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PRINCIPAL INVESTIGATOR: Subir Kumar Roy Chowdhury, M.D., Ph.D.

CONTRACTING ORGANIZATION: Hamilton Health Sciences Corporation
Hamilton, Ontario L8S 4J9 Canada

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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. Furthermore, both the glycolytic capacity and glycerophosphate-dependent ROS production was increased 1.68-4.44 fold and 5-7 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.

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 1,2.

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.

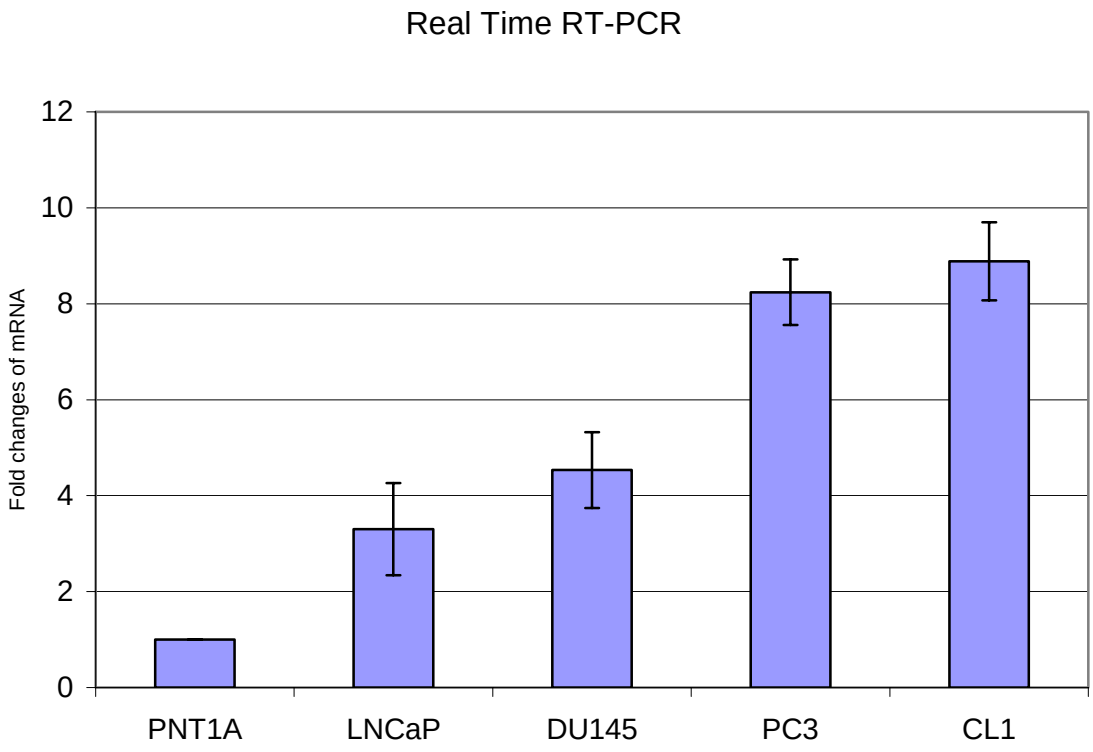
Cytoplasmic glycerophosphate dehydrogenase (cGPDH) activity: In order to determine that varying levels of cGPDH activity was not responsible for the observed differences in mGPDH activity, cGPDH activity was assessed. Spectrophotometric analysis revealed that the cGPDH activity of all cell lines used 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.

