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Increased glycogen stores due to γ-AMPK overexpression protects against ischemia and reperfusion damage

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ARTICLE INFO

Article history:
Received 19 August 2007
Accepted 26 December 2007

Keywords:
Ischemia reperfusion
Low-flow ischemia
Glycogen
AMP-activated protein kinase
Myocardium
Lactate

ABSTRACT

During ischemia, endogenous glycogen becomes the principal substrate for energy through glycolysis. Cardiac-specific manipulation of AMP-activated protein kinase (AMPK) by overexpression of its regulatory γ-subunit induces glycogen storage. The aim of this study was to examine whether heart glycogen in transgenic mice overexpressing *PRKAG2* may protect from ischemia and reperfusion injury. Isolated hearts were mounted on Langendorff apparatus and subjected to 30 min 'no-flow' or 'low-flow' ischemia and 60 min reperfusion. Hemodynamic measurements, tetrazolium staining, glycogen and lactate were used to monitor ischemia reperfusion damage. After low-flow ischemia, left ventricular pressure, coronary flow (CF) and the area of viable myocardium were 20–30% higher in *PRKAG2* mice compared to controls. The basal levels of glycogen in *PRKAG2* were 9.2 μg/g, markedly higher than in controls, but after low-flow ischemia they declined concomitantly with increased lactate washout in the coronary effluent. During no-flow ischemia there was neither protection nor consumption of glycogen in *PRKAG2* hearts. Cardioprotection was also eliminated when *PRKAG2* hearts were depleted of glycogen prior to low-flow ischemia. AMPK α Thr^{172} phosphorylation did not differ between *PRKAG2* hearts and controls either during low-flow ischemia or reperfusion. We conclude that *PRKAG2* hearts resist low-flow ischemia injury better than controls. Improved recovery was associated with increased consumption of glycogen, and was unrelated to AMPK activation. These findings demonstrate the potential of heart protection from ischemia and reperfusion injury through metabolic manipulation increasing the level and utilization of myocardial glycogen.

1. Introduction

AMP-activated protein kinase (AMPK) is a metabolic regulator that is responsible for adjusting the energy supply to match demands [1,2]. It has an essential role in activating glucose transport during hypoxia and ischemia. AMPK is a heterotrimer complex found in all eukaryotic cells comprising an α catalytic subunit and β and γ regulatory subunits. The catalytic AMPK α-subunit contains two functional regions: an N-terminal kinase domain and a C-terminal tail

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doi:10.1016/j.bcp.2007.12.011

that is responsible for autoinhibition and targeting other subunits [3,4]. Phosphorylation of the α-subunit at a threonine residue (Thr-172) by upstream kinases (AMPK Kinase, AMPKK) is ultimate for kinase activity [5]. The scaffold-like β-subunit contains a glycogen binding domain, and is N-terminally myristoylated and phosphorylated at multiple sites, but the functional significance of these is not yet known [6–8]. The γ-subunit of AMPK is composed of four sequence repeats called cystathionine β synthase (CBS) motifs, which associate in pairs to create two nucleotide binding modules [9,10]. These regulatory sites are capable of binding either AMP or ATP. AMPK is allosterically activated by AMP and inhibited by ATP. In other words, rising AMP coupled with falling ATP is a key signal that activates AMPK *in vivo*. Energy deficient states due to nutrient deprivation, exercise and anoxia activate AMPK by 2 mechanisms: (1) evoking a signaling cascade resulting in increased upstream kinase activity; (2) increasing the AMP/ATP ratio [5]. Following an ischemic episode, the heart becomes less efficient at converting energy into contractile function. Fatty acid oxidation dominates over glucose oxidation as the main source of mitochondrial metabolism. Glucose uptake and glycolysis accelerates, increasing proton production due to uncoupling of glycolysis from glucose oxidation [2]. Altering energy metabolism by either decreasing fatty acid oxidation or increasing glucose oxidation can improve cardiac recovery following ischemia [2,11].

Disruption of AMPK catalytic activity results in reduced glucose uptake and impaired tolerance to anoxia. Naturally occurring mutations in the γ-subunits result in glycogen storage in the heart (γ2) and skeletal muscles (γ3). These mutations influence the structure and activity of the γ-subunits, impairing their sensitivity to the nucleotides AMP and ATP [12]. As a result, there is a loss of inhibition by ATP and increased AMPK activity under resting conditions. Excess glucose entering the muscle cell is not paralleled by increased glycolytic flux and glycogen accumulation ensues. Human mutations in *PRKAG2* (encoding the regulatory γ2-subunit), causes unique cardiomyopathy characterized by myocardial hypertrophy, ventricular pre-excitation and progressive conduction system disease [13]. Glycogen storage and other features of this disease were completely recapitulated in transgenic mice overexpressing the human mutation in their hearts [14]. In contrast, transgenic mice overexpressing the wild-type *PRKAG2* gene have a milder increase in glycogen, which causes mild hypertrophy without cardiomyopathy. Because no AMPK activation could be demonstrated in total tissue extracts from 'wild-type *PRKAG2*' transgenic mice [14,15] we attributed this phenotype to differences in nucleotide sensitivity of specialized γ2 vs. housekeeping γ1-subunits leading to altered metabolic regulation. Remarkably, these hearts use their glycogen stores during stress [15,16]. Previous studies indicate that metabolic manipulations increasing tissue glycogen may result in improved ischemic tolerance [17]. We therefore hypothesized that altered metabolic regulation in the hearts of 'wild-type' *PRKAG2* transgenic mice, resulting in higher glycogen stores, may provide an advantage under conditions of metabolic stress. The aim of this study was to examine whether an increase in intracellular glycogen, triggered by the *PRKAG2* transgene, protects the heart from ischemia and reperfusion injury.

