

Abnormal glyceraldehyde-3-phosphate dehydrogenase binding and glycolytic flux in Autosomal Dominant Polycystic Kidney Disease after a mild oxidative stress

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Abstract

Aim: The aim of this study was, a) to investigate the effect of mild oxidative stress on glycolytic flux and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) binding in erythrocytes from patients with autosomal dominant polycystic kidney disease (ADPKD), and b) to examine whether the modulation of GAPDH-binding to the red cell membrane leads to changes in glycolytic flux.

Patients and methods: The rate of lactate production in intact erythrocytes and the GAPDH/actin ratio in erythrocyte ghost membranes were measured before and after treating cells with t-butyl hydroperoxide or N-ethylmaleimide (NEM) in 13 ADPKD patients and 12 controls.

Results: t-butyl hydro-peroxide had a significant effect on both lactate production and GAPDH/actin ratio in healthy subjects, but it had essentially no effect on ADPKD patients in which both parameters already resembled those of the peroxide-treated controls. NEM treatment after 300 sec had a very significant effect on both lactate production and GAPDH/actin ratio in both patient and control cells. However, after 10 sec the effect on GAPDH/actin ratio was only significant in the erythrocytes of ADPKD patients. In every experiment glycolytic lactate production correlated negatively with membrane-bound GAPDH/actin ratio.

Conclusions: We conclude that glycolytic flux and GAPDH binding in erythrocytes from ADPKD patients respond abnormally to both a mild oxidative stress and brief exposure to NEM. Hippokratia 2008; 12 (3): 162-167

Keywords: autosomal dominant polycystic kidney disease, glyceraldehyde-3-phosphate dehydrogenase, glycolysis, oxidative stress

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Autosomal dominant polycystic kidney disease (ADPKD) is a common hereditary disease characterised by the progressive expansion of multiple cystic lesions, which compromise the function of normal renal parenchyma¹. A number of observations, in models of inherited polycystic kidney disease in rodents, have implicated oxidative stress in the pathogenesis of ADPKD. Reduced expression of antioxidant enzymes and increased expression of heme oxygenase-1, a marker of oxidative stress, have been reported in cystic kidneys from cpk/cpk mice and Han:SPRD rats²⁻⁴. The modifier gene-2 for polycystic kidney disease in Jck mice has been recently identified as antioxidant protein-2 (Apo2), a thiol-specific antioxidant protein⁵. Targeted disruption of the Bcl-2 proto-oncogene, which has been shown to protect cells from apoptosis, caused by oxidative damage, results in renal cystic disease⁶. Torres et al have shown higher renal concentration of α -tocopherol in the cystic kidneys, consistent with a disturbance of redox metabolism associated with polycystic kidney disease in Han:SPRD Rats⁷. However,

no evidence has yet been reported in humans.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and several other glycolytic enzymes have been shown to bind to the acidic N terminus of the cytoplasmic domain of the erythrocyte membrane protein band 3 anion exchanger in vitro, and in contrast to earlier views⁸, recent evidence suggests that the interaction is also prominent in vivo⁹⁻¹¹. The intracellular membrane binding of glycolytic enzymes including GAPDH is important for the control of glycolysis. The bound enzyme is reported to be inactive and may form a functional reserve that becomes active when released from its binding site¹². Harrison et al reported that oxidizing agents, such as H₂O₂, promote the tyrosine phosphorylation of the GAPDH-binding site on band 3 and thereby stimulate both release of GAPDH and erythrocyte glycolysis¹³. Mallozzi et al suggested that the increase in glycolytic flux could provide reduced nucleotide (NADH) and energy for cells to cope with oxidative stress¹⁴.

In ADPKD patients, using the thiol-alkylating agent

N-ethylmaleimide (NEM), has been determined an abnormality in Na/Li CT (sodium-lithium counter-transport) kinetics and membrane lipid fluidity suggesting abnormal membrane lipid-cytoskeleton interactions^{15,16}. Watkins et al suggested that the NEM could modulate GAPDH binding to the red cell membrane altering, possibly, the association site on the cell membrane¹⁷.