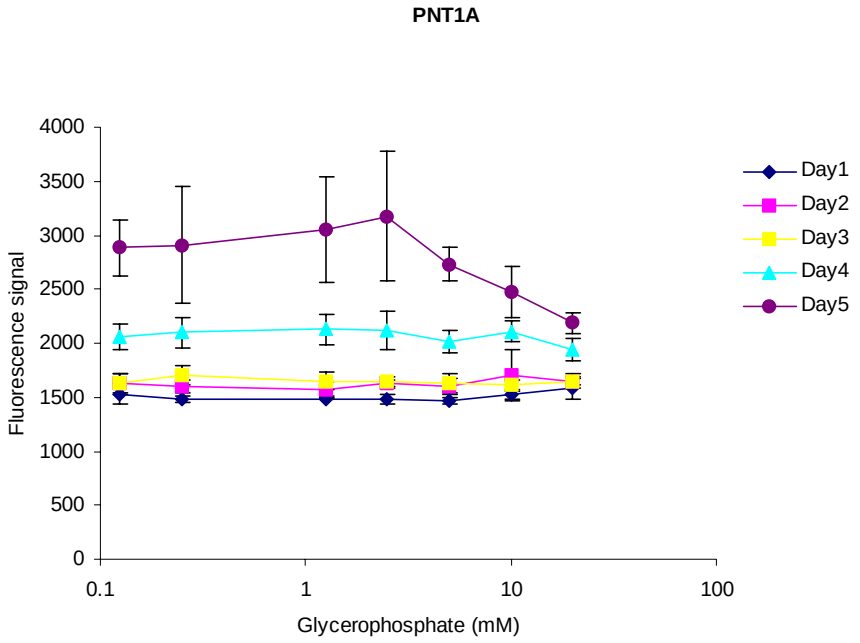
Our data are the first to suggest that mGPDH acts as an ROS generator in prostate cancer cells (Fig. 4). Moreover, glycerophosphate-dependent ROS production could be decreased with the addition of sodium oleate in DU145 cells, however this inhibitor had no effect on ROS production in PNT1A cells. These 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].

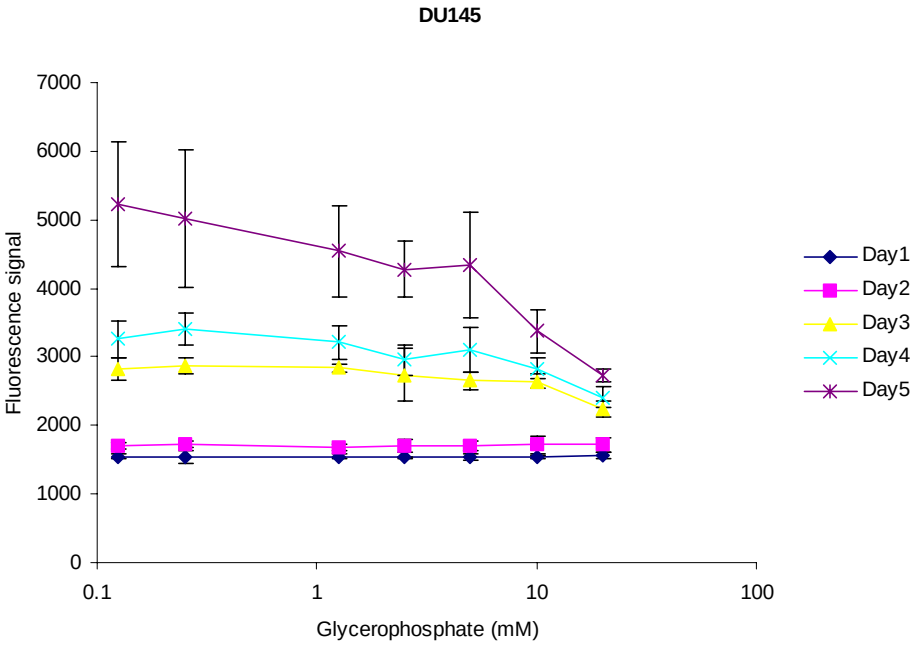
Evaluation of cell proliferation with glycerophosphate in prostate cancer cells. We further studied the effect of glycerophosphate on the cell proliferation of prostate cancer cell lines, DU145, LNCaP, and CL1 including normal prostate epithelial cells, PNT1A. All cell lines are stopped to proliferate 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