2. Materials and methods

2.1. Transgenic mice

The generation and phenotypic characterization of *PRKAG2* transgenic mice have previously been reported [14]. In brief, full-length human *PRKAG2* cDNA was subcloned into a pC126 expression vector between rabbit α-myosin heavy chain (αMHC) promoter and human growth hormone 3′-UTR. αMHC promoter becomes active in mouse ventricles postnatally, driving a robust, cardiac-specific expression of *PRKAG2* encoding the regulatory γ-subunit of AMPK. Heterozygous FVB transgenic males were used to establish a new colony at the Rabin Medical Center. All studies were approved and performed in accordance with the guidelines established by the Institutional Animal Care and Use Committee at the Tel Aviv University, Israel. Because mutant $PRKAG2^{N488I}$ mice suffer from severe glycogen storage cardiomyopathy, in current experiments we used $PRKAG2^{WT}$ mice (expressing the 'wild-type' transgene), which have elevated glycogen with preserved cardiac function.

Genotyping was done by extracting tail DNA and performing multiplex PCR reaction using transgene-specific (F 5′-GCCTGCTTT-CATGAAGCAGAA-3′ and R 5′-GCAGCCAGTGTT-CATGAGGCAAAAC-3′) and murine genomic DNA (F 5′-GAGAACTCGGCATGCC-AGATTC-3′ and R 5′-ACTCAGCAAG-CCTTC-CCATCTG-3′) primers. Transgenic mice gain two PCR products, a transgene-specific band of ~1000 base-pairs (bp) and another band of ~500 bp representing genomic DNA. In control mice, only genomic DNA band of ~500 bp was found.

2.2. Isolated heart perfusion

All experiments were done on 12-week-old heterozygous $PRKAG2^{WT}$ transgenic mice and their wild-type (control) littermates in FVB background. Mice were heparinized (500 U/kg), anesthetized (i.p.) with ketamine (100 mg/kg) and xylazine (10 mg/kg). The hearts were quickly removed, the aorta cannulated and the heart perfused in retrograde, according to Langendorff, at 96 cmH_2O pressure with oxygenated Krebs-Henseleit bicarbonate buffer solution (KHB) containing (mM): 118 NaCl, 2.4 KCl, 1.2 $MgSO_4$, 7× H_2O, 2.5 $CaCl_2$, 5 EDTA, 1.2 KH_2PO_4, 25 $NaHCO_3$, 4 glucose, 2 pyruvate at 37 °C [18]. The isolated heart was stabilized for 20 min at constant perfusion pressure and then subjected to 30 min of ischemia followed by 60 min reperfusion. No-flow ischemia was created by clamping the aortic cannula. Low-flow ischemia was created by providing 10% of baseline coronary flow (CF) through a peristaltic pump (P-1 Pharmacia, Fine Chemicals, Sweden). In an additional set of experiments, the heart was exposed to 30 min of KHB containing no glucose or pyruvate, to deplete the endogenous glycogen stores prior to ischemia. At all stages of the protocol, the left ventricular developed pressure (LVP), the rate of pressure development (±dP/dt) and the heart rate (HR), were continuously recorded by the CODAS data acquisition system (San Diego, CA, USA).

Table 1 – Cardiac function and coronary flow in Langendorff-perfused isovolumically contracting mouse hearts

	Control no flow ($n = 15$)	PRKAG2 no flow ($n = 13$)	Control low flow ($n = 8$)	PRKAG2 low flow ($n = 8$)	ANOVA
Weight (g)	26.4 ± 0.8	25.2 ± 0.7	26.3 ± 1.1	23.9 ± 1.0	0.217
Heart weight (g)	0.15 ± 0.01	0.2 ± 0.01	0.15 ± 0.01	0.22 ± 0.01	0.001
CF (ml/min)	3.4 ± 0.4	4.0 ± 0.3	2.6 ± 0.2	3.3 ± 0.5	0.022
CF nor. (ml/min g)	23.2 ± 2.9	21.1 ± 2.9	18.6 ± 1.7	15.9 ± 3.1	0.363
HR (bpm)	322 ± 15.6	263 ± 12.7	359 ± 26.1	174 ± 15.1	0.001
LVP (mmHg)	76.5 ± 3.12	90.7 ± 6.02	70.6 ± 6.93	119 ± 6.1	0.001
RPP (bpm × mmHg × 1000)	27.3 ± 1.7	22.7 ± 1.1	24.3 ± 1.4	20.9 ± 2.4	0.569
+dP/dt (mmHg × 1000/s)	2.6 ± 0.2	3.2 ± 0.2	2.8 ± 0.2	3.1 ± 0.2	0.186
−dP/dt (mmHg × 1000/s)	1.9 ± 0.1	2.7 ± 0.2	1.9 ± 0.2	2.4 ± 0.2	0.014

Baseline measurements are presented in absolute values as obtained after 20 min stabilization except for heart weight which was measured at the end of the experiment. Four groups were compared: hearts of wild-type control and the *PRKAG2*^{WT} group were subjected to 30 min no-flow or low-flow ischemia and 60 min of reperfusion. CF: Coronary flow, CF nor.: coronary flow normalized to heart weight, HR: heart rate, bpm: beats per minute, LVP: left ventricular developed pressure, RPP: rate pressure product (HR × LVP), ±dP/dt: positive and negative derivative of pressure development. ANOVA: Analysis of variance with repeated measurements using the multiple comparison option of Duncan, $p < 0.05$ was considered significant.

Rate pressure product (RPP), an index of myocardial workload, was calculated by multiplying LVP by HR.

2.3. Biochemical assays

Coronary flow was collected at various time points before, during and after ischemia (1 min aliquots). Glycogen content and lactate in tissue and effluent were determined as previously described [16]. At the end of reperfusion, the heart was weighed, frozen in liquid nitrogen, and kept at −70 °C for further analysis.

2.4. Histological examination

Sections were stained by Harris' Hematoxilin Eosine using light microscopy. Infarct size was measured with Triphenyltetrazolium chloride (TTC) staining. TTC stains the viable tissue in red while the necrotic tissues appear white. A digital image was obtained with a Nikon Coolpix 5000 digital camera and quantified in pixels using the computer software and IMAGE J 5.1. The measured infarct areas were compared to the entire area at risk [19].

2.5. AMPK activity

AMPK activity was estimated from α AMPK Thr^{172} phosphorylation. Protein extraction and Western blot were performed using 30 μg of protein lysate per lane. Primary polyclonal antibodies: rabbit anti-pan α AMPK, and rabbit anti-phospho α AMPK Thr^{172} were as previously reported [15,20]. HRP-conjugated goat anti-rabbit secondary antibody (Cell Signaling, Danvers, MA) and EZ-ACL solution (Biological Industries, Beit Haemek, Israel) were used for chemiluminescent detection.

