Published in final edited form as:

J Cell Physiol. 2011 September ; 226(9): 2457–2468. doi:10.1002/jcp.22592.

PKC ϵ Promotes Cardiac Mitochondrial and Metabolic Adaptation to Chronic Hypobaric Hypoxia by GSK3 β Inhibition

Joy McCarthy^{1,*}, Amanda Lochner², Lionel H. Opie¹, Michael N. Sack³, and M. Faadiel Essop⁴

¹Hatter Institute for Cardiovascular Research, University of Cape Town Medical School, Cape Town, South Africa ²Department of Medical Physiology, Stellenbosch University's Faculty of Health Sciences, Tygerberg, South Africa ³Translational Medicine Branch, National Heart Lung and Blood Institute, National Institutes of Health, Bethesda, Maryland ⁴Cardio-Metabolic Research Group (CMRG), Department of Physiological Sciences, Stellenbosch, South Africa

Abstract

PKC ϵ is central to cardioprotection. Sub-proteome analysis demonstrated co-localization of activated cardiac PKC ϵ (aPKC ϵ) with metabolic, mitochondrial, and cardioprotective modulators like hypoxia-inducible factor 1 α (HIF-1 α). aPKC ϵ relocates to the mitochondrion, inactivating glycogen synthase kinase 3 β (GSK3 β) to modulate glycogen metabolism, hypertrophy and HIF-1 α . However, there is no established mechanistic link between PKC ϵ , p-GSK3 β and HIF1- α . Here we hypothesized that cardiac-restricted aPKC ϵ improves mitochondrial response to hypobaric hypoxia by altered substrate fuel selection via a GSK3 β /HIF-1 α -dependent mechanism. aPKC ϵ and wild-type (WT) mice were exposed to 14 days of hypobaric hypoxia (45 kPa, 11% O₂) and cardiac metabolism, functional parameters, p-GSK3 β /HIF-1 α expression, mitochondrial function and ultrastructure analyzed versus normoxic controls. Mitochondrial ADP-dependent respiration, ATP production and membrane potential were attenuated in hypoxic WT but maintained in hypoxic aPKC ϵ mitochondria ($P < 0.005$, $n = 8$). Electron microscopy revealed a hypoxia-associated increase in mitochondrial number with ultrastructural disarray in WT versus aPKC ϵ hearts. Concordantly, left ventricular work was diminished in hypoxic WT but not aPKC ϵ mice (glucose only perfusions). However, addition of palmitate abrogated this ($P < 0.05$ vs. WT). aPKC ϵ hearts displayed increased glucose utilization at baseline and with hypoxia. In parallel, p-GSK3 β and HIF1- α peptide levels were increased in hypoxic aPKC ϵ hearts versus WT. Our study demonstrates that modest, sustained PKC ϵ activation blunts cardiac pathophysiologic responses usually observed in response to chronic hypoxia. Moreover, we propose that preferential glucose utilization by PKC ϵ hearts is orchestrated by a p-GSK3 β /HIF-1 α -mediated mechanism, playing a crucial role to sustain contractile function in response to chronic hypobaric hypoxia.

Adaptation of the heart to hypoxia evokes a protective program resulting in enhanced ischemic-stress tolerance, yet the underlying mechanisms remain unclear (Kopecky and Daum, 1958; Hurtado, 1960; Meerson et al., 1973; Sumeray et al., 2000). Hypoxia is a component of ischemia caused by reduced blood flow and tissue oxygen content, so understanding the molecular mechanisms underpinning hypoxia may elicit potential novel therapeutic targets. Previously, a role for PKC ϵ in this protective phenotype was implicated

since hypoxia increased the activation and translocation of PKC ϵ from the cytosol to the membrane fraction, whereas inhibition of PKC ϵ abolished hypoxia-induced oxidative stress tolerance in cardiomyocytes (Gray et al., 1997).

PKC ϵ is a downstream target of Akt (Ping, 2001) that phosphorylates and thus inactivates glycogen synthase kinase β (GSK3 β). However, PKC ϵ and Akt can also phosphorylate GSK3 β directly (Inagaki et al., 2006; Ping, 2002). GSK3 β regulates glucose availability in the cell by enhancing glycogen synthesis (Roach, 1990). Furthermore, a recent study found that GSK3 β inhibition during reperfusion promoted glycogen synthesis thereby decreasing glycolysis and associated harmful H⁺ production during reperfusion (Takeishi, 2000). Ca²⁺ overload was also reduced and mechanical function improved. Together this suggests that modulation of myocardial metabolism by the PKC ϵ -GSK3 β signaling cascade may represent a novel way to elicit cardioprotection in response to oxygen lack.

Interestingly, sub-proteome analysis of constitutively active PKC ϵ transgenic (aPKC ϵ) cardiac tissue demonstrated that PKC ϵ complexes several metabolic- and transcription-related peptides (Edmondson, 2002). Here PKC ϵ complexed with glycolytic enzymes such as enolase and glyceraldehyde-3-phosphate dehydrogenase, further supporting a role for PKC ϵ in glucose metabolism. Moreover, the presence of citric acid cycle enzymes, that is, succinate dehydrogenase and isocitrate dehydrogenase, further implicates PKC ϵ in broader myocardial metabolism. Multiple mitochondrial enzymes involved in ATP production and transport also reside in PKC ϵ protein complexes, emphasizing its pivotal role in regulating mitochondrial function and intracellular energy balance. Lastly, PKC ϵ co-localizes with heat shock proteins and hypoxia-inducible factor 1 α (HIF-1 α), previously identified as key mediators of cardioprotection (Edmondson, 2002).

We and others have previously shown that the acute cardiac ischemia-tolerance orchestrated by PKC ϵ is associated with improved mitochondrial and cellular bioenergetics (McCarthy, 2005; Murphy and Frishman, 2005; Inagaki et al., 2006). However, the mitochondrial response of aPKC ϵ mice to a chronic oxidant stress has not yet been explored. In light of this, we therefore hypothesized that the PKC ϵ cardioprotective program is also activated in response to chronic hypoxic stress and mediated by downstream targets GSK3 β and HIF-1 α to modulate myocardial metabolism and maintain mitochondrial homeostasis.