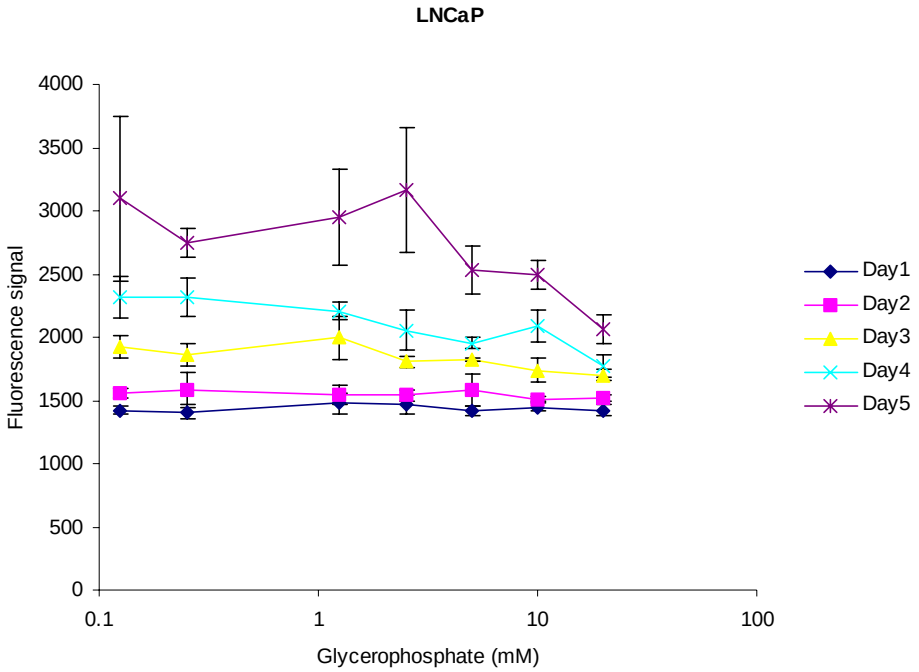
(A)


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

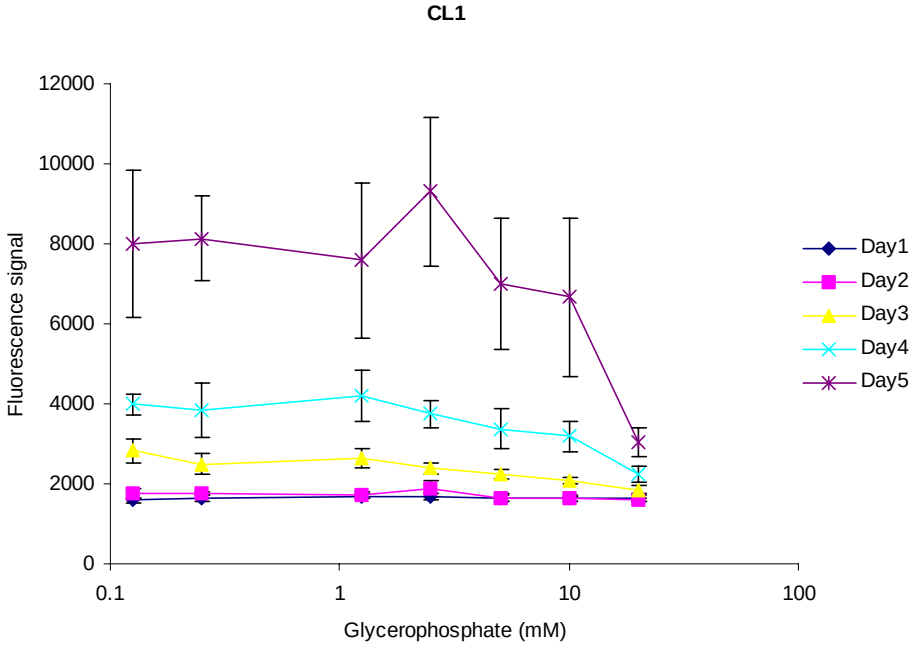
(B).


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) will be 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 will be transfected with prepared plasmid using Lipofectamine Plus (Invitrogen). Cell clones that stably express the antisense cDNA and lead to downregulation of mGPDH will be isolated by vector-mediated antibiotic-resistance and tested by western immunoblotting. Anchorage-independence will be determined by plating on soft agar. We also compare *in vitro* cell proliferation and anchorage-independent growth of cells between mGPDH-downregulated (antisense-transfected) and control (vector-transfected) cells. In Case we are not able to establish mGPDH-downregulated (antisense-transfected) and control (vector-transfected) cell lines using classical techniques, we will try to use the new technique, siRNA method to establish the down-regulated mGPDH cell line to investigate the role of mGPDH in production of ROS in prostate cancer lines.

In vivo determination of tumor formation in immune-incompetent mice: *In vivo* tumor formation in immune-incompetent mice will be determined by subcutaneous injection of Balb/C nude mice with $1x10^5$ of vector-transfected or antisense-transfected prostate cancer cells. Tumors will be scored for size every 1 or 2 days.

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 experiments outlined for the second year have begun and preliminary data have been obtained. We plan to present this work in the next six month.

Reportable outcomes:

The following publication describes 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).

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.

