (2006), 44-84.

[6] P. Jesina, D. Kholova, R. Bolehovska, Z. Cervinkova, Z. Drahota, J. Houstek, Glycerophosphate-dependent hydrogen peroxide production by rat liver mitochondria, Physiol. Res. 53 (2004) 305-10.

[7] Z. Drahota, S.K. Roy Chowdhury, D. Floryk, T. Mracek, J. Wilhelm, H. Rauchova, G. Lenaz, Houstek J, Glycerophosphate-dependent hydrogen peroxide production by brown adipose tissue mitochondria and its activation by ferricyanide, J. Bioenerg. Biomembr. 34 (2002) 105-113.

[8] S.K. Roy Chowdhury, A. Gemin, G. Singh, High activity of mitochondrial glycerophosphate dehydrogenase and glycerophosphate-dependent ROS production in prostate cancer cell lines, Biochem. Biophys. Res. Commun. 33 (2005) 1139-1145.

[9] T. Honzik, Z. Drahota, M. Bohm, P. Jesina, T. Mracek, J. Paul, J. Zeman, J. Houstek, Specific properties of heavy fraction of mitochondria from human-term placenta-glycerophosphate-dependent hydrogen peroxide production, placenta, 27 (2006), 348-356.

[10] S. Miwa, M.D. Brand, Mitochondrial matrix reactive oxygen species production is very sensitive to mild uncoupling. Biochem Soc Trans. 31 (2003), 1300-1311.

[11] Q. Chen , E. J. Vazquez , S. Moghaddas, C. L. Hoppel E. J. Lesnefsky, Production of reactive oxygen species by mitochondria: central role of complex III, J Biol Chem. 278 (2003), 36027-36031.

[12] S. Matoba, J.G. Kang, W.D.Patino, A. Wragg , M. Boehm , O. Gavrilova , P.J. Hurley, F. Bunz , P.M. Hwang , p53 regulates mitochondrial respiration, Science. 312 (2006), 1650-1653.

[13] O. Cussenot, P. Berthon, R. Berger, I. Mowszowicz, A. Faille, F. Hojman, P. Teillac, A. Le Duc, F. Calvo, Immortalization of human adult normal prostatic epithelial cells by liposomes containing large T-SV40 gene, J. Urol. 146 (1991) 881-886.

[14] O. Cussenot, P. Berthon, B. Cochand-Priollet, N.J. Maitland, A. Le Duc, Immunocytochemical comparison of cultured normal epithelial prostatic cells with prostatic tissue sections, Exp. Cell. Res. 214 (1994) 83-92.

[15] P. Berthon, O. Cussenot, L. Hopwood, A. Le Duc and N. Maitland. Functional expression of SV40 in normal human prostatic epithelial and fibroblastic cells: differentiation pattern of non-tumorigenic cell lines, Int. J. Oncol. 6 (1995) 333–343.

[16] S.K. Roy Chowdhury, Z. Drahota, D. Floryk, P. Calda, J. Houstek, Activities of mitochondrial oxidative phosphorylation enzymes in cultured amniocytes, Clin. Chim. Acta. 298 (2000) 157-173.

[17] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 25 (2001), 402-408.

[18] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976), 248-254.

Tables

Table 1. Pathophysiological description of rat animals

Animal number	Pathophysiological characteristics
40	Wild type
41	Wild type
43	Wild type
44	Hepatocellular carcinoma, lung carcinoma
51	Nodules in the spleen, liver and lungs; lymphoma
52	Hepatocellular carcinoma
54	Wild type
56	Dysplasia, early hepatocellular carcinoma
58	Wild type
60	Enlarged spleen, lung tumor, lymphoma
61	Lung tumor, lymphoma, carcinoid
66	Enlarged spleen, lung tumors, subcutaneous nodule on leg, metastasis on abdominal lymph nodes, multiple skin lumps, lymphoma
67	Spleen cancer, mild inflammation

68	Wild type

Table 2.

Spectrophotometric determination of glycerophosphate cytochrome *c* reductase (GCCR) related to succinate cytochrome *c* reductase (SCCR), citrate synthase (CS) and cytochrome *c* oxidase (COX) activity in normal prostate epithelial cells (PNT1A) and prostate cancer cells (LNCaP, DU145, PC3).

	Enzymatic activity (nmol/min/mg protein)			
Cells	GCCR/SCCR	GCCR/CS	COX	COX/CS
PNT1A	0.193 ± 0.035	0.021 ± 0.003	18.737 ± 0.231	0.240 ± 0.004
LNCaP	0.339 ± 0.026 *	0.044 ± 0.003 *	20.712 ± 2.460	0.208 ± 0.022
DU145	0.540 ± 0.010 *	0.094 ± 0.005 *	6.461 ± 0.324 *	0.086 ± 0.008*
PC3	1.282 ± 0.054 *	0.155 ± 0.011 *	5.793 ± 0.866 *	0.086 ± 0.022*

Enzymatic activity of GCCR and SCCR was determined with 20 mM glycerophosphate or 20 mM succinate in freshly harvested sonicated cells measuring the reduction rate of cytochrome *c* (nmol of cytochrome *c* oxidized per min per mg protein). The enzyme activity determination methodology of each individual enzyme is described in details under "Materials and Methods".

* $P < 0.05$ with respect to normal prostate epithelial cells, PNT1A.

Table 3. Enzymatic activity of GCCR, CS and ratio of GCCR and CS in rat liver tissues.

	Rat liver tissue	GCCR activity	CS activity	GCCR/CS
WT	40	3.05 ± 0.06	88.12 ± 1.61	0.034 ± 0.001
	41	2.17 ± 0.37	82.23 ± 3.67	0.024 ± 0.001
	43	1.83 ± 0.40	83.92 ± 5.23	0.020 ± 0.004
	54	3.93 ± 0.20	83.89 ± 0.36	0.046 ± 0.001
	58	2.68 ± 0.32	92.54 ± 1.67	0.027 ± 0.004
	68	3.21 ± 0.41	90.46 ± 0.45	0.033 ± 0.001
WT + Cancer	60	4.77 ± 0.28	93.94 ± 1.10	0.053 ± 0.003
	61	6.92 ± 0.83	100.70 ± 2.86	0.074 ± 0.005
	66	6.79 ± 0.49	65.72 ± 1.05	0.107 ± 0.003
	67	5.90 ± 0.39	89.71 ± 0.44	0.068 ± 0.002
WT + HCC	44	4.61 ± 0.33	85.80 ± 0.79	0.056 ± 0.001
	51	6.18 ± 1.03	75.42 ± 1.64	0.090 ± 0.004
	52	4.60 ± 0.65	80.74 ± 1.55	0.061 ± 0.005

Fig. 1. Roy Chowdhury SK et al., 2006

mRNA level of mGPDH in prostate cancer cell lines.