2.6. Data analysis and statistics

Results are expressed as means ± standard error of the mean (S.E.). Contractile measurements obtained in the course of the experiment were expressed as percent of baseline, defined as the end of stabilization period. A statistical difference between the groups was assessed by analysis of variance (ANOVA) with repeated measurements using the multiple comparison option of Duncan. $P < 0.05$ was considered significant. Post hoc comparisons were performed using Student's *t*-test if differences were established.

3. Results

3.1. Baseline data

Table 1 presents the basal data on mice and cardiac function prior to no-flow ischemia or low-flow ischemia in PRKAG2^{WT} and controls. Heart weight (HW) and CF were higher in *PRKAG2*^{WT} mice compared to controls but there were no differences in CF normalized for the HW. *PRKAG2*^{WT} mice have higher developed LVP but lower HR than the control group. The RPP representing the heart work did not differ between the groups. *PRKAG2*^{WT} mice also demonstrated faster left ventricular relaxation, represented by −dP/dt (Table 1).

3.2. No-flow ischemia

CF recovery after 30 min of global 'no-flow' ischemia was better in *PRKAG2*^{WT} compared to control mice (Fig. 1A). While CF deteriorated in the control group, it remained stable in the *PRKAG2*^{WT} hearts ($p < 0.05$). There were no significant differences in the recovery of HR, LVP, dP/dt, and RPP. In fact, recovery after ischemia appeared to be somewhat slower in *PRKAG2*^{WT} hearts but reached the same values at the end of the reperfusion period (Fig. 1).

Low-flow ischemia produced by reducing the CF to 10% of its basic value for 30 min, was followed by severe depression of heart rate and pressure development. There were no significant differences between the groups in the CF provided to the heart or in the contractile performance during the ischemic period (Fig. 2). The recovery of CF, LVP, dP/dt and RPP was significantly higher in *PRKAG2*^{WT} mice compared with control mice ($p < 0.001$). LVP and RPP product recovered to approximately 70% of baseline in PRKAG2^{WT} vs.

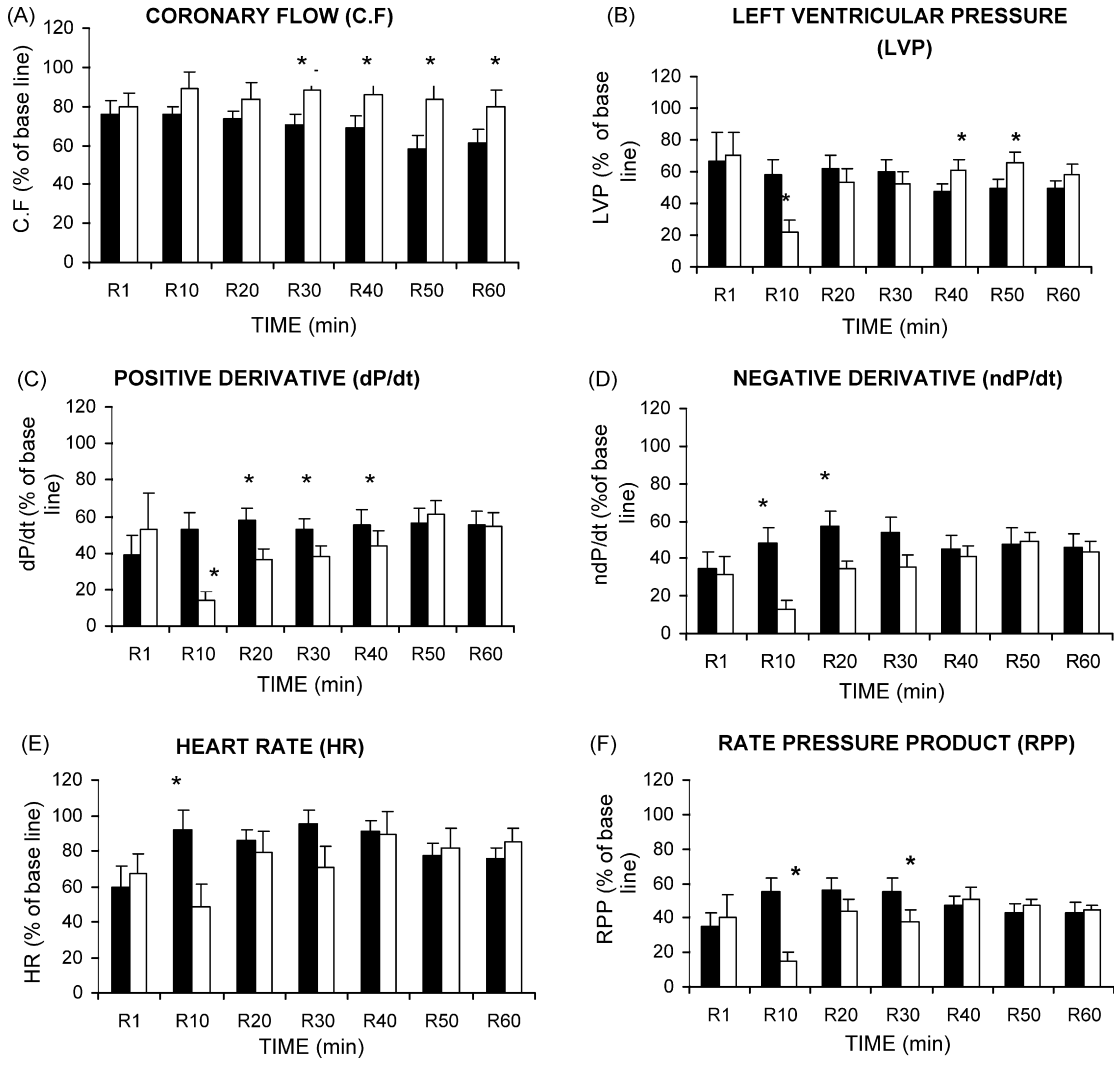

Fig. 1 – No-flow ischemia experiments. The hemodynamic measurements are presented as a percentage of baseline, mean ± S.E., n = 13–15/group. See Table 1 for baseline values group. The wild-type control is represented by black bars and the *PRKAG2*^{WT} group is represented by open bars. Langendorff-perfused hearts were subjected to a 20 min stabilization period, then to 30 min no-flow ischemia and 60 min of reperfusion. Coronary flow CF (A), left ventricular pressure LVP (B), dP/dt (C), −dP/dt (D), heart rate HR (E) and rate-pressure product RPP (F). R: Reperfusion (min). $^{*}p < 0.05$ (ANOVA).