This study was undertaken in order to determine whether t-butyl hydroperoxide, a well known agent inducing oxidative stress, could release GAPDH from erythrocyte membrane and therefore increase glycolytic flux in erythrocytes from ADPKD patients and healthy control subjects. Additional research was also carried out to investigate whether the modulation of GAPDH-binding to the red cell membrane of ADPKD patients by NEM could lead to changes in glycolytic flux.

Materials and methods

Patients and Healthy Control Subjects: Thirteen Caucasian patients with ADPKD, aged between 20 and 50 years were studied. The diagnosis of ADPKD was based on both the demonstration of renal cysts by abdominal ultrasound and having a family history of the disease. All had serum creatinine less than 2 mg/dl. Four patients had hypertension. Twelve healthy subjects from the laboratory staff were studied for comparison. The clinical data of healthy subjects and ADPKD patients are given in Table 1. All subjects gave informed consent to participate in the study.

Preparation of intact erythrocytes: Venous whole blood (10 ml) was collected in lithium heparin tubes, centrifuged, and the plasma and buffy coat were removed. The erythrocytes were washed twice with cooled phosphate buffered saline (PBS: 290 ± 2 mosmol/Kg, pH 7.4), and separated by centrifugation (3000 g, 5 min, 10° C).

Glycolysis assay of intact erythrocytes: Erythrocytes (0.5-ml packed cells) were incubated at 0° C for 10 sec and 300 sec and then equilibrated to room temperature for 20 min. Afterwards a (10%) red blood cell suspension in isotonic PBS (pH 7.4) was prepared. Two hundred fifty μ l of RBC suspension was mixed with 150 μ l PBS and 100 μ l arsenate (7.5 mmol/l in PBS) to prepare a 5% packed cell volume (PCV). D-glucose (500 μ l, 10 mM in PBS) was added (final volume 1ml) and the mixture was incubated at 37° C for 30 min, 60 min and 90 min. Arsenate, which uncouples glycolysis from substrate-level phosphorylation, was added to minimize the dependence of glycolysis rate on rate of ATP utilization in the erythrocytes. After the appropriate incubation period the suspension was rapidly centrifuged (10,000 g for 10 seconds), the supernatant removed and its content of L-lactate assayed.

L-lactate determination: Lactate, present in the spun supernatant after 30-90 min glycolysis, was determined using lactate dehydrogenase by measuring the coupled reduction of NAD^+ by absorbance at 340 nm in a CO-BAS® centrifugal analyser¹⁸. The lactate concentration was expressed in $\mu\text{mol/ml RBC/hr}$.

Tert-butyl hydroperoxide treatment of erythrocytes:

In order to cause a mild oxidative stress in erythrocytes, the previously described glycolysis assay was performed in the presence or absence of 0.06 mM t-butyl hydroperoxide. To determine the maximum lactate production with minimum concentration of t-butyl hydroperoxide various concentrations were used (0-5 mM) (Figure 1).

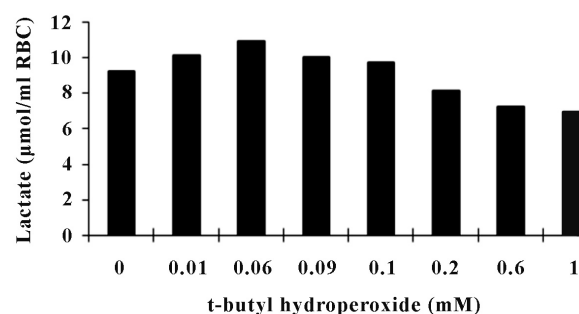


Figure 1: The effect of different concentrations of t-butyl hydroperoxide on rate of glycolytic lactate production by intact erythrocytes from healthy subjects.