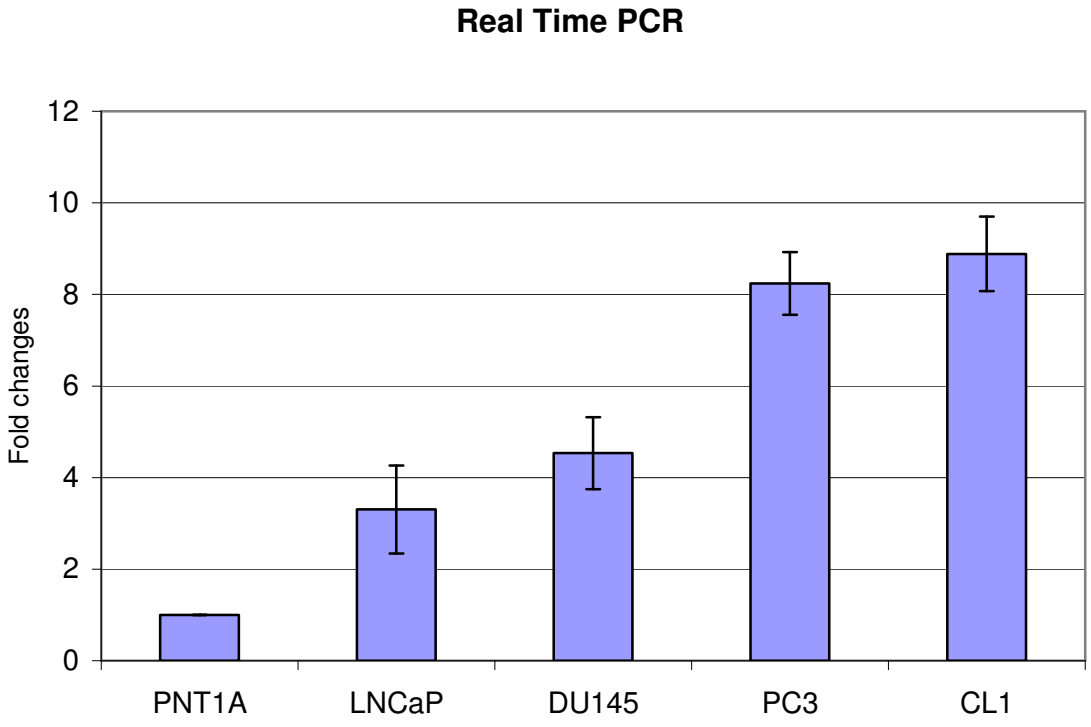


Fig. 2. Roy Chowdhury SK et al., 2006

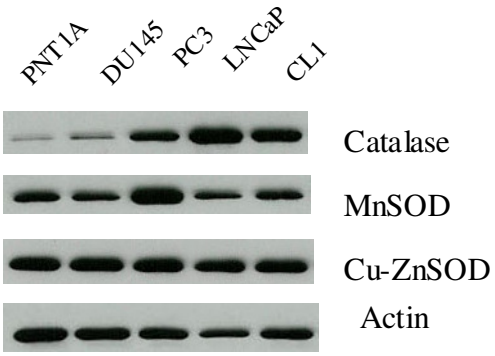


Fig. 3A. Roy Chowdhury SK et al., 2006

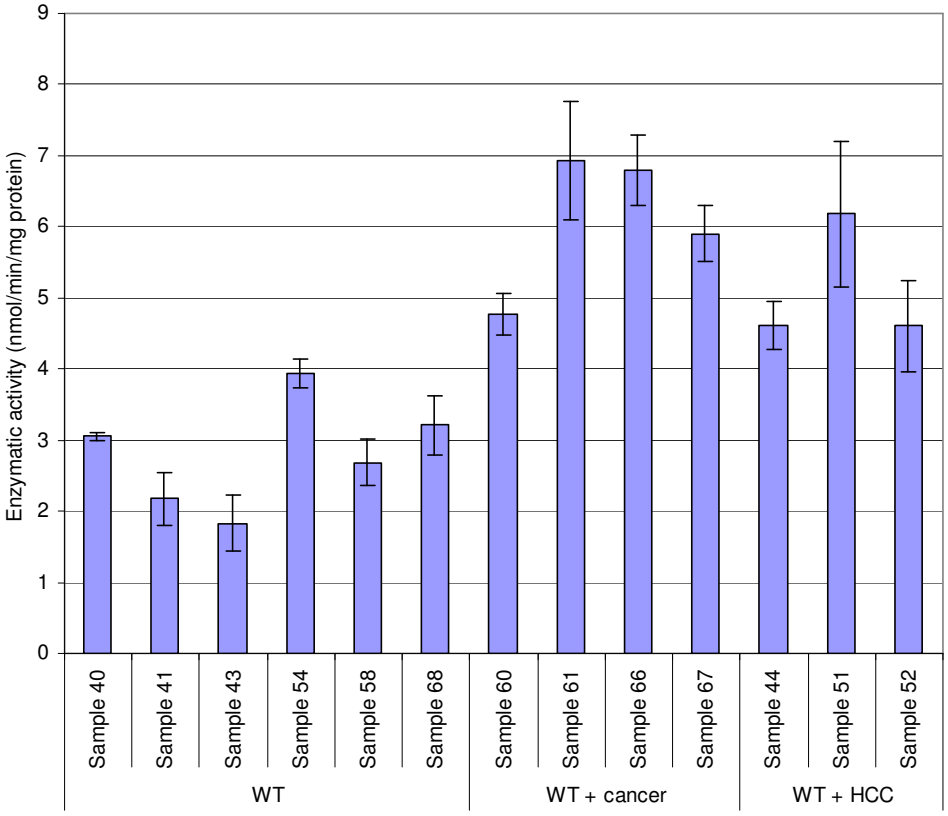
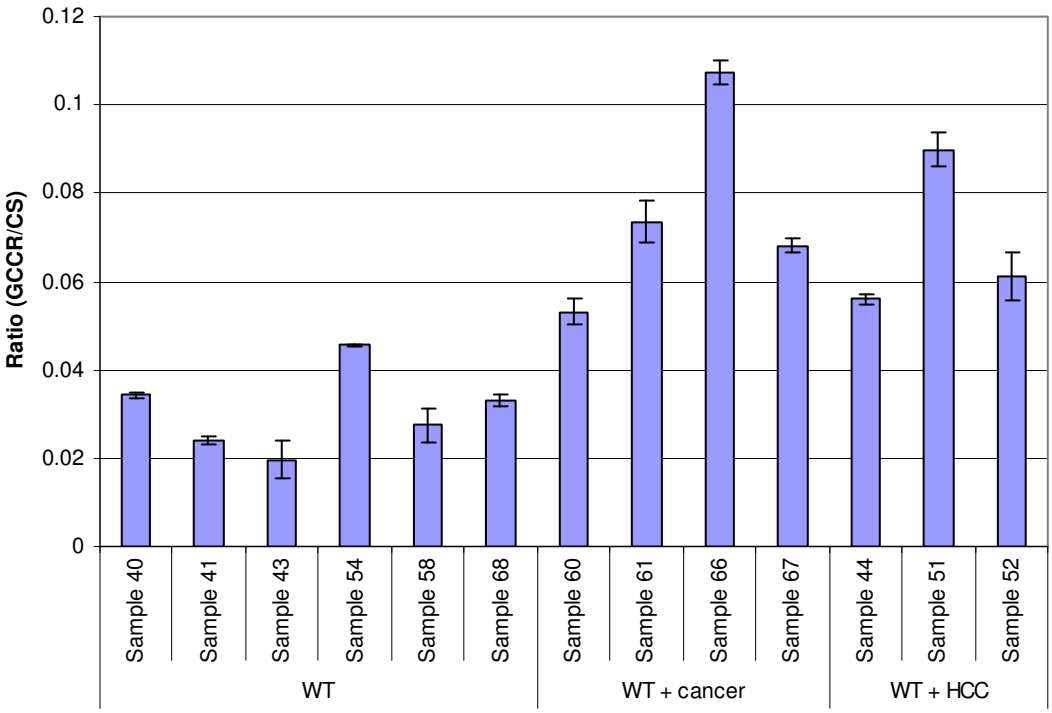


