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► **To cite this version:**

Karla Carvajal, Elham Zarrinpashneh, Ondrej Szarszoi, Frederic Joubert, Yoni Athea, et al.. Dual cardiac contractile effects of the alpha2-AMPK deletion in low-flow ischemia and reperfusion.. *AJP - Heart and Circulatory Physiology*, 2007, 292 (6), pp.H3136-47. 10.1152/ajpheart.00683.2006 . inserm-00151019

HAL Id: inserm-00151019

<https://inserm.hal.science/inserm-00151019v1>

Submitted on 10 Sep 2009

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Dual cardiac contractile effects of the alpha2-AMPK deletion in low-flow ischemia and reperfusion

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Running head: Role of AMPK α 2 in basal and ischemic heart function

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SUMMARY

In order to understand if $\alpha 2$ -AMPK isoform is a friend or a foe in the protection of the myocardium against ischemia-reperfusion injury, we studied the functional consequence of its deletion on the contractility, the energetics, and the respiration of the isolated perfused heart and characterized the response to low flow ischemia and reperfusion. Alpha2 deletion did not affect basal contractility, respiration and high energy phosphate contents despite a 2 fold reduction in glycogen content and a 3 fold reduction in glucose uptake. Low flow ischemia (LFI) increased AMPK phosphorylation and stimulated glucose uptake and phosphorylation in both $\alpha 2$ -KO and WT. The high sensitivity $\alpha 2$ -KO to the development of ischemic contracture was attributed to the constitutive impairment in glucose transport and glycogen content and not a perturbation of the energy transfer by creatine kinase (CK). The functional coupling of MM-CK to myofibrillar ATPase and the CK unidirectional fluxes were indeed similar in $\alpha 2$ -KO and WT. LFI impaired CK flux by 50% in both strains, suggesting that $\alpha 2$ -AMP does not control CK activity. Despite the higher sensitivity to contracture, the post ischemic contractility recovered to 60% of its control in both $\alpha 2$ -KO and WT. This could partly result from a low cost of contractility in the $\alpha 2$ -KO. In conclusion the $\alpha 2$ -AMPK is required for a normal glucose uptake and glycogen content which protects the heart from the development of the ischemic contracture but it is not required for optimal post ischemic recovery of systolic activity in the absence of fatty acids.

Key words: glucose uptake, energetics, rigor, creatine kinase, ^{31}P NMR spectroscopy, energetic cost of contractility

Introduction

AMPK is a major energetic sensor in the mammalian cell and regulator of cardiac metabolism, AMPK activation switches on the ATP synthesis pathways and inhibits the ATP consuming pathways (for recent reviews (14), (57)).

Despite numerous investigations, and as stated by (14) the question of “AMPK activation being an ally or an enemy in the protection against ischemic damage” is still controversial. On one side AMPK activation may be beneficial due to increased ATP production by stimulation of glucose uptake, glycogenolysis and glycolysis as well as fatty acid oxidation (14). As a result a chronic decrease in AMPK activity could induce energy deficiency and impair cardiac contractile function during an ischemia reperfusion challenge. However AMPK activation may be detrimental during post ischemic reperfusion due to the stimulation of fatty acid oxidation which inhibits glucose oxidation. It was indeed proposed that AMPK potentially contributes to post ischemic mechanical deficiency and reperfusion injury by uncoupling glycolysis from glucose oxidation (43): this would promote acidosis and could lead to overload the cell in sodium and calcium by sarcolemmal ionic exchanges. In this sense AMPK deficiency could be predicted to attenuate myocardial damage of the post ischemic heart. Activators of AMPK as AICAR are not fully selective, thus a knock out of the $\alpha 2$ catalytic unit, the main cardiac isoform is a model of choice for the study of the role of $\alpha 2$ AMPK in the cardiac response to ischemia.

The model of $\alpha 2$ -AMPK knockout, $\alpha 2$ -KO (55), results in complete abolition of the cardiac $\alpha 2$ activity without compensation by $\alpha 1$ isoform. In this mouse, $\alpha 2$ deletion accelerated the development of the ischemic contracture in global ischemia, but did not alter the post ischemic contractile recovery (58). An early rise in the ischemic contracture has already been observed in other transgenic models: the overexpression of dominant negative $\alpha 2$ kinase, DN (56) or the $\alpha 2$ kinase dead, KD, models (42). However, a major difference compared to our

accompanying study showing preserved contractile recovery (58), is the increased apoptosis and poor post ischemic contractility of the KD mutant which lead the authors to conclude that the activation of alpha2 AMPK plays an important protective role in limiting the damage of IR episodes.