~50% in control hearts. There were no differences between groups in the heart rate recovery which returned to baseline values.

3.3. *Glycogen breakdown and lactate production*

To test whether utilization of glycogen stores is associated with reduced ischemic injury, we examined glycogen and lactate levels in the heart tissue and the lactate levels in the coronary effluent (Fig. 3). Baseline glycogen levels in control mice were markedly lower than in *PRKAG2*^{WT} mice ($p < 0.05$). Following 30 min of 'no-flow' ischemia, only a small reduction of glycogen levels was observed (Fig. 3A). In contrast, in the 'low-flow' ischemia experiments, glycogen levels decreased in both groups but to a different extent: in the *PRKAG2*^{WT} hearts, average glycogen levels fell from 9.2 ± 1.3 μg/mg tissue at baseline, to 2.7 ± 0.7 μg/mg tissue following ischemia. Glycogen decreased to undetectable levels in control hearts but the capacity to consume glycogen was limited due to a very low basal content (0.5 ± 0.1 μg/mg tissue on average, Fig. 3B).

Tissue lactate was somewhat higher at baseline in transgenic hearts (Fig. 3C and D). No-flow ischemia (Fig. 3C) increased the lactate levels in the myocardium to the same extent in both groups. Lactate was effectively washed out at reperfusion. No lactate accumulation was found in heart tissue at the end of 30 min low-flow ischemia (Fig. 3D). Noteworthy, lactate was undetectable even in the hearts of *PRKAG2*^{WT} mice.

Lactate in the coronary effluent was detectable only in PRKAG2^{WT} hearts and only during ischemia. Remarkably, lactate washout increased with ischemia duration presumably reflecting the ongoing glycogen consumption and effective disposal from the myocardium (Fig. 3E).