To explore this, aPKC ϵ mice and their littermate controls were exposed to 14 days of chronic hypobaric hypoxia (45 kPa, 11% O₂). Mitochondrial function (mitochondrial respiration, membrane potential, ADP:O ratio, proton leak) and ultrastructure (electron microscopy), ATP synthesis, cardiac metabolism and function in ex vivo normoxically perfused working hearts were measured and compared to non-hypoxic controls. To gain additional insight into the underlying mechanisms we also evaluated myocardial peptide levels of p-GSK3 β and HIF-1 α .

Materials and Methods

Transgenic mouse and hypobaric hypoxia model

Constitutively active PKC ϵ transgenic mice (aPKC ϵ) (Ping, 2002) (a gift from Dr. Peipei Ping, Harvard, MA) were compared with littermate non-transgenic wild-type controls. Briefly, a full-length PKC ϵ cDNA was cloned from a rabbit heart cDNA library and a constitutively active PKC ϵ cDNA was generated through a single amino acid mutation (no. 159, A to E), driven by an α -MHC promoter to overexpress the gene in the heart only. This was used for microinjection into pronuclei of fertilized FVB mouse eggs. The presence of the transgene was confirmed by Southern analysis of genomic DNA. More than one strain of PKC ϵ overexpressing mouse was created, expressing differing copy numbers of the gene.

The strain employed for the current study moderately overexpresses (copy number = 20) constitutively active PKC ϵ (Takeishi, 2000; Pass et al., 2001b). PKC ϵ transgenic mice displayed an increase of $228 \pm 23\%$ in PKC ϵ activity in the particulate fraction compared to wild types (WT) (Takeishi, 2000). Furthermore, PKC ϵ protein levels were $9\times$ higher in the heart, a 6-fold increase in the membrane to cytosol ratio (indicating activation of PKC ϵ) and a 2.5-fold increase in PKC ϵ activity in the membrane fraction (Takeishi, 2000). There were no compensatory changes in other PKC isoforms. All mice (9–12 weeks of age) were housed, and experiments conducted, in accordance with the Guiding Principles in the Care and Use of Animals and National Institutes of Health Guidelines (NIH publication No. 85-22, revised 1996). All procedures were also approved by the Faculty of Health Sciences Animal Ethics Committee of the University of Cape Town. Only male mice were employed for this study and exposed to 14 days of hypobaric hypoxia (45 kPa, 11% O₂) as described previously (Smith et al., 2001). Normoxic transgenic and non-transgenic littermate control mice were maintained for 14 days in the same room as the hypoxic chamber and then sacrificed.

Characterization of mitochondrial function

Mitochondria were isolated from normoxic or hypoxic left ventricular (LV) and right ventricular (RV) cardiac tissue as described previously (McCarthy, 2005). As changes in RV were paralleled by changes in LV, only data for the LV is shown, unless otherwise stated. Mitochondrial respiration was polarographically determined using glutamate and malate as oxidative substrates (Sordahl, 1971). The respiratory control index (RCI), generally regarded as an index of mitochondrial quality and viability, was calculated as a ratio of state 3 respiration divided by state 4. Preparations with RCI values of less than 4 were discarded. The rate of ATP synthesis was measured by luminometry using a modification of the method of Budnikhov et al. (2002). Rate of ATP production was calculated against a standard curve using sodium ATP in concentrations of 10^{-5} – 10^{-9} M (Roche Biochemicals, Basel, Switzerland) (Ozcan, 2001). Inner mitochondrial membrane potential determination was performed on isolated normoxic and hypoxic mitochondria using two potentiometric dyes: DiOC₆ (3',3'-dihexyloxycarbocyanine iodide, Sigma, St. Louis, MO) (Macouillard-Poullietier de, 1998) and JC-1 (5,5',6,6'-tetrachloro-1, 1',3,3'-tetraethylbenzimidazolylcarbocyanine, Molecular Probes, Eugene, OR) and flow cytometry (Becton Dickinson, Franklin Lakes, NJ) as previously described (Minners, 2001). Phosphorylation ratios (ADP:O) were calculated as an indirect measure of oxygen consumption, and oxidative phosphorylation-independent proton kinetics were evaluated with the addition of oligomycin (1 μ g/ml) (Nogueira, 2001).

To confirm data generated from isolated mitochondrial preparations, we repeated mitochondrial anoxia/reoxygenation respiration experiments (glutamate and malate as oxidative substrates) as before using normoxic and hypoxic permeabilized LV cardiac fibers (Boudina, 2005) isolated from WT and aPKC ϵ hearts (McCarthy, 2005).

Electron microscopy for ultrastructural changes

Hearts were rapidly excised and arrested in ice-cold isolation buffer. A portion of the LV was dissected and fixed in glutaraldehyde for 2–4h at 4°C. The specimen was washed, dehydrated, embedded in resin and sectioned using an ultramicrotome. Longitudinal sections were placed onto copper grids were stained with heavy metals (uranyl acetate and lead nitrate) and visualized on a Jeol 1200 EX II electron microscope (JEOL, Tokyo, Japan). Assessment of grids was performed in a blinded manner. Firstly an overall assessment was done at $7,500\times$ magnification, then 3–4 fields were examined for each sample at $25,000\times$ magnification. Data shown are representative of the results obtained from $n = 3$ –5 for each group.

Quantitative RT-PCR

Metabolic gene changes after normoxia and 14 days hypobaric hypoxia were assessed by amplification of target genes by use of quantitative RT-PCR analysis. Hearts were harvested and right ventricle separated from left ventricle plus septum, rinsed in ice-cold isolation buffer and snap frozen in liquid nitrogen. The methods for the RNA extraction and real-time quantitative RT-PCR have been described previously (Sharma, 2004). The nucleotide sequences and probes have been previously published (Depre, 1998; Sharma, 2004) and the constitutive gene transcript 18S was used to normalize the data. Internal standards were prepared using T7 RNA polymerase method (Ambion, Austin, Texas).

Tissue glycogen content

Two to 10 mg of frozen heart tissue was pulverized under liquid nitrogen and heated at 95°C in 200 μ l 40% potassium hydroxide. Glycogen was precipitated at 4°C overnight, washed three times with 1 ml of 95% ethanol, then digested for 3 h in 200 μ l of 2 N HCl with heating at 95°C (Good and Somoygi, 1933). After cooling, samples were neutralized and a spectrophotometric glucose assay was performed.