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Appendices:

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

Subir K.R. Chowdhury, Adam Gemin, Gurmit Singh *

Juravinski Cancer Centre, 699 Concession St., Hamilton, Ont., Canada L8V 5C2
Department of Pathology and Molecular Medicine, McMaster University, 1200 Main St. West, Hamilton, Ont., Canada L8N 3Z5

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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.
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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].

* Corresponding author. Fax: +1 905 575 6330.
E-mail address: gurmit.singh@hrcc.on.ca (G. Singh).

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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

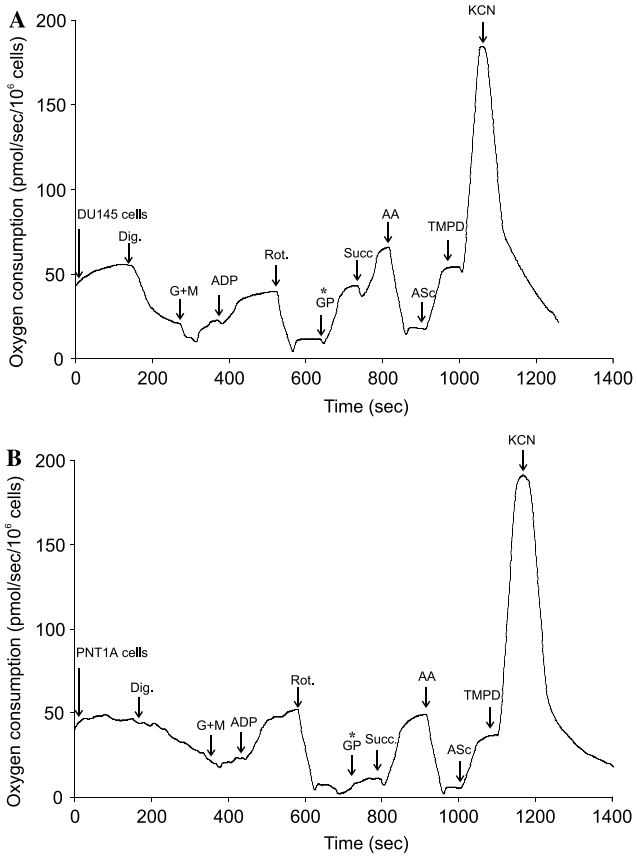

Fig. 1. Oxygen consumption using a multiple substrate-inhibitor analysis. Oxygen consumption was assessed in DU145 (A) and PNT1A cells (B) as described under Materials and methods. Dig, digitonin; G, glutamate (10 mM); M, malate (5 mM); ADP, adenosine diphosphate (1 μM); Rot, rotenone (1 μM); GP*, glycerophosphate (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.

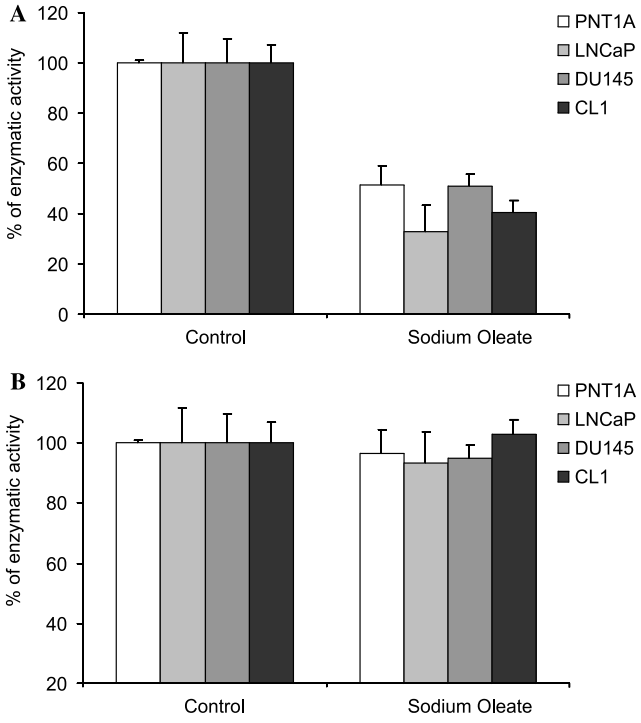

Fig. 2. Specific inhibition of mGPDH by sodium oleate. GCCR (A) and SCCR (B) activity was measured as described under Materials and methods. Samples were incubated in the presence or absence of 15 μM sodium oleate for 5 min prior to analysis of enzymatic activity.