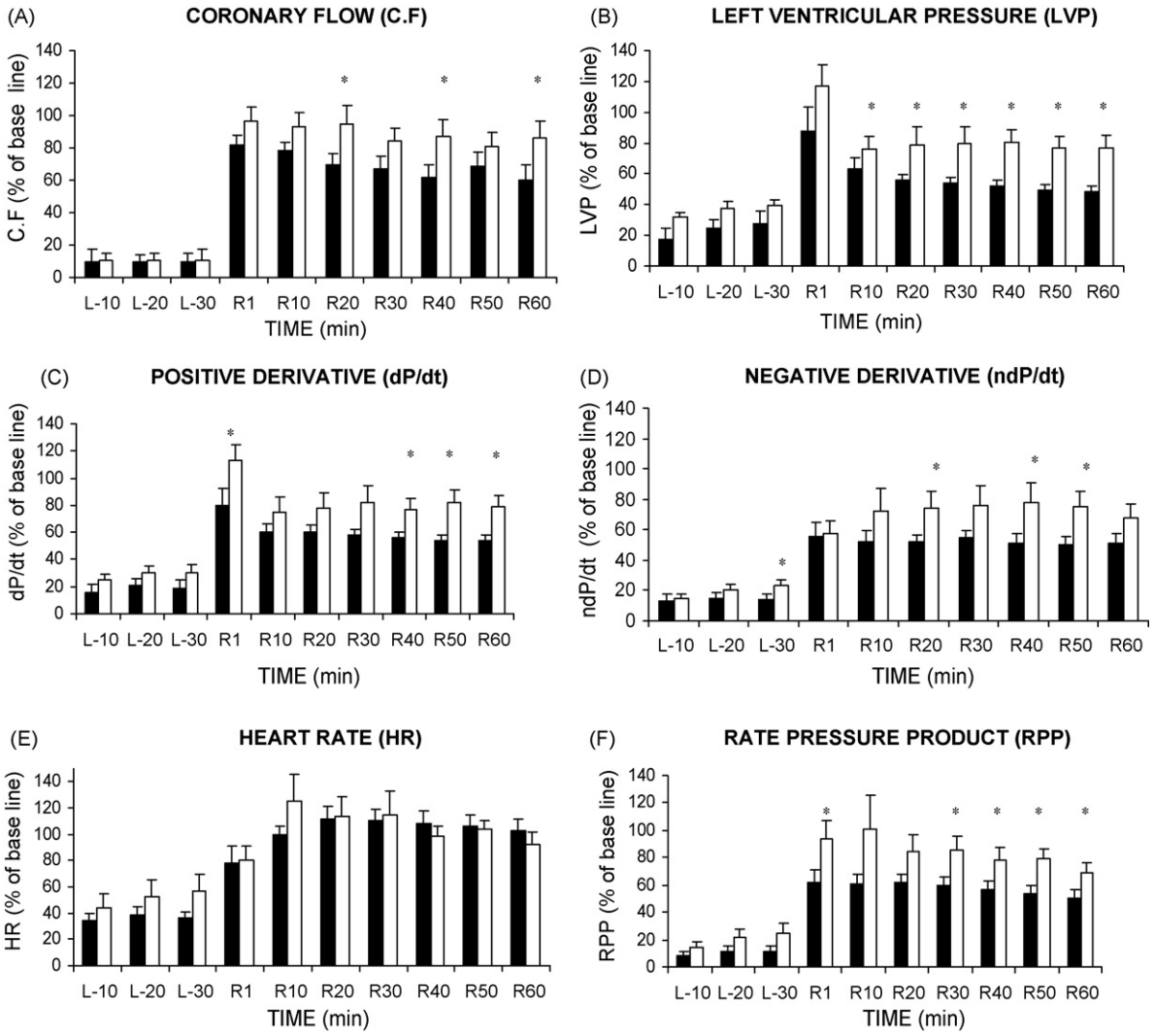

Fig. 2 – Low-flow ischemia experiments. The hemodynamic measurements are presented as a percentage of baseline ± S.E., *n* = 8/group. See Table 1 for baseline values group. The wild-type control is represented by black bars and the *PRKAG2*^{WT} group is represented by open bars. Langendorff-perfused hearts were subjected to a 20 min stabilization period, then to 30 min of low-flow (10% of base line perfusion) and 60 min of reperfusion. Coronary flow CF (A), left ventricular pressure LVP (B), dP/dt (C), −dP/dt (D), heart rate HR (E) and rate-pressure product RPP (F) were tested. L: low-flow ischemia (min), R: Reperfusion (min), LVP, ±dP/dt, CF and RPP were higher during reperfusion in the in PRKAG2^{WT} compared to control hearts. $^{*}p < 0.01$ (ANOVA).

3.4. *Myocardial necrosis*

Histological examination of the different groups using hematoxilin eosine staining revealed no differences (data not shown). TTC staining was used to assess irreversible injury (Fig. 4). PRKAG2^{WT} isolated heart subjected to 30 min low-flow ischemia and 60 min of reperfusion had a smaller region of irreversible ischemic injury compared with the other groups.

3.5. *AMPK activity*

We assessed the AMPK activity in control (WT) and *PRKAG2*^{WT} hearts after low-flow ischemia and after reperfusion, to determine whether differences in AMPK activity contribute to cardioprotection in *PRKAG2*^{WT}. Phosphorylation of the catalytic α-AMPK subunit was measured by Western blotting. As previously reported [14,15], there were no differences in AMPK activity in *PRKAG2*^{WT} hearts during isolated heart perfusion at baseline (Fig. 5). Fig. 5 displays representative blots as well as quantitative comparisons of α-subunit phosphorylation and of phosphor-α/pan-α ratio at the end of low-flow ischemic period and during reperfusion (n = 3–6/group). The levels of pan-α and pT172 α-AMPK were not significantly different between the groups.

3.6. *Glycogen depletion abolishes protection in PRKAG2*^{WT} *hearts*

To confirm the role of glycogen in protecting against low-flow ischemia, glycogen stores were depleted to 2.0 ± 0.4 μg/mg tissue by 30 min heart perfusion by oxygenated substrate-free solution. Hearts were then subjected to 30 min low-flow