Cardiac functional analysis

Heparin (100 U, i.p.) was administered to 9- to 12-week-old hypoxic and normoxic male mice 10 min prior to sacrifice. Mice were anesthetized with pentobarbitone sodium (60 mg kg⁻¹ i.p.) and hearts rapidly excised through a central sternotomy (Smith, 2002). After arrest in ice cold (4°C) modified Krebs–Henseleit (KH) (Aasum, 2008) buffer (NaCl 118.5 mM; NaHCO₃ 25.0 mM; KCl 4.7 mM; KH₂PO₄ 1.2 mM; CaCl₂ 2.25 mM; MgSO₄ 1.2 mM; di-sodium EDTA 0.5 mM; glucose 11 mM), hearts were mounted on an aortic cannula. Normoxic retrograde perfusion was initiated with modified KH buffer gassed with 95% O₂/5%CO₂ at 37°C and a constant pressure of 110 mm Hg. After 10 min of retrograde perfusion, hearts were perfused in working heart mode (Smith, 2002) at a preload of 12.5 mm Hg and afterload of 50 mm Hg for 40 min. Perfused hearts were allowed to beat spontaneously and pressures, aortic and coronary flow measurements were taken every 10 min. Cardiac output was calculated as the sum of the aortic and coronary flows. Peak systolic pressure was recorded in the afterload (Aasum et al., 2003) line, using a Statham pressure transducer (Transpac IV, Abbotts, Sligo, Ireland). Pressure signals were recorded in 10-sec pulses and analyzed using software developed by the University of Stellenbosch Electronic Department (SED) (Kannengiesser et al., 1979).

Cardiac metabolism

Substrate metabolism and contractile function were measured concurrently in working hearts as follows: 40 ml of KH buffer perfusate supplemented with 11 mM glucose or glucose plus 0.7 mM palmitate bound to 3% BSA. Radiolabeled tracer was added to buffer: 5-³H-glucose and U¹⁴C-glucose or U¹⁴C-glucose and 9, 10-[28] palmitate, which was recirculated for the duration of the experiment to determine rates of glycolysis and glucose oxidation or glucose oxidation plus fatty acid oxidation, respectively. Glycolysis was measured by the accumulation of ³H₂O in the circulating buffer over time compared with the specific activity of the tracer. Myocardial glucose oxidation was determined by trapping and measuring ¹⁴CO₂ released by the metabolism of glucose (Saddik and Lopaschuk, 1991; Belke, 1999). Palmitate oxidation was determined by measuring the amount of ³H₂O released from 9,10-[³H] palmitate as outlined by Saddik and Lopaschuk (1991). Metabolic rates were normalized using total dry mass of the hearts (Saddik and Lopaschuk, 1991; Belke, 1999; Aasum et al., 2003).

Cardiac hypertrophy

Hypobaric hypoxia is essentially a pressure overload model. The lower pressure in the chamber results in decreased pressure in the lungs, increasing the flow through the pulmonary vein (pulmonary hypertension) and hence increasing pressure in the right ventricle. With time, this continued pressure overload stimulus leads to RV hypertrophy as an adaptive response in the WT but not in the PKC ϵ hearts (Ostadal et al., 1999).

There are numerous references documenting changes in hearts and lungs of humans and rats living at high altitude (systemic hypoxia), where pressure and oxygen is reduced. In two instances, systemic hypoxia can be considered as physiological:

- i. the fetal myocardium adapted to hypoxia corresponding to an altitude of 8,000 m;
- ii. the myocardium of subjects living permanently at high altitudes.

In both situations the myocardium is significantly more resistant to acute oxygen deficiency (Meerson et al., 1973; Opie et al., 1978; Essop, 2007; Ostadal and Kolar, 2007), recovers better from ischemia-reperfusion injury and subjects have a lower incidence of heart disease.

Hearts were excised, RV separated from LV plus septum, blotted and weighed. Respective weights of a PKC ϵ heart chambers were compared with WT, and as a function of body weight.

Immunoblotting analysis

GSK3 β , a protein regulating glucose partitioning (Omar et al., 2010), maybe responsible for the metabolic changes observed in the aPKC ϵ hearts. As HIF1 α , a protective transcription factor also elevated by hypoxia (Czibik, 2009), is a downstream target of GSK3 β , it may also play a signaling role in the cardioprotection observed in hypoxic aPKC ϵ mice. Normoxic and hypoxic hearts from either group were separated into RV and LV plus septum, snap frozen in liquid nitrogen and stored at -80°C . Only a portion of LV was used for Western blotting analysis.

Nuclear and cytosolic extracts from hypoxic and normoxic LV were extracted by pulverizing the tissue under liquid nitrogen then homogenizing in a lysis buffer (Deryckere and Gannon, 1994). One hundred twenty micrograms of nuclear and cytosolic protein was analyzed by SDS-PAGE for phosphorylated and total GSK3 β (1:3,000 in TBS-T plus 3% fat free milk; Cell Signaling Technology, Danvers, MA), as GSK3 β is a downstream target of PKC ϵ and influences the half-life of HIF1- α (1:200 in TBS-T plus 3% BSA; Santa Cruz Biotechnology, Santa Cruz, CA). Potential HIF-1 α downstream targets, that is, nuclear respiratory factor 1 (NRF1) (1:500 in TBS-T; Santa Cruz Biotechnology) and peroxisome proliferator-activated receptor gamma coactivator-1 α (PGC1 α) (1:500 in TBS-T; Santa Cruz Biotechnology) were also assayed together with peptide levels of GLUT1 (1:250 in TBS-T plus 5% milk; Santa Cruz Biotechnology) and GLUT4 (1:1,000 in TBS-T; Cell Signaling Technology). Equal loading was verified with β -tubulin (1:1,000 in TBS-T; Santa Cruz Biotechnology). Relative densitometry was determined using computerized software package (UVI Soft, UVI Band, UVI Tech, Cambridge, UK and Image-J) and all peptide data normalized to β -tubulin. A minimum of six hearts was used per group.

Immunohistochemical co-localization of PKC ϵ and HIF-1 α

To confirm greater association and co-localization of PKC ϵ with HIF1- α in the transgenic aPKC ϵ hearts compared with wild-type littermates, cardiac tissue was fixed in 10% paraformaldehyde at 4°C then processed using a Tissue Tek II automated ultramicrotome. In brief, samples were dehydrated using 70%, 90%, 95%, and 100% ethanol, then cleared using xylol. Histotec wax was used for wax impregnation. Paraffin embedded sectioning was

performed using an R Jung microtome (Heidelberg, Germany) to generate 5 μm sections, which were collected on slides coated with poly-L-lysine (P8920, Sigma).