Fig. 3B. Roy Chowdhury SK et al., 2006



Legends to Figures

Fig. 1. mRNA level of mGPDH in prostate cancer cell lines.

Real-time PCR was used to measure the relative abundance of steady-state mRNA levels of mGPDH in prostate cancer cell lines. Real-time PCR was performed using ABI PRISM 7700 Sequence Detection System from Applied Biosystem.

Fig. 2. Immunodetection of antioxidant enzymes in prostate cancer cell lines.

Cellular lysates (5 μg/lane) from cultured cell lines PNT1A, DU145, PC3, LNCaP and CL1 were analyzed by SDS-PAGE and western blotting using anti catalase, anti manganese superoxide dismutase (MnSOD) and anti copper-zinc superoxide dismutase (CuZnSOD). β-actin serves as a control of protein loading and integrity.

Fig. 3. Enzymatic activity of glycerophosphate cytochrome *c* reductase (GCCR) [A] and GCCR activity related to citrate synthase [B] in mouse liver tissues.

Enzymatic activity of GCCR and GCCR activity related to citrate synthase were measured in liver tissues from mice with and without cancer either in liver tissue or other organs.

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High activity of mitochondrial glycerophosphate dehydrogenase and glycerophosphate-dependent ROS production in prostate cancer cell lines

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Abstract

Most malignant cells are highly glycolytic and produce high levels of reactive oxygen species (ROS) compared to normal cells. Mitochondrial glycerophosphate dehydrogenase (mGPDH) participates in the reoxidation of cytosolic NADH by delivering reducing equivalents from this molecule into the electron transport chain, thus sustaining glycolysis. Here, we investigate the role of mGPDH in maintaining an increased rate of glycolysis and evaluate glycerophosphate-dependent ROS production in prostate cancer cell lines (LNCaP, DU145, PC3, and CL1). Immunoblot, polarographic, and spectrophotometric analyses revealed that mGPDH abundance and activity was significantly elevated in prostate cancer cell lines when compared to the normal prostate epithelial cell line PNT1A. Furthermore, both the glycolytic capacity and glycerophosphate-dependent ROS production was increased 1.68- to 4.44-fold and 5- to 7-fold, respectively, in prostate cancer cell lines when compared to PNT1A cells. Overall, these data demonstrate that mGPDH is involved in maintaining a high rate of glycolysis and is an important site of electron leakage leading to ROS production in prostate cancer cells.

Keywords: Mitochondrial glycerophosphate dehydrogenase; Reactive oxygen species; Glycolysis; Glycerophosphate shuttle; Prostate cancer

Prostate cancer is the most commonly diagnosed malignant disease and the second leading cause of cancer-related mortality in men in most Western countries [1]. A number of risk factors such as age, cigarette smoking, obesity, high fat diet, and environmental toxins have been associated with both prostate cancer and increased ROS generation [2]. However, mechanisms by which they increase the risk of this disease are not clear. Prostate cancer etiology is in part related to oxidative stress. ROS has been proposed as a mediator in several human pathologies [3], ageing [4], necrotic and apoptotic processes [5,6] as well as cancer in general [7]. In addition, ROS plays an important role in regulating both the growth and survival of prostate cancer [8]. Oxidative stress is associated with an increased generation of ROS derived from oxygen and oxidants, and/or impaired cellular antioxidant mechanisms. Mitochondria are the main site of ROS production, converting up to 4% of intracellular molecular oxygen to ROS as byproducts of oxidative phosphorylation [9]. It has been previously demonstrated that ROS formed during this process of energy production results from the inefficient flow of electrons through Complexes I and III of the respiratory chain [9]. However, more recent studies have shown that mitochondrial glycerophosphate dehydrogenase also participates in the production of significant amounts of ROS when glycerophosphate is supplied as a respiratory substrate [10,11].

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Mitochondrial FAD-dependent glycerophosphate dehydrogenase (mGPDH, EC 1.1.99.5) is encoded in the nucleus and is located on the outer surface of the inner mitochondrial membrane. This enzyme acts in concert with the cytoplasmic NAD-linked glycerophosphate dehydrogenase (cGPDH, EC 1.1.1.8) to form the glycerophosphate shuttle [12]. This shuttle interconverts glycerol-3-phosphate and dihydroxyacetone phosphate, transferring reducing equivalents into the electron transport chain, in order to reoxidize cytosolic NADH generated from glycolysis. The activity of mGPDH is low in most mammalian tissues resulting in a low glycerophosphate shuttle activity [11]. However, brown adipose tissue contains mGPDH and cGPDH in equimolar proportions resulting in high shuttle activity [13]. The glycerophosphate shuttle is also active in tissues that metabolize glucose rapidly, such as skeletal muscle [14], brain [15], and insect flight muscle [16]. In addition, high mGPDH activity has also been reported in rat spermatozoa [17], pancreatic β cells [18], heart muscle [19], placenta [20], testis [21], and the rat liver after hormonal induction [22]. The glycerophosphate shuttle also contributes to thermogenic pathways [23], the regulation of lipid synthesis [24], the energy dissipating system due to bypassing one phosphorylation site at Complex I [25], and the control of glycolysis by shuttling electrons from NADH into the mitochondria [13]. mGPDH appears to play a crucial role in fetal development as Brown et al. [26] reported an ~50% reduction in viability of mGPDH knockout mice when compared to wild-type littermates. However, the multiple roles of mGPDH in cellular metabolism have not been fully elucidated.

In this study, we investigate the role of mGPDH in the production of ROS and its function in the reoxidation of cytosolic NADH; thereby, allowing the high rate of glycolysis observed in prostate cancer cells.