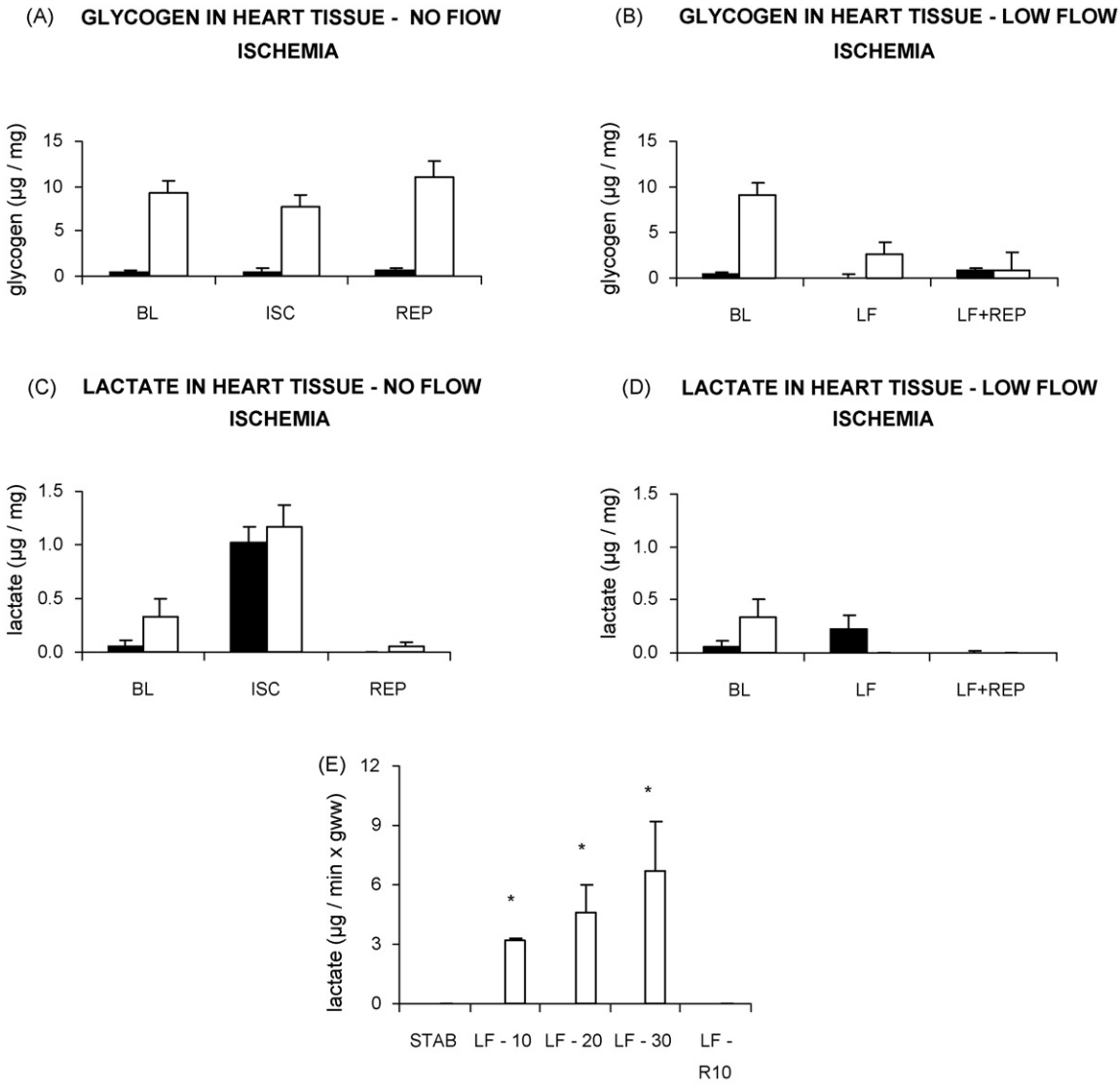

Fig. 3 – Levels of glycogen and lactate during ischemia experiments. Measurements in tissue are presented in panels A–D (mean ± S.E.). No-flow ischemia groups: Control (n = 6 hearts), black bars; *PRKAG2*^{WT} no-flow group (n = 12), white bars. Low-flow ischemia groups: Wild type, black bars; *PRKAG2*^{WT}, white bars (n = 6–7/group). Langendorff-perfused hearts were subjected to 20 min stabilization period, then to 30 min ischemia and 30 min of reperfusion. BL: End of stabilization period (baseline); ISC: after 30 min of no-flow ischemia; LF: after 30 min of low-flow ischemia; REP: after 30 min of reperfusion of no-flow ischemia; LF + REP: after 30 min of reperfusion of low-flow ischemia. Panel E shows the level of lactate in the coronary effluent during low-flow ischemia experiments (mean ± S.E., n = 3/group). The *PRKAG2*^{WT} group: open bars. No lactate levels are detected in the CF of the wild-type hearts. In the PRKAG2^{WT} group lactate was detectable during low-flow ischemia. STAB: After 20 min of stabilization; LF 10, LF 20, LF 30: samples obtained after 10, 20 and 30 min of low-flow ischemia, respectively. LF R10: Sample obtained after 10 min of reperfusion following low-flow ischemia. $^{*}p < 0.05$ (ANOVA).

ischemia and reperfusion as previously described. As shown in Fig. 6, no significant difference between *PRKAG2*^{WT} and control was found. Postischemic recovery was markedly attenuated by prior glycogen depletion in *PRKAG2*^{WT} hearts (being below 50% of baseline) but not in wild-type controls (Fig. 6).

4. Discussion

Using genetically engineered mice we demonstrated that upregulating myocardial glycogen significantly improves ischemic tolerance. Protection is associated with glycogen breakdown through the glycolytic pathway and takes place as long as there is sufficient residual coronary flow to prevent lactic acidosis.

We have previously found that mutations in *PRKAG2* gene cause cardiomyopathy characterized by myocardial glycogen storage [13]. Remarkably, unlike classical glycogen storage diseases, in this disease entity glycogen accumulation arises from dysregulated metabolism but not an enzymatic defect in glycogen breakdown pathway [12,21]. Transgenic mice over-expressing the 'wild-type' *PRKAG2* gene (*PRKAG2*^{WT}) also

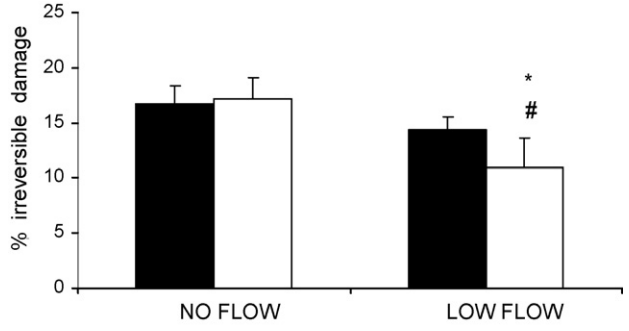

Fig. 4 – Irreversible damage in the heart tissue after no/low-flow ischemia: the percentage of irreversible damage following I/R is presented as mean ± S.E., $n = 6–11$. The wild-type groups: black bars; *PRKAG2*^{WT} groups: white bars. $^{*}p < 0.05$ in ANOVA and between *PRKAG2*^{WT} and control low-flow groups.

develop high levels of myocardial glycogen, but not to the extent of causing cardiac dysfunction. Glycogen turnover rates are increased in *PRKAG2* transgenic hearts during metabolic stress [16]. We hereby demonstrate that hearts of *PRKAG2*^{WT} transgenic mice resisted ischemic injury better than controls.

Excess glycogen was associated with a higher heart-to-body weight ratio in *PRKAG2*^{WT} transgenic mice compared to controls ($p = 0.009$). Baseline coronary flow of *PRKAG2*^{WT} mice group was higher than in controls due to higher heart weight, but normalizing CF to heart weight abolished the difference. *PRKAG2*^{WT} mice had higher developed pressure, a slower spontaneous sinus heart rate but a similar rate pressure product (LVP × HR). Basal phenotypic differences could account for the different response to ischemic injury. However, previous studies suggest impaired ischemic tolerance in hypertrophic hearts [22,23]. We therefore assume that hypertrophy did not benefit *PRKAG2* ^{WT} hearts and in fact could diminish the advantage afforded by glycogen.

The contribution of anaerobic glycolysis to ischemic tolerance as opposed to its potential damage through tissue