Samples were then processed and rehydrated using a xylol-ethanol dilution range. For antigen retrieval, slides were incubated for 15 min in pre-warmed (37°C) 0.1% trypsin in phosphate-buffered saline (PBS), followed by a rinsing step with distilled water. Samples were then blocked 5% donkey serum and stained with anti-PKCe and anti-HIF1- α antibody, followed by FITC-conjugated anti-goat antibody or rhodamine-conjugated anti-rabbit antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Nuclei were then stained with Hoechst 33342 at a final concentration of 50 $\mu\text{g/ml}$. Slides were mounted with fluorescent mounting medium (Dako Cytomation, Glostrup, Denmark) and sealed.

Image acquisition was performed on an Olympus CellR system, attached to an IX81 inverted fluorescence microscope equipped with an F-view-II cooled CCD camera (Soft Imaging Systems, Muenster, Germany). Using a Xenon-Arc burner (Olympus Biosystems, Hamburg, Germany) as a light source, images were acquired using the 360, 472, or 572 nm excitation filter. Emission was collected using a UBG triple-bandpass emission filter cube (Chroma Technology, Rockingham, VT). For the Z-stack image frame acquisition, a step width of 0.5 μm , an Olympus Plan Apo N60 $\times$ /1.4 oil objective and the CellR imaging software were used. Images were processed, background-subtracted and the signal co-localized using the CellR software. The data represent four normoxic hearts in each group with eight images per panel.

Statistical analysis

Results are expressed as means $\pm$ SEM. Significance ($P < 0.05$) was determined for discrete variables using the Student's *t*-test and a Welch correction that assumes samples may have different standard deviations. Two-way ANOVA analysis was performed when appropriate.

Results

aPKC ϵ mitochondria display increased mitochondrial membrane potential at baseline

At baseline, normoxic WT mitochondria showed no significant differences in mitochondrial respiration (Fig. 1a) or rate of mitochondrial ATP production (Fig. 1b) when compared to aPKC ϵ . However, mitochondrial membrane potential for aPKC ϵ mitochondria was significantly increased at baseline versus WT controls (Fig. 1c).

Activation of PKC ϵ prevents mitochondrial dysfunction and energy reduction in cardiac mitochondria in response to hypobaric hypoxia

Initial mitochondrial ATP concentration differs between the transgenic aPKC ϵ mice and their wild-type littermate controls, albeit with a similar rate of production (Fig. 1b). Unlike WT mitochondria, aPKC ϵ mitochondria maintain basal ADP-dependent respiration following 14 days of hypobaric hypoxia (Fig. 1d). In agreement, the rate of mitochondrial ATP production (Fig. 1e) and the relative mitochondrial membrane potential (Fig. 1f) were elevated in response to hypoxia versus WT controls. Furthermore, the ADP:O ratio was increased (Fig. 2a) and the oxidative phosphorylation-independent proton leak (Fig. 2b) diminished in aPKC ϵ mitochondria compared to WT. These data therefore demonstrate that aPKC ϵ mitochondria are more tightly coupled than WT in response to a chronic hypobaric hypoxia insult, reinforcing the improved mitochondrial energetics found in the aPKC ϵ mice.

Functional assessment of isolated mitochondria does not necessarily reflect changes that occur in situ. We therefore also evaluated mitochondrial respiration in permeabilized cardiac fibers (Boudina, 2005) from normoxic and hypoxic WT and aPKC ϵ hearts and found that

these data compared well with results obtained in isolated mitochondria (Supplementary Data Fig. S1). In addition, infarct size after 45 min of global ischemia was compared between aPKC ϵ and WT, using triphenyltetrazolium (TTC) staining (Sumeray et al., 2000). Here aPKC ϵ hearts exhibited reduced infarct size and robust cardioprotection ($P < 0.03$ vs. WT, Supplementary Data Fig. S2).

Right ventricular hypertrophy

RV and LV plus septum weights of aPKC ϵ and WT normoxic and hypoxic hearts were examined. Hypoxic aPKC ϵ RV exhibited a significantly attenuated hypertrophic response at 14 days hypobaric hypoxia compared to WT littermate controls (Supplementary Data Fig. S3). LV +septum weights did not differ significantly.

Activation of PKC ϵ prevents hypobaric hypoxia-mediated perturbations of the cardiac myofilament-mitochondrial ratio

Maintenance of mitochondrial and contractile function in the aPKC ϵ transgenic mice in response to hypobaric hypoxia may have resulted from mitochondrial biogenesis. To further assess this, ultrastructural differences between LV sections of normoxic and hypoxic aPKC ϵ and WT hearts were directly visualized using electron microscopy (25,000 $\times$ magnification). At baseline, the myofilament to mitochondrial ratio appeared similar in aPKC ϵ versus WT strains (Fig. 3a,b) with no ultrastructural differences noted. With the progressive duration of hypoxia WT mice displayed a progressive alteration in the myofilament to mitochondrial ratio (Fig. 3c,e). In stark contrast, the aPKC ϵ cardiac ultrastructure was better preserved in response to 14 days of hypobaric hypoxia (Fig. 3d,f).

Transcript levels of key target genes

The metabolic response of aPKC ϵ hearts to hypobaric hypoxia was further explored by performing quantitative RT-PCR for potential target genes (Fig. 4) in hypoxic and normoxic RV and LV tissue from both groups. For clarity only LV data are shown, but these changes were paralleled by similar changes in the RV. Uncoupling protein 3 (UCP3) is expressed in the heart and thought to uncouple mitochondrial respiration, thereby reducing mitochondrial efficiency (Yu, 2000; Echta, 2001; Skarka, 2003). However, it is also implicated in the regulation of fatty acid oxidation (Garcia-Martinez, 2001; Harper and Himms-Hagen, 2001; Himms-Hagen and Harper, 2001). UCP3 transcript levels were not significantly different at baseline in aPKC ϵ mice compared with WT, but markedly declined with hypoxia (Fig. 4a, $P < 0.0005$ vs. WT hypoxia) which may account for the increased mitochondrial efficiency observed in aPKC ϵ heart mitochondria.