Materials and methods

Cell culture. The normal immortalized human prostate epithelial cell line, PNT1A, was a generous gift from Prof. Norman J. Maitland (University of York, UK) [27,28]. This cell line was maintained in RPMI 1640 (Invitrogen Life Technologies, Ontario, Canada) supplemented with 10% fetal bovine serum (Invitrogen Life Technologies), 1% penicillin–streptomycin (Invitrogen Life Technologies), and 1.0 mM glutamine. The androgen-sensitive human prostate cancer cell line, LNCaP, and androgen-insensitive DU145 and PC3 cell lines were obtained from the American Type Culture Collection (Manassas, VA). These cell lines were maintained in RPMI 1640 supplemented with 10 mM Hepes, 1.0 mM sodium pyruvate, 10% fetal bovine serum, and 1% penicillin–streptomycin. The CL1 androgen-independent cells derived from LNCaP cells were a generous gift from Dr. A.S. Belldegrun (University of California Los Angeles Medical School, Los Angeles, CA). CL1 cells were maintained in RPMI 1640 supplemented with 10% charcoal-stripped fetal bovine serum and 1% penicillin–streptomycin. Cell lines were cultured at 37 °C in a humidified atmosphere with 5% CO_2. All other reagents used in this investigation were purchased form Sigma–Aldrich unless otherwise stated.

Polarographic measurements. Oxygen consumption was determined at 37 °C using the OROBOROS oxygraph (Oroboros, Innsbruck, Austria) [29]. Cells were trypsinized, resuspended in KCl medium (80 mM KCl, 10 mM Tris–HCl, 3 mM $MgCl_2$, 1 mM EDTA, and 5 mM potassium phosphate, pH 7.4) at a concentration of 1.5×10^6 cells/ml, and permeabilized by digitonin (12.5 μg/1×10^6 cells). Various respiratory substrates and inhibitors were used as indicated in Figs. 1A and B. Oroboros DatLab software was used to calculate oxygen consumption and Microsoft Excel for graphic presentation of experimental data. Oxygen consumption is expressed as pmol/s/1×10^6 cells.

Spectrophotometric measurements. Both glycerophosphate cytochrome *c* reductase (GCCR) and succinate cytochrome *c* reductase (SCCR) activity were measured in a reaction mixture containing 10 mM potassium phosphate (pH 7.4), 2 mM EDTA, 0.01% bovine serum albumin (fatty acid free), 0.2 mM ATP, 1 mM KCN, 5 μM rotenone, and 20 mM glycerophosphate or succinate, respectively. After two washes in PBS, cell samples were sonicated for 20 s and protein content was measured according to the method of Bradford using bovine serum albumin as a standard [30]. Sonicated cells (0.2 mg protein) were incubated in the reaction mixture for 3 min, after which time 40 μM oxidized cytochrome *c* was added and changes in

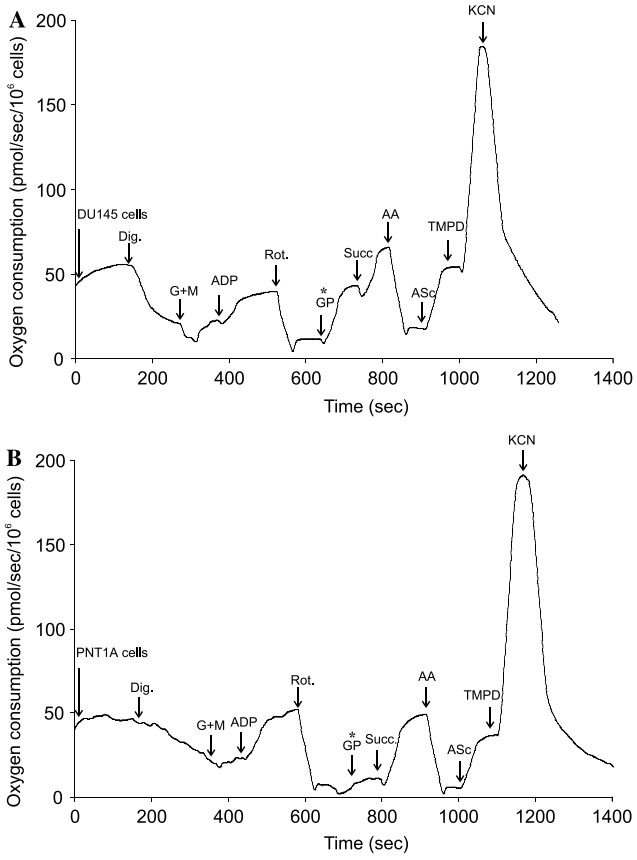

Fig. 1. Oxygen consumption using a multiple substrate-inhibitor analysis. Oxygen consumption was assessed in DU145 (A) and PNT1A cells (B) as described under Materials and methods. Dig, digitonin; G, glutamate (10 mM); M, malate (5 mM); ADP, adenosine diphosphate (1 μM); Rot, rotenone (1 μM); GP*, glycerophosphate (10 mM); Succ, succinate (10 mM); AA, antimycin A (1 μg/ml); ASc, ascorbate (5 mM); TMPD: *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine dihydrochloride (0.5 mM); KCN, potassium cyanide (0.25 mM).

absorbance were monitored for 5 min at 550 nm. All spectrophotometric measurements were conducted at 30 °C using a Beckman DU-640 spectrophotometer affixed with an internal temperature control unit (Beckman Instruments, Fullerton, CA).

Cytoplasmic glycerophosphate dehydrogenase (cGPDH) activity was measured in a reaction mixture containing Hepes–NaOH buffer (pH 7.5), 1 mM dihydroxyacetone phosphate, 0.1 mM NADH, 1 mM KCN, and 1 μM rotenone [31]. Cells were sonicated for 20 s in 500 μl of Hepes–NaOH buffer (pH 7.4) containing 60 mM sucrose, 190 mM mannitol, 15 mM KCl, 3 mM KH_2PO_4, 1 mM $MgCl_2$, and 0.5 mM EGTA. Protein content was determined as described above and 100 μg of sonicated cells was added to the reaction mixture at a final volume of 1 ml. The absorbance of NADH was monitored for 3 min at 340 nm.

Lactate dehydrogenase activity was evaluated by the addition of 1×10^5 cells to a reaction mixture containing 0.1 M Tris–HCl (pH 7.1), 0.25% Triton X-100, 10 mM pyruvate, and 0.3 mM NADH in a final volume of 1 ml [32]. The absorbance of NADH was monitored for 3 min at 340 nm.

Western blot analysis. Whole cell lysates were prepared in a buffer containing 15% NP40, 5 M NaCl, 2 M Tris base (pH 7.4), and 0.5 M EDTA. Protein concentrations were determined as described above and 40 μg of protein was loaded onto a 10% SDS–PAGE gel. Following electrophoresis, separated proteins were transferred onto nitrocellulose membranes and blocked with 5% skimmed milk in TBST for 1 h. Membranes were then incubated overnight at 4 °C with a polyclonal goat anti-mGPDH antibody in TBST (generous gift from Dr. J.M. Weitzel University Hospital Hamburg-Eppendorf, Germany) [33]. After three washes in TBST, membranes were incubated in horseradish peroxidase-linked goat anti-rabbit IgG antibody for 1 h (Santa Cruz Biotechnology), and proteins were detected using the enhanced chemiluminescence Western blotting detection kit (Amersham Biosciences).