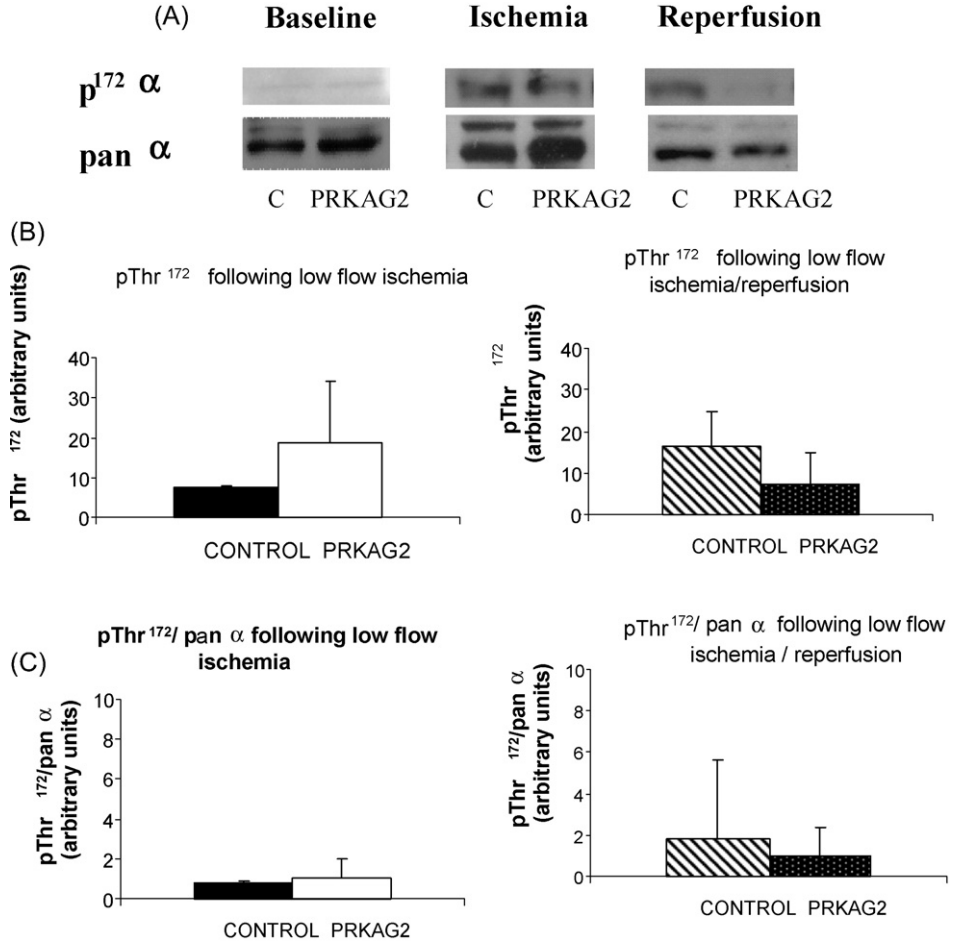

Fig. 5 – AMPK activity in the heart tissue. Western blot with antibodies against PThr^{172}-α-subunit and pan-α AMPK during baseline perfusion, low-flow ischemia and post-ischemic reperfusion are shown in panel (A). Protein extracts (30 μg/sample) were run on a SDS polyacrylamide gel (12%) under denaturing conditions and transferred onto nitrocellulose for 1 h at 100 V. The α-subunit phosphorylation was barely detectable during normal isolated heart perfusion. Panel B represents densitometry results for PThr^{172}-α-subunit. In panel C the ratio of PThr^{172}-α/pan-α AMPK following low-flow ischemia and reperfusion is presented (means ± S.E., $n = 3–6$/group). There were no significant differences between groups in the levels of pan-α, PThr^{172} and their ratio.

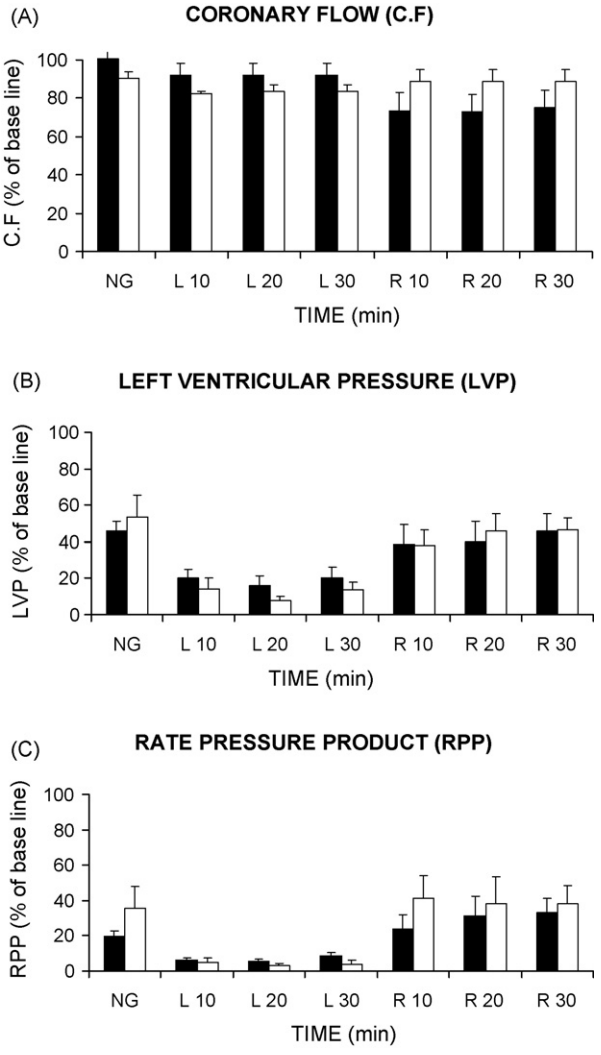

Fig. 6 – Hemodynamic measurements following glycogen depletion and low-flow ischemia experiments. Values are presented as a percentage of baseline (mean ± S.E., $n = 5–6$/group). Wild-type control: black bars; *PRKAG2*^{WT} hearts: open bars. Using the Langendorff preparation hearts of *PRKAG2*^{WT} and wild-type mice were subjected to a 20 min stabilization period, then to 30 min glucose and pyruvate-free KHB solution, 30 min low-flow (10% basal flow) ischemia - and 30 min of reperfusion. No differences in the contractile measurements between the groups (CF (A), LVP (B), RPP (C)) were found. NG: No glucose; L: low-flow ischemia; R: reperfusion time.

acidosis is dependent on several factors including lactate disposal and the generation of oxygen-derived free radicals at reperfusion, which inhibit different ionic pumps, induce a mitochondrial calcium release, and block the glycolytic flux [24]. It was previously reported that increasing glycogen levels prior to ischemia may improve recovery of function, lessen membrane damage, and prevent the loss of adenine nucleotides [17]. Moreover glycogen and glycogen metabolizing enzymes are colocalized with the sarcoplasic reticulum and play a major role in metabolic signaling systems of cell survival and calcium homeostasis [25]. We therefore used 2 experimental protocols: no-flow ischemia and low-flow ischemia where the coronary flow was reduced to 10% of baseline values. Improved functional recovery and myocardial viability were associated with consumption of glycogen stores and massive release of lactate to the coronary effluent. Low-flow ischemia allows a residual supply of some oxygen and nutrients and washout of toxic metabolites and is therefore compatible with ongoing glycolytic flux and continuous glycogen utilization to create energy through anaerobic and aerobic pathways [26]. Once PRKAG2^{WT} hearts were depleted of glycogen, protection from ischemic injury was eliminated, confirming the importance of generating energy through glycolysis in maintaining myocardial viability. Lactate removal in *PRKAG2*^{WT} mice during low-flow ischemia was remarkably effective (Fig. 3D and E), possibly due to accelerated lactate transporter activity [28,29]. In the no-flow ischemia experiments where the heart could not remove lactate and adequately consume its glycogen (Fig. 3), *PRKAG2*^{WT} did not benefit from functional or myocardial viability compared to control hearts. 'No flow' leads to the accumulation of lactate and the reduction in the intracellular pH. Acidosis inhibits glycolysis, leading to severe depletion of high-energy compounds and inhibition of function of cell organelles [26]. Interestingly, recovery of CF during reperfusion was better in the *PRKAG2*^{WT} mice than controls in either model of ischemia, suggesting an attenuation of endothelial-capillary injury and of no-reflow phenomenon, independently of glycolysis.