MHC β is a fetal gene protein that confers slow, oxygen sparing contractions. MHC β transcript levels were elevated in the aPKC ϵ normoxic and hypoxic samples, suggesting a contractile and/or energetic advantage compared to the WT hearts (Fig. 4b). Transcript levels of GLUT1 (non-insulin responsive transporter) were robustly increased in aPKC ϵ hearts under normoxic conditions, but reduced with hypoxia to WT levels (Fig. 4c), correlating well with observed glycogen changes (see below).

Cardiac glycogen levels

Counterintuitively, cardiac glycogen levels were significantly lower in normoxic aPKC ϵ hearts, yet significantly elevated in hypoxic aPKC ϵ cardiac tissue when compared to WT littermates (Fig. 5), reinforcing a significant role for p-GSK3 β during hypoxia.

Cardiac metabolism

Perfusion with glucose only—Normoxic aPKC ϵ hearts oxidized more glucose than WT (0.29 ± 0.09 $\mu\text{mol/g dry weight/min}$ vs. 0.092 ± 0.014 $\mu\text{mol/g dry weight/min}$; * $P < 0.05$, Fig. 6). For WT hearts, exposure to hypoxia demonstrated the expected increased glucose oxidation (0.19 ± 0.02 compared to 0.12 ± 0.02 $\text{mmol/g dry weight/min}$, Fig. 6) whereas glucose oxidation in hypoxic aPKC ϵ mice decreased to levels comparable with WT. The rate of glycolysis was similar in both groups at normoxia (Fig. 7), although there was a tendency towards higher glycolytic rates in the aPKC ϵ hypoxic mice.

Perfusion with palmitate/glucose—Perfusion of normoxic aPKC ϵ and WT hearts with glucose and moderately high palmitate (0.7 mM) resulted in no significant difference in palmitate oxidation (Fig. 8). However, glucose oxidation remained significantly higher in the aPKC ϵ hearts, both during normoxia and hypoxia, compared to WT hearts (Fig. 9).

Hypoxia-mediated disruption of cardiac function is abrogated by PKC ϵ

In light of bioenergetic distinctions observed between aPKC ϵ and WT mice, we next compared the cardiac contractile response to chronic hypobaric hypoxia by utilizing a working heart model to study LV hemodynamics.

Perfusion with glucose only—Normoxic aPKC ϵ hearts perfused with glucose only demonstrated similar cardiac function compared to the WT, producing 1.47 ± 0.2 mJ of work/dry weight/min versus 1.61 ± 0.2 in the WT (Supplementary Data Table 1a). Hypoxic aPKC ϵ hearts exhibited a distinct energetic advantage over WT hearts (1.8 ± 0.1 mJ work/g dry weight/min vs. 1.3 ± 0.2 mJ work/g dry weight/min in WT, $P < 0.05$, Supplementary Data Table 2a) but at a lower heart rate, suggesting reduced oxygen consumption to produce the same amount of cardiac work. WT mice exposed to hypobaric hypoxia exhibited significantly blunted cardiac output, aortic flow and heart work, with no significant change in coronary blood flow and heart rate. In contrast, these parameters were enhanced in hypoxic aPKC ϵ versus hypoxic WT hearts (Supplementary Data Table 2a).

Perfusion with glucose + moderately high palmitate (0.7 mM)—Normoxic aPKC ϵ hearts demonstrated elevated function, producing 1.31 ± 0.2 mJ of work/dry weight/min, compared to 0.76 ± 0.1 in the WT ($P < 0.05$, Supplementary Data Table 1b). However, aPKC ϵ -mediated protection was lost after exposure to chronic hypobaric hypoxia (glucose and palmitate perfusion), as evidenced by diminished function in the aPKC ϵ hearts (0.95 ± 0.21 mJ work/g dry weight/min vs. 1.25 ± 0.15 mJ work/g dry weight/min in WT, ** $P < 0.01$, Supplementary Data Table 2b) compared to WT.

Percentage contribution of fuel substrates to ATP generation

To investigate why aPKC ϵ hearts display attenuated function on exposure to moderately high palmitate plus glucose, we calculated the relative percentage contribution of glucose and palmitate to ATP production (Opie, 1998). Under normoxic conditions palmitate contributed the majority of ATP produced in WT and aPKC ϵ hearts (Fig. 10). Under hypoxic conditions glucose oxidation increased for both WT and aPKC ϵ hearts (Fig. 11). However, aPKC ϵ hearts displayed higher rates of glucose oxidation compared to WT under these conditions.

Increased peptide levels of downstream PKC ϵ targets following chronic hypobaric hypoxia

We found increased p-GSK3 β peptide levels in hypoxic aPKC ϵ hearts (Fig. 12a). In agreement, the ratio of p-GSK3 β /total GSK was increased from 0.31 ± 0.02 to 0.48 ± 0.01 ($P < 0.05$). As expected, nuclear HIF-1 α was also increased compared to hypoxic wild types

(Fig. 12b), with a concomitant increase in its downstream target PGC1 α (Fig. 12c). The cytosolic fraction exhibited similar elevations in protein levels of p-GSK3 β , HIF-1 α , PGC1 α , and NRF-1 in hypoxic aPKC ϵ tissue compared to WT (Fig. 13). However, here the ratio of p-GSK3 β /total GSK was not significantly altered. We are of the opinion that significant ratio changes would be detected for the mitochondrial fraction since GSK3 β is closely associated with it. This is part of ongoing investigations in our laboratory. GLUT1 levels were increased in aPKC ϵ hearts at baseline but similar to WT after exposure to chronic hypoxia (data not shown), whereas GLUT4 expression was significantly elevated in hypoxic aPKC ϵ compared to WT (Fig. 13). Additional Western blotting data that compare phospho- and total GSK3 β peptide levels during hypoxia are included in the Supplementary Data Figure S4.

Immunohistochemical co-localization of PKC ϵ with HIF-1 α

Co-localization studies using fluorescent antibodies show significantly elevated co-localization of PKC ϵ with HIF-1 α in the aPKC ϵ hearts compared to wild-type littermates ($4.8 \pm 0.9\%$ vs. $21.4 \pm 1.2\%$, $P < 0.0001$) (Fig. S5), reinforcing our hypothesis that PKC ϵ exerts its cardioprotective effects against oxygen lack via a HIF-1 α -PKC ϵ mechanism.