N-ethylmaleimide (NEM) treatment of erythrocytes: Erythrocytes (0.5 ml packed cells) were suspended in 3 ml of choline medium (139 mmol/l choline chloride, 1 mmol/l MgCl_2 , 10 mmol/l glucose, 10 mmol/l Tris MOPS, pH 6.0, 290 ± 2 mosmol/Kg). NEM (3 μmol in 100 μl of choline medium) was added and the suspension incubated at 0° C for the indicated times. The reaction was stopped by the addition of a 5-fold excess of mercaptoethanol in choline medium and immediate centrifugation (3000 rpm, 3 min, 10° C). Afterwards the erythrocytes were washed 3 times with PBS, pH 7.4, and equilibrated to room temperature for 20 min. A range of incubation times (0-1000 seconds), different pH values (6 and 7.4), various concentrations of NEM (0-5 mM) and temperatures (0° and 21° C) as well as choline or PBS buffer were used.

The previously described glycolysis assay was performed in the presence or absence of NEM treated erythrocytes from normal subjects and ADPKD patients

Glycolysis assay in saponin-permeabilized erythrocytes: The glycolysis rate of permeabilized cells was assayed as for intact cells except there was added phosphate buffer, saponin, NAD^+ , ATP, and MgCl_2 . The final concentrations in the 1 ml assay volume were: cells, 2.5% (v/v), sodium phosphate (pH 7.4), 10 mM; KCl, 139 mM; sodium arsenate, 0.75 mM; NAD^+ , 0.1 mM; ATP, 0.5 mM; MgCl_2 , 1 mM; D-glucose, 5 mM. Saponin was added to the glycolysis assay in increasing amounts to find the concentration that gave maximum rate of lactate production. With a 5 min pre-incubation and 2.5% packed cell volume, maximum lactate rate was obtained with 4 μg saponin ml^{-1} . The same procedure was also carried out on the NEM treated erythrocytes.

Preparation of erythrocyte ghost membranes: Packed erythrocytes (50 μl) from untreated, NEM treated and t-butyl hydroperoxide treated cells were washed twice with

1.5 ml of phosphate buffered saline and cooled to 2°C in ice. The erythrocytes were then lysed at 2°C in 1.5 ml hypotonic PBS [20 mmol/l PBS (40 mosm/kg) containing 4 mmol/l MgSO₄, PMSF (230 µmol/l), EDTA (1 mmol/l), pepstatin (1 µmol/l), leupeptin (1 µmol/l) and benzamidine (2.5 mmol/l)]. The ghost membranes were collected by centrifugation at 10,000g for 10 minutes at 2°C and washed a further twice with the lysing buffer. The pellet of erythrocyte ghost membranes was resuspended in 50 µl of hypotonic PBS and stored at -80°C.

SDS-PAGE and GAPDH determination: Ghost membrane proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using the Laemmli discontinuous system under reducing conditions. Gels (1mm thick) of 10.5% total acrylamide were used in a Bio-Rad mini-protean system and run at 10-15 mA per gel. Gels were stained in Coomassie brilliant blue (200 mg Coomassie brilliant blue dissolved in 10:25:65 acetic acid: industrial methylated spirit water) for 2 hours. The gel was then destained in 5:5:90 acetic acid: industrial methylated spirit: water.

Analysis of Coomassie blue stained gels: Scan analysis was performed on dried, Coomassie stained, gels using a BioImage Visage Electrophoresis Gel Analysis System, version 4.6Q (Copyright 1993 Millipore Corporation). The GAPDH/actin ratio was determined from the integrated optical density using Whole Band Analysis, version 2.4. This ratio probably depended on exact conditions of preparation and washing of ghosts; it was constant between tracks in any one experiment, but somewhat variable between experiments.

Statistical analysis: All values are reported as means ±SD. The significance of differences between groups was assessed using an unpaired t test. The significance of differences within groups was assessed using a paired t test.

Results

The clinical data of patients with ADPKD and normal control subjects are given in Table 1. Four patients were hypertensive otherwise there were no statistically significant differences between the two groups.