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

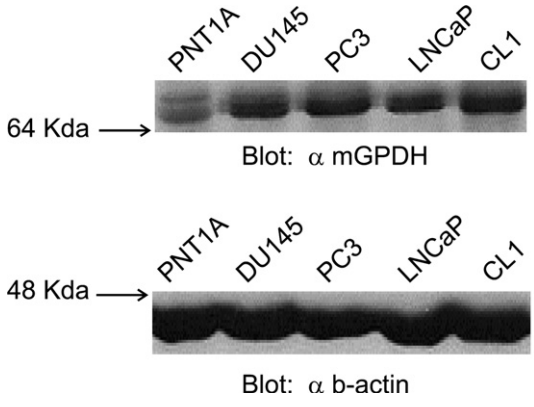

Fig. 3. Western blot analysis of mGPDH expression. Total cellular extracts were prepared from PNT1A, DU145, PC3, LNCaP, and CL1 cells prior to subsequent immunoblot analysis to monitor the level of mGPDH as described under Materials and methods.

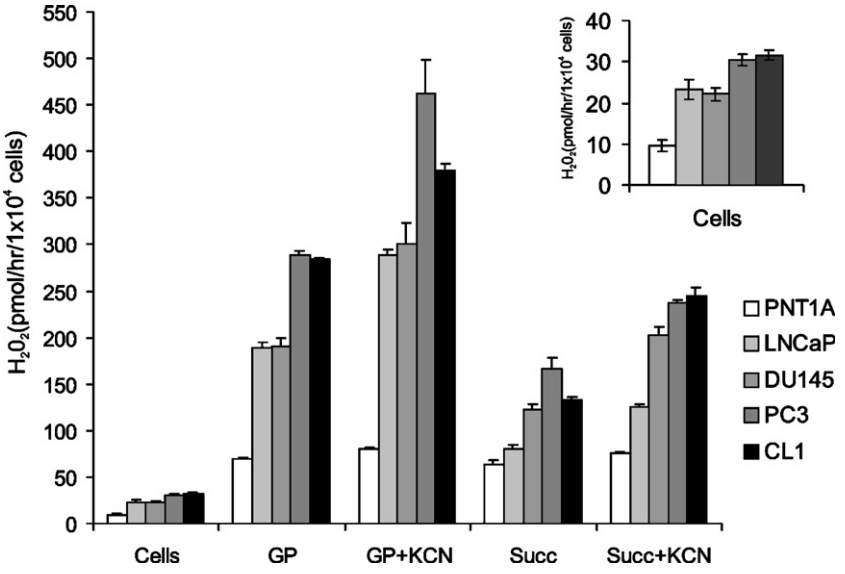

Fig. 4. Fluorometric detection of H_2O_2. H_2O_2 production by PNT1A, DU145, PC3, LNCaP, and CL1 was performed spectrofluorometrically in the presence and absence of GP, GP + KCN, Succ, Succ + KCN as described under Materials and methods.

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.

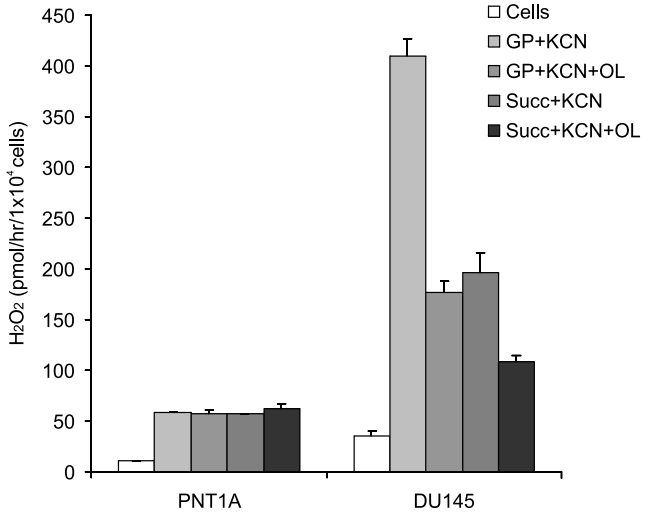

Fig. 5. Inhibition of GP-dependent generation of H_2O_2 by sodium oleate. H_2O_2 generation by PNT1A and DU145 cells was performed spectrofluorometrically in the presence and absence of 15 μM sodium oleate as described under Materials and methods.

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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