Discussion

Previous studies show that the acute cardiac ischemia-tolerance orchestrated by PKC ϵ is associated with improved mitochondrial and cellular bioenergetics (McCarthy, 2005; Murphy and Frishman, 2005; Inagaki et al., 2006). However, its protective role in response to chronic oxygen lack remains unclear. Here we tested the hypothesis that PKC ϵ orchestrates prolonged cardioprotection in constitutively active PKC ϵ transgenic mice exposed to 14 days of hypobaric hypoxia versus matched normoxic controls.

The novel findings of this study are:

- Cardiac aPKC ϵ mitochondria are robustly protected against chronic oxygen lack compared to wild-type mice. aPKC ϵ mitochondria maintain an energetic advantage after hypoxia, that is, higher respiratory capacity, increased ATP production, lower proton leak and are tightly coupled.
- Hypoxic working aPKC ϵ hearts perfused with glucose alone produce more cardiac work at a lower heart rate, suggesting reduced oxygen consumption compared to wild types.
- Hypoxic working aPKC ϵ hearts perfused with glucose plus moderately high palmitate as substrates exhibit reduced cardiac work compared to wild types, suggesting that aPKC ϵ hearts preferentially utilize carbohydrates as major fuel substrates to maintain cardioprotection.
- Hypoxic aPKC ϵ hearts display significantly elevated levels of phospho-GSK3 β and HIF-1 α compared to wild types that likely contributed to the preferential glucose utilization observed in aPKC ϵ hearts.

Since mitochondrial function and efficiency directly affect cardiac function and contractility (Lesnefsky, 2001; Neubauer, 2007; Rosca and Hoppel, 2009; Bugger, 2010), our data demonstrate that maintenance of state 3 ADP-dependent respiration and mitochondrial energetics confer enhanced recovery of cardiac LV function in aPKC ϵ hearts after exposure to a chronic lack of oxygen supply. Further, intermittent or chronic hypoxia activates PKC ϵ (Kopecky and Daum, 1958; Hurtado, 1960; Meerson et al., 1973; Gray et al., 1997; Rafiee, 2002) and HIF-1 α (Kim, 2006), but inactivates GSK3 β (Juhászova, 2004) as part of the cardioprotective response to hypoxia. PKC ϵ inactivation of GSK3 β limits mitochondrial

permeability transition pore (mPTP) opening (Bopassa, 2005) and improves cell survival (Juhaszova, 2004). In agreement, we found elevated mitochondrial membrane potential in the aPKC ϵ hearts both with normoxia and hypoxia. There is support for this concept from other studies. For example, incubation of isolated cardiac mitochondria with recombinant PKC ϵ resulted in a significant inhibition of Ca²⁺-induced mitochondrial swelling, an index of mPTP opening (Baines, 2003). Furthermore, in cardioprotected aPKC ϵ mice, inhibition of Ca²⁺-induced pore opening was observed (Baines, 2003). In contrast, cardiac expression of kinase-inactive PKC ϵ did not affect pore opening. Finally, administration of atractyloside (mPTP opener) significantly attenuated the infarct-sparing effect of PKC ϵ transgenesis (Baines, 2003), demonstrating the influence of calcium on PKC ϵ cardioprotection.

As transgenesis is an extreme condition, results in such an animal model should be viewed with caution. Overexpression or knockout models are often used to explore the role of a particular protein, but such physiological extremes are now reconsidered in favor of moderate overexpressing or partial knock-down models. This is also the case with our PKC ϵ overexpression mouse model where several strains were established expressing low, moderate, and high gene copy numbers. We employed the moderately overexpressing mouse model that displays no negative contractile effects. This is unlike data published by Pass et al. (2001a) and Kooij et al. (2010) who employed the higher copy number strain that shows progression to hypertrophy and heart failure with age with pronounced calcium insensitivity.

Additional proof of the cardioprotective effects of elevated activated PKC ϵ is shown in the hypothyroid rat model of Cokkinos DV et al. (Pantos, 2003). Here hearts exhibit increased MHC β contractile protein isoforms, elevated glycogen stores, reduced rate of ATP hydrolysis and increased protection from ischemia-reperfusion induced arrhythmias (Pantos, 2003; Mourouzis, 2009). These changes were observed in parallel with an increase in activated PKC ϵ levels.

During normoxia GSK3 β controls HIF-1 α levels by phosphorylation, targeting it for degradation (Murphy, 2004). However, under hypoxic conditions this would abrogate HIF-1 α -induced cardioprotection (Semenza, 1996; Seagroves, 2001). aPKC ϵ inactivation of GSK3 β would therefore facilitate elevated HIF-1 α protein levels to confer cardioprotection. Our immunoblotting data confirmed increased protein levels of inactivated p-GSK3 β in aPKC ϵ hypoxic and normoxic hearts compared to wild types. The effect of GSK3 β inhibition was first demonstrated by Tong et al. (2002) where its inhibition mimicked the cardioprotective effect of ischemic preconditioning. Moreover, Omar et al. (2010) conclusively demonstrated that specific pharmacological inhibition of GSK3 β during ischemia or early reperfusion attenuates calcium overload and proton accumulation independently of LV work. This enhanced post-ischemic functional recovery occurred possibly by partitioning glucose away from glycolysis, reducing proton accumulation and hence calcium overload (Omar et al., 2010).

HIF-1 α is a recognized downstream target of GSK3 β , promoting efficient mitochondrial function by reducing oxygen consumption and elevating mitochondrial ATP production (Kim, 2006). In agreement, our data highlight both elevated p-GSK3 β and HIF-1 α as downstream targets of the PKC ϵ cardioprotective signaling pathway. In support, we have demonstrated a lower oxygen requirement in aPKC ϵ mitochondria to produce more ATP, as well as reduced proton leak. Furthermore, HIF-1 α increases transcription and protein levels of GLUT1 and GLUT4 (Seagroves, 2001), as demonstrated in both normoxic and hypoxic aPKC ϵ hearts, suggesting increased myocardial glucose utilization. GLUT1 mRNA changes shown (Fig. 4c) matched protein expression (Fig. 13): mRNA was elevated during normoxia and decreased in hypoxia. The same was true for the GLUT4: protein levels were elevated in

aPKC ϵ hearts with hypoxia, but not with normoxia. The latter may indeed be the reason for the significantly decreased glycogen content found in normoxic aPKC ϵ hearts.