Treatment of erythrocytes from normal controls with

Table 2: Effect of t-butyl hydroperoxide on lactate production rate and membrane GAPDH/actin ratio in control and ADPKD erythrocytes

	Healthy controls (n=12)				ADPKD patients (n=13)			
	Lactate rate		GAPDH/actin		Lactate rate		GAPDH/actin	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Untreated	8.62	1.01	0.86	0.49	9.36	1.85	0.47 [‡]	0.17
Peroxide-treated	9.32	0.96	0.59	0.30	9.37	1.71	0.46	0.09
	p<10 ⁻⁵		p<0.016		N.S.		N.S.	

[‡] p=0.028 compared with healthy control.

Lactate production rate is given as µmol h⁻¹ ml⁻¹ (packed erythrocytes), membrane-bound GAPDH is given as ratio with membrane bound actin. The significance (paired t-test; N.S. = not significant) of the comparison of treated and untreated cells from the same individual is shown below each column.

high doses of t-butyl hydroperoxide inhibited glycolysis. However, low doses significantly increased the rate of lactate production compared with native erythrocytes (Figure 1). Corresponding with the increase in glycolytic rate, there was a significant decrease in the GAPDH/actin ratio, there was a significant decrease in the GAPDH/actin ratio (Table 2). By contrast, in ADPKD patients after the same treatment, there was no significant change in either the rate of lactate production or the GAPDH/actin ratio (Table 2). However, the rate of lactate production was already higher and the GAPDH/actin ratio was significantly lower in erythrocytes from ADPKD patients compared with normal controls (Table 2).

The effects of NEM were opposite to that of mild oxidative stress, but here also the same negative correlation was observed between lactate rate and ratio of membrane associated GAPDH/actin. In control erythrocytes 1 mM NEM caused a significant decrease in lactate production in 10 sec and 300 sec (Figure 2), and a significant in-

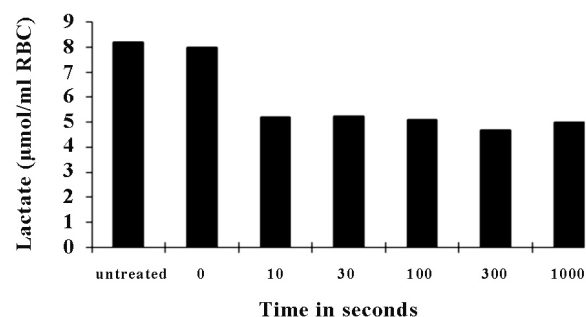


Figure 2: Time-course of the effect of 1 mM N-ethylmaleimide (NEM), at pH 6 and 0°C, on rate of glycolytic lactate production in intact healthy erythrocytes.

Table 1: Clinical data of healthy control subjects and ADPKD patients

	Healthy subjects	ADPKD
Sex (male/female)	6/6	7/6
Age (y)	40 ± 4	42 ± 3
Hypertension	0	4
Serum creatinine (mg/dl)	1.0 ± 0.1	1.2 ± 0.2
Fasting blood sugar (mg/dl)	82 ± 2	83 ± 2

Note. Values are means ± SD.

Abbreviation: ADPKD, autosomal dominant polycystic kidney disease.

crease in the GAPDH/actin ratio (Table 3). In erythrocytes from ADPKD patients the effects were similar to the controls except that the GAPDH/actin ratio was significantly more sensitive to NEM, it responded significantly more rapidly and to a significantly greater extent (Table 3).

Figure 3 shows the effect of different concentrations of NEM on lactate production rate in intact healthy erythrocytes. An

Table 3: The effect of N-ethylmaleimide (NEM) on lactate production rate and membrane GAPDH/actin ratio in control and ADPKD erythrocytes

	Healthy controls (n=12)				ADPKD patients (n=13)			
	Lactate rate		GAPDH/actin		Lactate rate		GAPDH/actin	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Untreated	8.62	1.01	0.41	0.264	9.36	1.85	0.33	0.112
NEM 10 s	4.87	0.71	0.44	0.271	5.24	1.52	0.48	0.211
	p<10 ⁻⁸		p: 0.12		p<10 ⁻¹¹		p: 0.04	
NEM 300 s	4.51	0.82	0.673	0.375	5.32	1.53	0.63	0.142
	p<10 ⁻⁹		p<10 ⁻³		p<10 ⁻¹⁰		P<10 ⁻⁴	

Lactate production rate is given as $\mu\text{mol h}^{-1} \text{ml}^{-1}$ (packed erythrocytes), membrane-bound GAPDH is given as ratio with membrane bound actin. The significance (paired t-test) of the comparison of treated and untreated cells from the same individual is shown below each comparison

increase from 0.75 to 5 mM NEM concentration did not bring any lower the rate of glycolysis.