The myocardium depends for its contractility on the continuous adequacy in the flux of ATP synthesis, ATP transport (mainly by creatine kinase) and ATP utilization (mainly by the ATPases of the myofilaments and sarcoplasmic reticulum). The ischemic contracture results from the imbalance between the anaerobic ATP synthesis and ATP utilization which inhibits the myofibrillar ATPase activity by increasing ADP concentration (52), although calcium overload may contribute to its development (47). The ADP concentration is controlled at the level of myofibrils by the activities of the ATPase and of the MM Creatine kinase (MM-CK) bound to the myofibrils in its vicinity (52). Early rise in contracture can thus be related to a higher energetic requirement of the ATPases, to a decreased rate of glycolysis, to a decreased glycogen content or glycogenolytic flux, to an impaired transfer of energy by creatine kinase or to a defect in bound MM CK (15), (51). Because activation of AMPK by ischemia stimulates glucose transport at the sarcolemma and glycolysis by activation of 6-phosphofructo-2-kinase (PFK2) leading to PFK1 stimulation (41), (35), the decreased in AMPK activation in the a2-KO might impair the stimulation of glycolytic ATP synthesis and be harmful to the myocardium.

During early reperfusion, AMPK helps fatty acid oxidation to predominate over glucose oxidation. AMPK phosphorylates and inactivates acetyl-CoA carboxylase (ACC), decreasing the concentration of malonyl-CoA, the inhibitor of fatty acid transport into the mitochondria. In this sense the decreased activation of AMPK may be beneficial to the post ischemic contractile function.

Our aim was to characterize the consequences of $\alpha 2$ -AMPK deletion on the basal and ischemic heart function and to investigate some of the mechanisms involved in its response to an ischemia reperfusion (I-R) insult. We chose the low flow ischemia (LFI) model which better simulates the conditions prevailing in an infarcted region than global ischemia: during an acute occlusion of the left anterior descending coronary artery collateral flow still represents 10-40% of the baseline flow in the infarct zone. Pyruvate was used as substrate because its oxidation is efficient in LFI (33) and moreover as an antioxidant pyruvate allows to study the role of AMPK $\alpha 2$ in conditions minimizing the complex and not fully understood interference of oxygen and nitrosyl radicals with AMPK activity.

The consequences of $\alpha 2$ deletion on the contractility, oxygen consumption and energetics were firstly assessed in basal normoxic condition: we found uncompromised energetics and performance of the myocardium. In order to characterize the response of the $\alpha 2$ -KO to low flow ischemia (LFI) and reperfusion we followed the time change of phosphorus metabolites and the stimulation of glucose transport and phosphorylation by LFI. Because Creatine Kinase (CK) has early been shown to be potentially regulated by AMPK activation both in vitro and in isolated skeletal muscle myofibrils (39); it could have been expected that AMPK deletion would alter its activity. However the deletion of $\alpha 2$ -AMPK had no consequence on the global CK flux, measured by NMR magnetization transfer in normoxia, on the ischemic impairment of CK flux or on the function of MM-CK bound to myofibrils. Finally our results suggest that the maintenance of contractile performance of the $\alpha 2$ -KO heart might be related to the lower energetic cost of its contractility.

Methods

Animals

Nine months old male $\alpha 2$ AMPK KO (-/-) and WT (+/+) were obtained by crossing heterozygote $\alpha 2$ AMPK (-/+) (55). The genotype of the offspring was checked by polymerase chain reaction PCR on DNA extracted from the tail. The animals were fed ad libitum a standard chow except for the estimation of glucose transport and phosphorylation in which the animals were fasted for 12 to 18 hours. The investigation was conducted in accordance with our institutional guidelines defined by the European Community guiding principles in the care and use of animals and French decree no. 87/848 of October 19, 1987. Authorizations to perform animal experiments according to this decree were obtained from the French Ministry of Agriculture, Fisheries and Food (no. 7475, May 27, 1997).