PGC1 α is a recognized downstream target of HIF-1 α . Transcription of PGC1 α is induced in response to acute oxidant stress, in parallel with increased electron transport chain (ETC) proteins and increased ETC activity (McLeod, 2004). Thus activation of PGC1 α elevates mitochondrial respiration. With chronic hypoxia we demonstrated a similar elevation of PGC1 α protein levels in aPKC ϵ hearts together with maintained mitochondrial respiration. Conversely, wild-type hearts exhibit lower PGC1 α levels and decreased mitochondrial respiration, thus reinforcing our hypothesis that PKC ϵ cardioprotection is mediated at least in part by a p-GSK3 β and HIF-1 α signaling cascade.

Mitochondrial biogenesis in response to hypoxia depends on the extent/duration of the stress and whether oxygen lack is continuous or intermittent (Finck and Kelly, 2006; Lynn, 2007). The heart displays an increase in mitochondrial content and number in response to intermittent and chronic hypobaric hypoxia (Meerson et al., 1973; Costa, 1988). In this study, wild-type mice exhibited increased, smaller mitochondria with structural disarray following 14 days of oxygen deficit. Such excessive mitochondrial number is usually associated with a diminished cardiac contractile function (Finck and Kelly, 2006) due to mechanical disruption of the normal sarcomeric architecture. We found aPKC ϵ mitochondria display decreased bioenergetic function together with a reduction in overall cardiac work and contractile function. These robust data therefore highlight the ameliorating effect of aPKC ϵ on cardiac bioenergetic functioning in response to hypobaric hypoxia. Lack of cardiac biomechanical deficits with activation of PKC ϵ emphasizes its important role in the cardiac hypobaric hypoxia adaptive program. Moreover, it demonstrates a functional interaction between PKC ϵ and activation of cytochrome oxidase with hypoxia (Barnett, 2008), raising the possibility that PKC ϵ -mediated maintenance of mitochondrial bioenergetic homeostasis during hypoxia may itself attenuate signals driving the mitochondrial biogenesis program. The molecular program evoked by PKC ϵ activation that confers these beneficial adaptations has been elusive. In addition, whether PKC ϵ targets modulate mitochondrial bioenergetic efficiency and oxygen biology to prevent the hypobaric hypoxia-mediated perturbations, or whether this effect is due to a more global modulation in the control of mitochondrial function and number also needs to be directly explored. Putative mitochondrial targets that are known to be activated by PKC ϵ include mitochondrial proteins involved in oxidative phosphorylation, electron transfer, and ion transport proteins (Yabe, 2000; Edmondson, 2002; Baines, 2003).

An interesting and unexpected aspect of the study is the effect of substrate utilization on cardiac work in previously robustly protected aPKC ϵ hearts. The disparate ability of hypoxic aPKC ϵ hearts to produce cardiac work in the presence of glucose and moderately high fatty acids (palmitate) indicates that the presence of fatty acids or its breakdown products interfere with the aPKC ϵ cardioprotective signaling cascade. This would be expected to occur despite its ability to oxidize fatty acids at the same rate as wild types during normoxia and hypoxia. It is unclear what the precise mechanisms are whereby this occurs and further studies are required to delineate this interesting phenomenon. Since PGC1 α is integral to fatty acid metabolism and hypoxic aPKC ϵ hearts exhibit elevated PGC1 α peptide levels, this may potentially explain why aPKC ϵ hearts metabolize fatty acids as efficiently as WT, despite the reduced cardiac function.

However, all the parameters examined in this study indicate that aPKC ϵ hearts are preferentially glucose utilizing. Here aPKC ϵ hearts display: (a) higher rates of glucose oxidation in the presence of palmitate under both normoxic and hypoxic conditions; (b) a higher percentage contribution to ATP generation from glucose; (c) elevated protein levels

of glucose transporters (GLUT1 and GLUT4); and (d) greater HIF-1 levels—activated HIF-1 α activates hexokinase II, promoting glucose utilization (Lambert et al., 2010).

The results from this study thus highlight important pathological changes in wild-type mice and demonstrate phenotypic modifications operational following the modest activation of PKC ϵ . In wild-type mice, 2 weeks of hypobaric hypoxia results in the development of multiple cardiac deficits: reduced mitochondrial metabolism, mitochondrial biogenesis with myofilament/mitochondrial ultrastructural disarray, RV hypertrophy and a modest reduction in LV contractile function and work. In stark contrast the modest activation of PKC ϵ ameliorates these adverse ventricular responses to hypobaric hypoxia.

In conclusion, this study shows that modest and sustained activation of PKC ϵ abrogates both the mitochondrial energetic and numerical perturbations observed in response to chronic hypoxia, as well as conferring metabolic modulation. These data therefore suggest that the induction of PKC ϵ may have therapeutic potential to ameliorate the cardiac contractile response to hypoxia, possibly via a p-GSK3 β /HIF-1 α /PGC1 α signaling cascade.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The University of Cape Town Electron Microscopy Unit for assistance, Ms Sonia Genade for the working mouse heart perfusions and Dr. Heinrich Taegtmeier (Houston, TX, USA) for quantitative RT-PCR analysis performed in his laboratory. We further acknowledge the South African MRC and the South African National Research Foundation for financial support, and Dr. Uthra Rajamani for formatting of the figures.

Contract grant sponsor: South African MRC.

Contract grant sponsor: South African National Research Foundation.

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Fig. 1.

Comparison of mitochondrial function under normoxic and hypoxic conditions. a: State 3 and 4 are similar in mitochondria isolated from normoxic wild-type and aPKCε mice. b: The rate of ATP synthesis is initially enhanced in PKCε versus wild-type cardiac mitochondria (normoxic conditions) ($^{\dagger}P < 0.05$ vs. WT). c: Increased mitochondrial membrane potential in aPKCε heart mitochondria compared to wild types (under normoxic conditions) ($^{\#}P < 0.0001$ vs. WT). d: Following 14 days of hypoxia, state 3 respiration is sustained in aPKCε cardiac mitochondria but blunted in wild types ($^*P < 0.05$ vs. WT). e: Decreased rate of ATP synthesis in wild-type mitochondria versus aPKCε following 14 days of hypoxia ($^*P < 0.005$ vs. WT). f: Higher in inner mitochondrial membrane potential found in aPKCε mitochondria under normoxic conditions was sustained under hypoxic conditions ($^{**}P < 0.005$ vs. WT).