The rate of lactate production increased significantly after the treatment of red cells with saponin (Table 4).

Table 4: Rate of lactate production in saponin-permeabilized healthy erythrocytes after NEM treatment

	Lactate production rate		
	$\mu\text{mol/h/ml RBC}$		
	No NEM	NEM (10s)	% Change
No saponin (n: 5)	8.35±0.97	5.15±0.46	39.40±3.60
Saponin-treated (n: 5)	10.35±0.65	5.30±0.26	49.82±4.10
	p<0.001	p<0.008	p<0.001

Note: Values are mean \pm SD. The significance (paired t-test) of the comparison of treated and untreated cells from the same individual is shown below each column. Abbreviations: NEM, N-ethylmaleimide.

Discussion

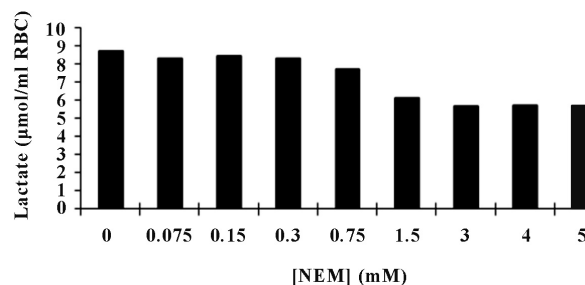
In all the experiments reported here the same inverse correlation was observed between glycolytic lactate production and membrane-bound GAPDH/actin ratio. Tert-butyl hydroperoxide lowered the GAPDH/actin ratio in erythrocyte ghosts from healthy controls but stimulated glycolysis, while in erythrocytes from ADPKD patients neither effect occurred. N-ethylmaleimide, on the other hand, raised the GAPDH/actin ratio but inhibited glycolysis. However, there was no quantitative relationship between the two effects, and in the erythrocytes from ADPKD patients the effect of NEM on glycolysis preceded that on the GAPDH/actin ratio. These results are therefore qualitatively consistent with the experiments cited above¹²⁻¹⁴ one has only to postulate that NEM can inhibit glycolysis by a second mechanism in addition to the increased binding of GAPDH to the membrane. NEM at low pH and temperature is a moderately specific thiol-alkylating reagent, and it is known that glycolysis is inhibited by thiol alkylation both at hexokinase and at GAPDH¹⁹. However, neither a 3 fold increase in exposure time (Figure 2) nor a 5 fold increase in NEM concentration (Figure 3) brought the rate of glycolysis any lower than 50%, and whatever the target of NEM attack it

would seem therefore not to be a catalytic group on a glycolytic enzyme.

It is generally agreed that glucose entry is considerably faster than its subsequent metabolism in the red blood cell, especially in man where entry is quoted as 250 times as fast as glycolysis²⁰. However, if glycolysis is speeded by arsenate uncoupling, and glucose entry inhibited by NEM, it seemed possible that the rate of glycolysis in whole erythrocytes could be limited to some extent by glucose entry and that the effect of NEM on the rate of lactate production could have been on glucose transport rather than glycolysis itself. The forgoing experiments were therefore repeated on (healthy control) cells treated with saponin where it was expected that glucose entry would exercise very little or negligible rate-control²¹. The results (Table 4) show that saponin treatment does indeed increase the rate of lactate production, although it is not clear whether that is the effect of maintaining nucleotide concentrations or the removing a rate-limiting glucose entry. However, the inhibitory effect of NEM on rate of lactate production is greater (relative to cells not treated with NEM), which rules out transport as a significant site of NEM action in these experiments.