Fluorometric detection of H_2O_2. Cells were plated in triplicate at a density of 10,000 cells/well in a 96-well plate, allowed to adhere, and washed once with Dulbecco's phosphate-buffered solution (DPBS). The Amplex Red Hydrogen peroxide Assay kit (Molecular probes, Oregon) was used to determine extracellular H_2O_2 production. Briefly, 50 μM of Amplex red and 0.2 U/ml of horseradish peroxidase in DPBS were added to cells and fluorescence readings were obtained using a CytoFluor Series 4000 plate reader (PerSeptive Biosystems, Framingham, MA, Ex/Em = 530/590 nm). A standard curve with known amounts of H_2O_2 was used to determine extracellular H_2O_2 concentrations.

Statistical analysis. Data are presented as means ± SD. Statistical significance was determined through the use of a Student's *t* test.

Results

This is the first study to conduct a systematic evaluation of mGPDH using two complementary functional methods, namely polarography and spectrophotometry, in normal prostate epithelial cells (PNT1A) and prostate cancer cell lines. Like mGPDH, succinate dehydrogenase is also flavoprotein-dependent and transfers electrons directly to coenzyme Q of the electron transport chain [10]. Therefore, the activity of succinate dehydrogenase was measured in each sample and compared directly to that of mGPDH activity. Oxygen consumption measurements were done in intact permeabilized cells to ensure mitochondrial and intracellular integrity as well as interorganelle structural communication [34]. Spectrophotometric measurements, on the other hand, characterize activities of isolated complexes in disintegrated membranes. Both methods provide complementary data about mitochondrial functions.

Polarographic analysis

Representative measurements of oxygen consumption of DU145 and PNT1A cells are depicted in Figs. 1A and B, respectively. Following the addition of glycerophosphate (GP*), oxygen consumption was significantly increased in DU145 cells (Fig. 1A) when compared to PNT1A cells (Fig. 1B). Moreover, this observation appears to be a characteristic of all prostate cancer cell lines evaluated as LNCaP, DU145, PC3, and CL1 cells consumed 2.5- to 6.0-fold more oxygen after the addition of glycerophosphate when compared to PNT1A cells (Table 1). In contrast, oxygen consumption following the addition of succinate in prostate cancer cells and normal prostate cells showed no significant difference (Table 1).

Spectrophotometric measurements

GCCR activity

The enzymatic activity of mGPDH was evaluated in cell lysates by monitoring the rate of reduced cytochrome *c* formation at 550 nm when glycerophosphate was used as a substrate, termed glycerophosphate cytochrome *c* reductase (GCCR) activity. Using this assay, prostate cancer cells showed 2- to 6-fold higher GCCR activity when compared to normal PNT1A cells (Table 2). To evaluate the specific inhibition of mGPDH, cell lysates were incubated with sodium oleate for 5 min prior to the evaluation of GCCR activity. Sodium oleate diminished GCCR activity by 50–67% in both prostate cancer cell lines and normal prostate epithelial cell lysates (Fig. 2A).

SCCR activity

The enzymatic activity of succinate dehydrogenase was evaluated in cell lysates by monitoring the rate of

Table 1
Oxygen consumption by prostate cancer cells with glycerophosphate or succinate in PNT1A, LNCaP, DU145, PC3, and CL1 cells

Cells	O_2 consumption (pmol/s/1×10^6 cells)	
	Glycerophosphate	Succinate
PNT1A	12.19 ± 1.86	61.33 ± 6.85
LNCaP	30.46 ± 5.46*	69.21 ± 23.31
DU145	46.63 ± 3.59*	70.28 ± 11.91
PC3	69.65 ± 23.50*	78.46 ± 28.90
CL1	72.80 ± 16.66*	65.77 ± 9.84

Oxygen consumption was measured with 10 mM glycerophosphate or 10 mM succinate in the presence of 1 mM ADP and 1 μM rotenone as described under Materials and methods.

* $P < 0.005$ with respect to normal prostate epithelial cells, PNT1A.

Table 2
Spectrophotometric determination of glycerophosphate cytochrome *c* reductase (GCCR), succinate cytochrome *c* reductase (SCCR), cytoplasmic glycerophosphate dehydrogenase (cGPDH), and lactate dehydrogenase (LDH) activity in PNT1A, LNCaP, DU145, PC3, and CL1 cells as described under Materials and methods

Cells	Enzymatic activity (nmol/min/mg protein)			
	GCCR	SCCR	cGPDH	LDH
PNT1A	1.95 ± 0.29	8.95 ± 1.13	91.86 ± 6.54	392.53 ± 22.91
LNCaP	$3.65 \pm 0.88^{*}$	11.86 ± 1.63	91.51 ± 2.51	$902.02 \pm 146.94^{*}$
DU145	$6.82 \pm 0.47^{*}$	12.79 ± 0.78	71.71 ± 8.32	$657.47 \pm 42.09^{*}$
PC3	$11.10 \pm 0.92^{*}$	8.60 ± 1.47	79.89 ± 6.73	$1743.20 \pm 172.62^{*}$
CL1	$11.37 \pm 1.53^{*}$	11.04 ± 1.94	98.03 ± 5.35	$1261.25 \pm 83.40^{*}$

* $P < 0.005$ with respect to normal prostate epithelial cells, PNT1A.

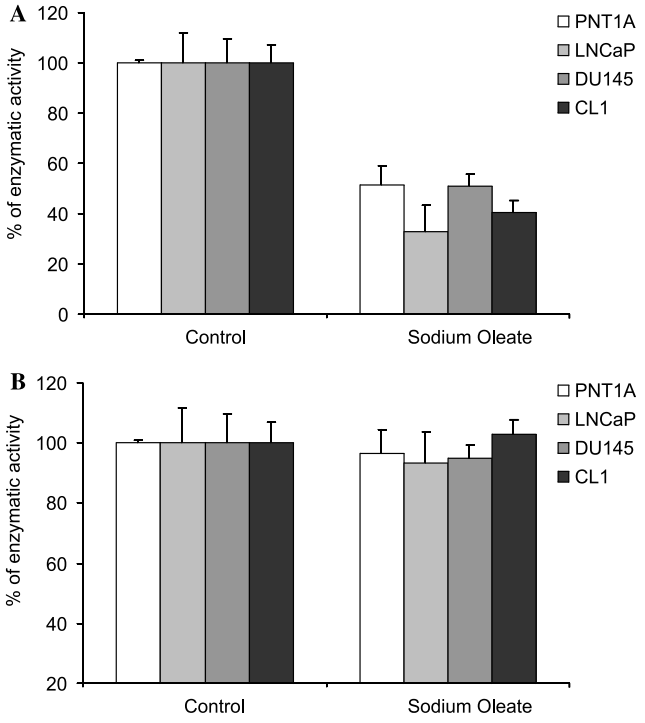

Fig. 2. Specific inhibition of mGPDH by sodium oleate. GCCR (A) and SCCR (B) activity was measured as described under Materials and methods. Samples were incubated in the presence or absence of 15 μM sodium oleate for 5 min prior to analysis of enzymatic activity.

reduced cytochrome *c* formation at 550 nm when succinate was used as a substrate, termed succinate cytochrome *c* reductase (SCCR) activity. Using this assay, prostate cancer cells showed no significant difference in SCCR activity when compared to PNT1A cells (Table 2). Unlike GCCR activity, SCCR activity was not altered significantly in any cell line upon the addition of sodium oleate (Fig. 2B).

cGPDH activity

cGPDH activity was measured by monitoring the formation of NAD during the cGPDH-catalyzed reduction of dihydroxyacetone phosphate. Spectrophotometric analysis revealed that the cGPDH activity of all cell lines used in this investigation was not significantly different from each other (Table 2).