Fig. 2.

(a) ADP:O ratio is the amount of oxygen used to phosphorylate a fixed amount of ADP and is an indication of the efficiency and degree of coupling of mitochondria. Normoxic aPKCε mitochondria have similar ADP:O ratios to wild types. However, after 14 days of hypoxia, aPKCε mitochondria have adapted to use ~50% less oxygen to convert the same amount of ADP to ATP (* $P < 0.0005$ vs. matched WT control; $n = 6$). This demonstrates increased efficiency and coupling after the stress. (b) Oxidative phosphorylation-independent proton kinetics estimate proton leak across the inner mitochondrial membrane in the presence of oligomycin. Wild-type mitochondria are clearly disadvantaged by the chronic oxidant stress, as shown by ~300% increase in proton leak while aPKCε mitochondria remain unaffected (* $P < 0.0005$ vs. matched WT control; $n = 6$).

Fig. 3. Electron microscopic assessment of ventricular ultrastructure. Under normoxic conditions the ratio of mitochondria to myofilaments was similar in the wild-type (WT) left ventricles (a) and aPKCε ventricular tissue. At 7 (c) and 14 (e) days of hypobaric hypoxia the number of mitochondria increased several-fold in ventricles of wild-type mice with disruption of the mitochondrial to myofilament ratio. By contrast, the number of mitochondria and the ratio to myofilaments remain unchanged in aPKCε ventricles after 7 (d) and 14 (f) days of hypobaric hypoxia.

Fig. 4. mRNA transcript levels in hypoxic left ventricle after 14 days of hypobaric hypoxia. These changes were paralleled by similar transcript level changes in the right ventricle. (a) UCP3, uncoupling protein 3 (b) MHCβ, β myosin heavy chain (c) insulin independent glucose transporter, GLUT1. (* $P < 0.0005$ vs. WT normoxic, # $P < 0.0005$ vs. WT hypoxic, ◆ $P < 0.05$ vs. aPKCε normoxic, ** $P < 0.05$ vs. WT normoxic; $n = 4-5$).

Fig. 5.

Cardiac glycogen levels following chronic hypobaric hypoxia. Cardiac glycogen content in normoxic aPKCε hearts was significantly lower than wild types, whereas hypoxic aPKCε hearts displayed elevated glycogen levels, consonant with inhibition of GSK3β during hypoxia (^{#,*} $P < 0.05$ vs. WT normoxia and hypoxia, respectively).

Fig. 6.

Glucose oxidation in hearts perfused with glucose only—under normoxic conditions and in response to 14 days of hypobaric hypoxia. Glucose oxidation was elevated in aPKCε versus wild-type hearts at baseline ($0.09 \pm 0.01 \mu\text{mol/dry wt/min}$ vs. $0.29 \pm 0.09 \mu\text{mol/dry wt/min}$) but similar to wild types after hypobaric hypoxia ($0.16 \pm 0.03 \mu\text{mol/dry wt/min}$ vs. $0.18 \pm 0.02 \mu\text{mol/dry wt/min}$). Values were calculated at 40 min of perfusion (* $P < 0.05$ vs. WT; $n = 7-9$).

Fig. 7.

Glycolysis in hearts perfused with glucose only under normoxic conditions and in response to 14 days of hypobaric hypoxia. aPKCε hearts exhibited higher rates of glycolysis versus wild types (WT) during normoxia (2.2 ± 0.25 $\mu\text{mol/dry wt/min}$ vs. 3.19 ± 0.46 $\mu\text{mol/dry wt/min}$), that was reduced with hypoxia, but not statistically significant (2.06 ± 0.2 $\mu\text{mol/dry wt/min}$ vs. 2.16 ± 0.2 $\mu\text{mol/dry wt/min}$). The rate of glycolysis in wild types was unaltered by hypoxia (values calculated at 40 min; n = 8–11).

Fig. 8.

Palmitate oxidation in the presence of glucose under normoxic conditions and in response to 14 days of hypobaric hypoxia. Palmitate oxidation rates were similar in aPKCε and wild types (WT) during normoxia (189.4 ± 20 nmol/g dry wt/min vs. 172.6 ± 17 nmol/g dry wt/min; $n = 14$). Despite elevated glucose oxidation rates during hypoxia, aPKCε hearts did not exhibit diminished palmitate oxidation rates as would be expected from the Randle hypothesis, that is, palmitate oxidation rates were similar to wild types (219 ± 14 nmol/g dry wt/min vs. 196 ± 16 nmol/g dry wt/min; $n = 8-9$, values taken at 40 min of perfusion).

Fig. 9.

Glucose oxidation in hearts perfused with glucose plus palmitate under normoxic conditions and in response to 14 days of hypobaric hypoxia. Despite the presence of palmitate, aPKCε hearts persistently oxidized more glucose under both normoxic (0.011 ± 0.004 vs. 0.051 ± 0.017) and hypoxic conditions (0.432 ± 0.12 vs. 0.845 ± 0.17) (values calculated at 40 min) (* $P < 0.05$ vs. matched WT controls; $n = 7-9$).

Fig. 10. Percentage contribution to ATP under normoxic conditions (palmitate/glucose perfusions). Fatty acid oxidation is the major contributor to ATP production under these conditions.

Fig. 11. Percentage contribution to ATP in response to 14 days of hypobaric hypoxia (palmitate/ glucose perfusions). aPKCε hearts display a shift from fatty acid to glucose oxidation with chronic hypoxia (* $P < 0.05$ vs. WT; $n = 8-9$).

Fig. 12. Nuclear extracts from hypoxic aPKCε hearts demonstrate increased protein levels of (a) phospho-GSK3β, (b) HIF1-α, and (c) PGC1α compared to wild types ($^{\#}P < 0.005$ vs. matched WT control, $^*P < 0.05$ vs. matched WT control, $^{\dagger}P < 0.05$ vs. aPKCε hypoxic; $n = 6$).

Fig. 13. Cytosolic extracts from hypoxic aPKCε hearts exhibit elevated protein levels of (a) HIF-1α and (b) phospho-GSK3β. Expression of (c) NRF1 and (d) PGC1α, downstream targets of HIF-1α, were also significantly increased in a PKCε hearts but not in wild types. e: GLUT4 peptide levels were also higher (* $P < 0.05$ vs. WT; # $P < 0.005$ vs. WT; $n = 6$).