At high concentration, tert-butyl hydroperoxide decreases erythrocyte protein thiol content and inactivates GAPDH²². However, at low concentration as used here, it had an insignificant effect on overall thiol content and caused an increase in enzymatic activity of GAPDH in human lung carcinoma cells²³. It has been suggested that t-butyl hydroperoxide has its effect by rather specific thiol oxidation (and thus inhibition) of the thiol-dependent enzyme tyrosine phosphatase. This would increase phosphorylation of band 3 (as well as other proteins) and reduce GAPDH binding at this major site^{24,25}.

In their response to the mild oxidative stress represented by a low concentration (0.06 mM) of t-butyl

**Figure 3:** The effect of different concentrations of N-ethylmaleimide on lactate production rate in intact healthy erythrocytes.

hydroperoxide two related differences were observed between erythrocytes from healthy control subjects and those from ADPKD patients. While healthy erythrocytes responded to the peroxide with increased glycolysis and decreased GAPDH binding, those from cystic patients did not. However, untreated erythrocytes from ADPKD patients showed the same high rate of glycolysis and low membrane binding of GAPDH as was observed in erythrocytes from healthy subjects after mild oxidative stress. Perhaps the simplest explanation is that erythrocytes from ADPKD are already subjected *in vivo* to a mild oxidative stress. Alternatively, it could be postulated that the control of membrane binding of GAPDH (and possibly other enzymes) is abnormal in ADPKD even in the absence of oxidative stress. In this connection it will be remembered that the effect of NEM on GAPDH/actin ratio is significantly faster in ADPKD than in healthy controls.

The relationship between these results and the pathophysiology of ADPKD is not known. However, there are a number of results in the literature that point to a possible relevance^{26,27}. It has been observed that, in patients with ADPKD, hypertension is prevalent even in early stages of the disease²⁸. The reason for this hypertension has not been fully explained^{29,30}. Studies on erythrocytes from patients with essential hypertension have shown an increased rate of phosphorylation of band 3 protein by membrane-bound protein kinases³¹. Moreover tyrosine phosphorylation of human platelet plasma membrane Ca(2+)-ATPase is essential in pathophysiology of hypertension^{32,33}. One interesting possibility arising out of our work is that the mild oxidative stress that we have inferred in ADPKD patients may inhibit protein phosphatases. This could release GAPDH from red cell membrane and increase glycolytic rate causing hypertension^{34,35}.

It is of interest that reagents such as hydrogen peroxide have long been known to mimic insulin action³⁶. Since the insulin receptor is a protein kinase, many of insulin effects are thought to be mediated through tyrosine phosphorylation³⁷. Reports have shown that hydrogen peroxide increases the phosphotyrosine content of several putative cellular substrates of the insulin receptor kinase possibly by inhibiting a protein tyrosine phosphatase³⁸. In this study we show an increase in glycolytic flux after inducing mild oxidative stress in erythrocytes from healthy subjects but not from ADPKD patients. This could explain in part the insulin resistance that has been reported very early in the course of the cystic disease in these patients^{39,40}. Chronically high levels of protein phosphorylation could contribute to an insulin resistance characteristic of both ADPKD and hypertension.

The controlled induction of oxidative stress and the maintenance of the cellular redox state are essential for the regulation of cell proliferation and for many metabolic processes important in the pathogenesis of Polycystic Kidney Disease (PKD)^{41,42}. Although our experiments refer to erythrocytes, if similar defects exist in the renal tubule cells of individuals with ADPKD they could provide a possible mechanism for downstream pathogenic events

in the expansion of renal cysts^{43,44}. Recent studies show that the change of tubular cell to a hyperproliferative state in PKD involves oxidative stress and kinase phosphorylation cascades^{45,46}.

In conclusion, the GAPDH binding and glycolytic flux responds abnormally in erythrocytes from ADPKD patients to both a mild oxidative stress and brief exposure to NEM. This abnormality may suggest either a chronic pre-existing mild oxidative stress or an abnormal membrane organization and function in this disease. More studies are necessary to clarify whether our findings have any causative role in major phenotypes of ADPKD such as cyst formation and expansion, hypertension and insulin resistance

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