Lactate dehydrogenase activity

LDH catalyzes the last step in glycolysis. LDH activity was therefore used as a quantitative marker of glycolytic activity in intact cells by monitoring the formation of NAD as pyruvate is converted to lactate [32]. Using this method, LDH activity was 1.68- to 4.44-fold higher in prostate cancer cells when compared to normal prostate epithelial cells (Table 2). These results confirmed that prostate cancer cells are more glycolytic than normal prostate cells.

Western blot analysis

Mitochondrial glycerophosphate dehydrogenase protein levels were elevated in prostate cancer cell lines when compared to the normal prostate cell line, PNT1A (Fig. 3). Moreover, mGPDH appears to be less abundant in the androgen-sensitive LNCaP cell line when compared to DU145, PC3 or CL1 cells that are all androgen insensitive.

Glycerophosphate-dependent H_2O_2 production by prostate cancer cells

Hydrogen peroxide production was evaluated in both prostate cancer cell lines and PNT1A cells. Prostate cancer cells produced 2- to 3-fold more H_2O_2 when compared to PNT1A cells under basal conditions (Fig. 4). LNCaP, DU145, PC3, and CL1 cell lines generated 2.7- to 4.1-fold higher H_2O_2 than PNT1A cells, when glycerol-3-phosphate was used as a substrate (Fig. 4). The Complex IV inhibitor, KCN, dramatically increased H_2O_2 production rates. Glycerophosphate-induced H_2O_2 production in the presence of KCN was elevated 5- to 7-fold in prostate cancer cells when compared to PNT1A cells. Moreover, glycerophosphate-induced

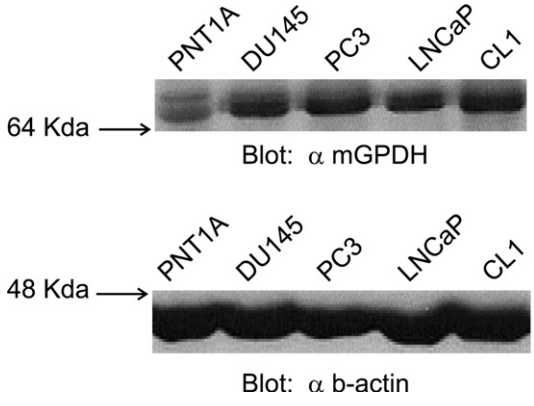

Fig. 3. Western blot analysis of mGPDH expression. Total cellular extracts were prepared from PNT1A, DU145, PC3, LNCaP, and CL1 cells prior to subsequent immunoblot analysis to monitor the level of mGPDH as described under Materials and methods.

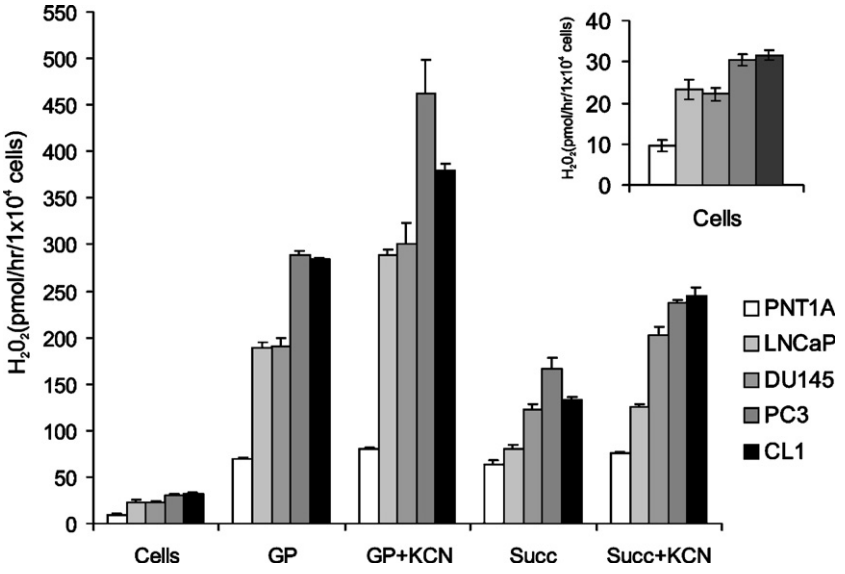

Fig. 4. Fluorometric detection of H_2O_2. H_2O_2 production by PNT1A, DU145, PC3, LNCaP, and CL1 was performed spectrofluorometrically in the presence and absence of GP, GP + KCN, Succ, Succ + KCN as described under Materials and methods.

H_2O_2 production in the presence of KCN exceeded H_2O_2 production when both succinate and KCN were administered in combination (Fig. 4).

H_2O_2 production following administration of the GCCR inhibitor sodium oleate

GP-dependent generation of H_2O_2 in DU145 was decreased 57% by sodium oleate (Fig. 5). In contrast, GP-dependent H_2O_2 generation in PNT1A cells was unaffected by the addition of sodium oleate.

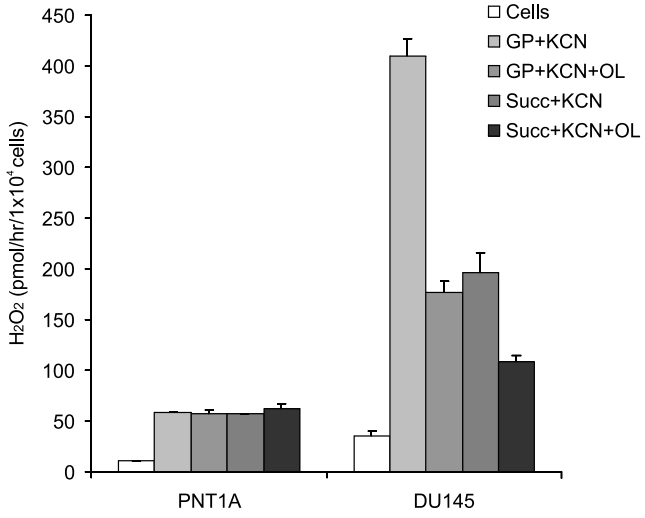

Fig. 5. Inhibition of GP-dependent generation of H_2O_2 by sodium oleate. H_2O_2 generation by PNT1A and DU145 cells was performed spectrofluorometrically in the presence and absence of 15 μM sodium oleate as described under Materials and methods.