An advantage of increased muscle glycogen in the R70Q γ1-AMPK mice manifests as increased exercise capacity [27]. Deficient activity of cardiac glucose transporters is responsible for decreased ischemic tolerance in GLUT 4 knockout mice [30]. Transgenic mice overexpressing the GLUT I transporter demonstrated improved mechanical performance and resisted pressure-overload cardiomyopathy [31]. AMPK activation is associated with increased glucose uptake, possibly mediated through increased GLUT4 activity [21,32,33]. Previous studies with dominant negative AMPK α transgenic mice reported decreased ischemic tolerance in hearts with dysfunctional AMPK, attributed to decreased glucose uptake. It was recently reported that α2-AMPK deletion induced a twofold reduction in glycogen content and a threefold reduction in glucose uptake [32,33,35]. α2-AMPK appears to be required for regulation of glucose uptake, glycogen contents and glycolysis, postponing the development of the ischemic contracture. The exact role played by AMPK stimulation during ischemia-reperfusion may be dependent on the availability of fatty acids in the medium [34–36]. AMPK increases fatty acid oxidation through inhibition of acetyl CoA carboxylase and decreasing malonyl CoA. Fatty acids inhibit glucose oxidation, uncoupling it from the glycolytic flux, thereby causing acidosis and increased energy expenditure on lactate disposal [36]. Despite these divergent mechanisms, AMPK activation is considered to be a necessary component in mounting a stress response [20] and was reported to be associated with improved outcome after ischemia-reperfusion injury, even when fatty acids were present in the medium [32,35]. AMPK may also mediate suppression of protein synthesis concomitant with translocation of GLUT 4 to

sarcolemma, leading to increased tolerance to the next period of ischemia, i.e. ischemic preconditioning [37].

Unlike previous studies which showed decreased cardiac tolerance with shutting down of AMPK subunits, we demonstrate that overexpression of an AMPK subunit may benefit the heart under ischemic conditions. Unlike mutant *PRKAG2*^{N488I}, *PRKAG2*^{WT} hearts used in this study show no significant increase in AMPK activity, although they do demonstrate a mild increase in glucose uptake [15,21]. Previous results [14,15] and our measurements of α-subunit phosphorylation (Fig. 5) indicate the absence of increased AMPK activation in *PRKAG2*^{WT} hearts compared to controls. During ischemia AMPK appears to be activated to a similar degree in both groups. Improved recovery was associated with increased consumption of glycogen and effective removal of lactate, unrelated to AMPK activation. Thus, this model demonstrates the importance of the ability to store and metabolize glycogen in improving the heart's tolerance to metabolic stress. We cannot completely rule out the contribution of AMPK upregulation to improved ischemic tolerance in *PRKAG2*^{WT} hearts. Absence of a significant difference in levels of α AMPK phosphorylation between *PRKAG2*^{WT} and controls during low-flow ischemia could result from improved metabolic status of *PRKAG2* hearts. Because AMPK activation leads to glycogen storage in *PRKAG2*^{N488I} hearts, we may therefore expect that subtle activation in *PRKAG2*^{WT} is responsible for the mild increase in glucose uptake and glycogen synthesis [21]. Such a change might be below the threshold of detection using conventional methods and/or limited to certain subcellular locations. This is supported by the fact that PRKAG2^{WT} mice bred with 'dominant-negative' α2 transgenic reduced their glycogen to near normal levels [16].

This study used an artificial perfusion system to assess heart tolerance to ischemia and reperfusion. The Langendorff isolated heart system is often utilized to perfuse isolated hearts with KHS containing pyruvate to test the effects of drugs, cardioplegic solutions, metabolic and genetic manipulations on cardiac function and ischemic damage [18,15,32]. Glucose becomes the main substrate for oxidative metabolism of the heart when fatty acid levels are low, when the concentrations of glucose and insulin are high and during oxygen deprivation. A concentration of 2 mM pyruvate appears to be a sufficient physiological substitute for fatty acids in the retrograde Langendorff perfusion system, as a good source of metabolizable substrate [35]. The oxygen consumption peaks at 70% of the actual coronary flow rates obtained in the Langendorff system, independent of fatty acids in the medium [38]. It may be therefore assumed that the low-flow ischemic conditions in our study are likely to resemble the metabolic status of the border-zone of acutely ischemic myocardium supplied through collaterals.

In summary: this study shows the potential for heart protection from ischemia and reperfusion injury through metabolic manipulation increasing the level and utilization of myocardial glycogen. Improved recovery was associated with increased consumption of glycogen, and was unrelated to AMPK activation. These findings imply that increasing heart glycogen by pharmacological or physiological means can increase the tolerance to ischemia and extend the time-window of intervention in clinical situations involving acute ischemia and minimal residual coronary flow.

Disclosures

None.

Acknowledgements

We are grateful to Frosa Katsis for the preparation of AMPK antibodies.

This work was supported by BEK is an Australian Research Council (ARC) Federation Fellow supported by the ARC, NHMRC and National Heart Foundation of Australia and the Hamer Foundation, Sackler Faculty of Medicine, Tel Aviv University, Israel.

Dr. Arad was supported in part by the 876/05 grant from the Israel Science Foundation.

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