Discussion

In 1930 Otto Warburg [35] hypothesized that cancer cells may have an impaired respiratory capacity resulting in elevated rates of glycolysis. Although the impairment of respiratory capacity is not established in cancer, the high rate of glycolysis is a common metabolic phenotype in most cancer cells. However, the maintenance of glycolysis is dependent on the continual reoxidation of cytosolic NADH to NAD. One cellular system capable of catalyzing this reaction is the glycerophosphate shuttle which is composed of two enzymes, namely mGPDH and cGPDH. mGPDH ensures unidirectionality of electron transfer into the coenzyme Q pool, while the formation of glycerophosphate from dihydroxyacetone and NADH is catalyzed by cGPDH and is reversible [12]. In this study, we have evaluated the abundance and activity of mGPDH as well as glycerophosphate-dependent ROS production in various prostate cancer lines and normal prostate epithelial cells.

A previous study by MacDonald et al. [36] demonstrated that mGPDH activity is elevated in insulinomas, carcinoid, and other tumors that are derived from tissue that belong to the amine precursor uptake decarboxylation system. In addition, this study also observed that a proportion of breast carcinomas and melanomas also had elevated mGPDH activity. Likewise, we have determined through both polarographic and spectrophotometric analyses that mGPDH activity is similarly elevated in a number of human prostate cancer cell lines when compared to normal prostate cancer epithelial cells (Fig. 1B; Tables 1 and 2). MacDonald et al. also noted that succinate dehydrogenase activity was elevated when compared to that of mGPDH in a number of

normal tissues, which is in concordance with findings from this study (Figs. 1A and B; Tables 1 and 2). Results from both polarographic and spectrophotometric analyses of mGPDH and succinate dehydrogenase activity show a high degree of similarity indicating that the results of either analysis are likely not spurious.

In order to determine that varying levels of cGPDH activity was not responsible for the observed differences in mGPDH activity, cGPDH activity was assessed. Spectrophotometric analysis revealed that the cGPDH activity of all cell lines used in this investigation was not significantly different from each other and, therefore, cannot account for the observed differences in mGPDH activity (Table 2).

To assess the amount of mGPDH in normal prostate cells and prostate cancer cells, immunoblot analysis was performed on whole cell lysates utilizing an mGPDH specific antibody. Cell lines expressing high levels of mGPDH also had elevated mGPDH activity, while cell lines expressing lower amounts of this protein had decreased enzymatic activity (Fig. 3; Tables 1 and 2). This observation would indicate that there is a positive correlation between mGPDH expression and activity, at least in the cell lines evaluated in this study. In the same fashion, MacDonald et al. also reported a positive correlation between mGPDH expression and activity in various rat tissues [37]. It is of interest to note that there may also be a correlation between androgen sensitivity and mGPDH expression as androgen-sensitive LNCaP cells had decreased amounts of mGPDH when compared to androgen-insensitive DU145, PC3, and CL1 cells.

Our data are the first to suggest that mGPDH acts as an ROS generator in prostate cancer cells (Fig. 4). Moreover, glycerophosphate-dependent ROS production could be decreased with the addition of sodium oleate in DU145 cells, however, this inhibitor had no effect on ROS production in PNT1A cells. These findings suggest a direct involvement of mGPDH in ROS generation in prostate cancer lines, but not in normal cells. Sodium oleate does not act as an antioxidant as there was no effect on ROS production under any condition tested in PNT1A cells (Fig. 5). It is not yet clear why sodium oleate has no observable effect on succinate dehydrogenase activity (Fig. 2), but is capable of decreasing succinate-dependent ROS production (Fig. 5).

Fluorometric analysis revealed that glycerophosphate-dependent ROS production exceeded that of succinate-dependent ROS production (Fig. 4). This observation may be attributable to the absence of a CoQ-binding protein in mGPDH. This CoQ-binding protein evidently has a natural protection of ubisemiquinone formed during CoQ reduction by succinate dehydrogenase [38].

The data presented in this paper suggest a coordinated higher activity and abundance of mGPDH and an increased rate of glycerophosphate-dependent ROS production in prostate cancer cells in comparison to normal prostate epithelial cells. The elevated activity of mGPDH and GP-dependent ROS production may be a characteristic of neoplastic cells. The elevated expression and activity of mGPDH in prostate cancer cells indicate that the glycerophosphate shuttle is activated along with the malate–aspartate shuttle to maintain highly glycolytic environment in cancer cells. This is the first report to demonstrate that mGPDH is an important source of ROS in prostate cancer cells that could be used as a strategy to target their destruction.

Acknowledgments

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References

[1] S.H. Landis, T. Murray, S. Bolden, P.A. Wingo, Cancer statistics, CA Cancer J. Clin. 49 (1) (1999) 8–31.
[2] S.K. Pathak, R.A. Sharma, W.P. Steward, J.K. Mellon, T.R. Griffiths, A.J. Gescher, Oxidative stress and cyclooxygenase activity in prostate carcinogenesis: targets for chemopreventive strategies, Eur. J. Cancer 41 (2005) 61–70.
[3] B. Halliwell, Oxidants and human disease: some new concepts, FASEB J. 1 (1987) 358–364.
[4] G. Barja, Mitochondrial oxygen radical generation and leak: sites of production in states 4 and 3, organ specificity, and relation to aging and longevity, J. Bioenerg. Biomembr. 31 (1999) 347–366.
[5] G. Kroemer, B. Dallaporta, M. Resche-Rigon, The mitochondrial death/life regulator in apoptosis and necrosis, Annu. Rev. Physiol. 60 (1998) 619–642.
[6] P.L. Pedersen, Mitochondrial events in the life and death of animal cells: a brief overview, J. Bioenerg. Biomembr. 31 (1999) 291–304.
[7] A.L. Jackson, L.A. Loeb, The contribution of endogenous sources of DNA damage to the multiple mutations in cancer, Mutat. Res. 477 (2001) 7–21.
[8] S.S. Brar, Z. Corbin, T.P. Kennedy, R. Hemendinger, L. Thornton, B. Bommarius, R.S. Arnold, A.R. Whorton, A.B. Sturrock, T.P. Huecksteadt, M.T. Quinn, K. Krenitsky, K.G. Ardie, J.D. Lambeth, J.R. Hoidal, NOX5 NAD(P)H oxidase regulates growth and apoptosis in DU 145 prostate cancer cells, Am. J. Physiol. Cell Physiol. 285 (2003) C353–C369.
[9] G. Lenaz, C. Bovina, M. D'Aurelio, R. Fato, G. Formiggini, M.L. Genova, G. Giuliano, P.M. Merlo, U. Paolucci, C.G. Parenti, B. Ventura, Role of mitochondria in oxidative stress and aging, Ann. NY Acad. Sci. 959 (2002) 199–213.
[10] Z. Drahota, S.K. Chowdhury, D. Floryk, T. Mracek, J. Wilhelm, H. Rauchova, G. Lenaz, J. Houstek, Glycerophosphate-dependent hydrogen peroxide production by brown adipose tissue mitochondria and its activation by ferricyanide, J. Bioenerg. Biomembr. 34 (2002) 105–113.

[11] P. Jesina, D. Kholova, R. Bolehovska, Z. Cervinkova, Z. Drahota, J. Houstek, Glycerophosphate-dependent hydrogen peroxide production by rat liver mitochondria, Physiol. Res. 53 (2004) 305–310.
[12] M. Klingenberg, Mitochondria metabolite transport, FEBS Lett. 6 (1970) 145–154.
[13] J. Houstek, B. Cannon, O. Lindberg, Glycerol-3-phosphate shuttle and its function in intermediary metabolism of hamster brown-adipose tissue, Eur. J. Biochem. 54 (1975) 11–18.
[14] E.S. Cole, C.A. Lepp, P.D. Holohan, T.P. Fondy, Isolation and characterization of flavin-linked glycerol-3-phosphate dehydrogenase from rabbit skeletal muscle mitochondria and comparison with the enzyme from rabbit brain, J. Biol. Chem. 253 (1978) 7952–7959.
[15] I.R. Cottingham, C.I. Ragan, The reconstitution of L-3-glycerophosphate-cytochrome *c* oxidoreductase from L-3-glycerophosphate dehydrogenase, ubiquinone-10 and ubiquinol–cytochrome *c* oxidoreductase, Biochem. J. 192 (1980) 19–31.
[16] R.S. Sohal, Hydrogen peroxide production by mitochondria may be a biomarker of aging, Mech. Ageing Dev. 60 (1991) 189–198.
[17] J.B. Schenkman, D.A. Richert, W.W. Westerfeld, alpha-glycerophosphate dehydrogenase in rat spermatozoa, Endocrinology 76 (1965) 1055–1061.
[18] H. Ishihara, M. Nakazaki, Y. Kanegae, K. Inukai, T. Asano, H. Katagiri, Y. Yazaki, M. Kikuchi, J. Miyazaki, I. Saito, Y. Oka, Effect of mitochondrial and/or cytosolic glycerol 3-phosphate dehydrogenase overexpression on glucose-stimulated insulin secretion from MIN6 and HIT cells, Diabetes 45 (1996) 1238–1244.
[19] T.D. Scholz, S.L. Koppenhafer, C.J. TenEyck, B.C. Schutte, Developmental regulation of the alpha-glycerophosphate shuttle in porcine myocardium, J. Mol. Cell. Cardiol. 29 (1997) 1605–1613.
[20] J. Swierczynski, P. Scislowski, Z. Aleksandrowicz, High activity of alpha-glycerophosphate oxidation by human placental mitochondria, Biochim. Biophys. Acta 429 (1976) 46–54.
[21] J.M. Weitzel, N.B. Shiryaeva, R. Middendorff, M. Balvers, C. Radtke, R. Ivell, H.J. Seitz, Testis-specific expression of rat mitochondrial glycerol-3-phosphate dehydrogenase in haploid male germ cells, Biol. Reprod. 68 (2003) 699–707.
[22] S. Muller, H.J. Seitz, Cloning of a cDNA for the FAD-linked glycerol-3-phosphate dehydrogenase from rat liver and its regulation by thyroid hormones, Proc. Natl. Acad. Sci. USA 91 (1994) 10581–10585.
[23] W.N. Aldridge, B.W. Street, Mitochondria from brown adipose tissue, Biochem. J. 107 (1968) 315–317.
[24] R.R. Chaffee, J.R. Allen, Y. Cassuto, R.E. Smith, Biochemistry of brown fat and liver of cold-acclimated hamsters, Am. J. Physiol. 207 (1964) 1211–1214.
[25] V. Bobyleva, L. Pazienza, U. Muscatello, N. Kneer, H. Lardy, Short-term hypothermia activates hepatic mitochondrial *sn*-glycerol-3-phosphate dehydrogenase and thermogenic systems, Arch. Biochem. Biophys. 380 (2000) 367–372.
[26] L.J. Brown, R.A. Koza, C. Everett, M.L. Reitman, L. Marshall, L.A. Fahien, L.P. Kozak, M.J. MacDonald, Normal thyroid thermogenesis but reduced viability and adiposity in mice lacking the mitochondrial glycerol phosphate dehydrogenase, J. Biol. Chem. 277 (2002) 32892–32898.
[27] O. Cussenot, P. Berthon, R. Berger, I. Mowszowicz, A. Faille, F. Hojman, P. Teillac, A. Le Duc, F. Calvo, Immortalization of human adult normal prostatic epithelial cells by liposomes containing large T-SV40 gene, J. Urol. 146 (1991) 881–886.
[28] O. Cussenot, P. Berthon, B. Cochand-Priollet, N.J. Maitland, A. Le Duc, Immunocytochemical comparison of cultured normal epithelial prostatic cells with prostatic tissue sections, Exp. Cell Res. 214 (1994) 83–92.
[29] S.K. Chowdhury, Z. Drahota, D. Floryk, P. Calda, J. Houstek, Activities of mitochondrial oxidative phosphorylation enzymes in cultured amniocytes, Clin. Chim. Acta 298 (2000) 157–173.
[30] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[31] J. Rasschaert, W.J. Malaisse, Hexose metabolism in pancreatic islets. Glucose-induced and Ca(2+)-dependent activation of FAD-glycerophosphate dehydrogenase, Biochem. J. 278 (Pt. 2) (1991) 335–340.
[32] K. Renner, A. Amberger, G. Konwalinka, R. Kofler, E. Gnaiger, Changes of mitochondrial respiration, mitochondrial content and cell size after induction of apoptosis in leukemia cells, Biochim. Biophys. Acta 1642 (2003) 115–123.
[33] J.M. Weitzel, S. Kutz, C. Radtke, S. Grott, H.J. Seitz, Hormonal regulation of multiple promoters of the rat mitochondrial glycerol-3-phosphate dehydrogenase gene: identification of a complex hormone-response element in the ubiquitous promoter B, Eur. J. Biochem. 268 (2001) 4095–4103.
[34] T.G. Frey, C.A. Mannella, The internal structure of mitochondria, Trends Biochem. Sci. 25 (2000) 319–324.
[35] O. Warburg, On respiratory impairment in cancer cells, Science 124 (1956) 269–270.
[36] M.J. MacDonald, T.F. Warner, R.J. Mertz, High activity of mitochondrial glycerol phosphate dehydrogenase in insulinomas and carcinoid and other tumors of the amine precursor uptake decarboxylation system, Cancer Res. 50 (1990) 7203–7205.
[37] M.J. MacDonald, L.J. Brown, Calcium activation of mitochondrial glycerol phosphate dehydrogenase restudied, Arch. Biochem. Biophys. 326 (1996) 79–84.
[38] H. Rauchova, R. Fato, Z. Drahota, G. Lenaz, Steady-state kinetics of reduction of coenzyme Q analogs by glycerol-3-phosphate dehydrogenase in brown adipose tissue mitochondria, Arch. Biochem. Biophys. 344 (1997) 235–241.