Perfused heart

Mice were anesthetized by intraperitoneal injection of urethane (2 mg/g). The heart dissection was performed in a solution containing low Calcium (0.4mM) and high glucose (15mM). The perfusion solution contained (in mM): Na 143; K 6; Ca 1.8; Mg 1; mannitol 1.1; Hepes 20, glucose (11 mM) and pyruvate (2 mM) and was oxygenated with 100 % O₂. pH was adjusted to 7.35 at 36.5 °C. Hearts were perfused at a constant flow of 2.5mL.min⁻¹ the average flow value measured in the same hearts perfused at a constant pressure (see (58)). To ensure adequate oxygenation and thermoregulation the silicon perfusion line was surrounded by circulating water continuously bubbled with 100% O₂ at 36.5°C down to the heart. A latex balloon inserted in the left ventricle chamber was inflated to maximal isovolumic condition of work (end diastolic pressure of 5-8 mmHg). The on-line measured parameters were heart rate (HR, in beats.min⁻¹), left ventricle systolic developed pressure (LVP), end diastolic pressure (EDP), coronary pressure (all pressure expressed in mmHg). Contractility was estimated as the rate pressure product (RPP, expressed in 10⁴.mmHg.beats.min⁻¹). Hearts were freeze clamped to measure their ATP, Phosphocreatine (PCr) and glycogen contents (in

nmol.mg.prot⁻¹) and to assess the state of AMPK phosphorylation at the end of control, LFI and reperfusion periods.

NMR spectroscopy

³¹P NMR spectra were acquired on a Inova Varian at 9.4 T wide bore magnet in 10 mm diameter tubes using a 80° pulse angle α , 4 K data points acquisition, a spectral width of 10000 Hz, an acquisition time of 0.205 s, a repetition time of 2s, 32 scans, zero filling to 8 K and line broadening of 30 Hz. After 10 min of equilibration, 4 partially saturated spectra and a fully relaxed spectra (repetition time 10s) were acquired. Stability of the preparation was checked by comparing control spectra acquired before and after the magnetization transfer experiment: any heart showing more than 10 % variation in its metabolite content was discarded. In a pilot study we found the kinetics of depletion of PCr during global normothermic ischemia to be too fast for accurate detection and PCr content too low to allow the determination of CK flux. WT and KO hearts, freeze clamped in control, showed similar ATP contents measured by spectrofluorimetry, the average ATP content, (19.0 ± 0.5 nmol.mg prot⁻¹, n=10) was used as a reference for the NMR quantification of PCr, Pi and 2DG6P concentrations after correction for incomplete magnetic relaxation as described previously (23). A cytosolic water volume of 2.72 μ L. mg. protein⁻¹ was used to calculate the concentrations. Free cytosolic ADP was calculated from the equilibrium of CK (apparent equilibrium constant $K_{eqCK} = 166 \cdot 10^{-0.87(pH-7)}$). Free AMP concentration was $[AMP] = [ADP]^2 \cdot K_{eqAK} / [ATP]$ (using the adenylate kinase equilibrium constant $K_{eqAK} = 1.05$ (31) and the free energy of ATP hydrolysis was $A_{ATP} = A_0 + RT \ln [ATP] / [ADP] \cdot [Pi]$.

Kinetics of Creatine kinase

CK forward flux (PCr \rightarrow ATP) was assessed by Time Dependent Saturation Transfer (TDST) of γ ATP under control as previously described (6), [(49). Briefly 24 scans fully

relaxed spectra repetition time =10s) were acquired by blocs of 8 scans cycling 3 times through the whole protocol. Six spectra acquired with γ ATP saturation (duration 0 to 4s) allowed measuring the PCr relaxation time, T_{1PCr} and the apparent rate constant k_{for} of the CK reaction. The average control T_{1PCr} values were similar in both groups (3.1 ± 0.5 and 3.2 ± 0.4 s in WT, n=12 and KO, n=11 respectively). To reduce experimental time and because T_{1PCr} is unchanged by low flow ischemia, steady state saturation of γ ATP was applied in low flow ischemia. k_{for} was determined from the ratio $M_{ss}/M_0 = 1/[1+k_{for} T_{1PCr}]$ (M_{ss} and M_0 were the magnetization of PCr in the presence and absence of a 4s saturation of γ ATP, $T_{1PCr} = 3.1$ s). CK forward flux, the product of k_{for} and PCr concentration, was expressed in $mM.s^{-1}$. Glucose transport and phosphorylation was assessed by following the transport and phosphorylation of 2-D-deoxyglucose (2DG, 5mM) in presence of pyruvate (5mM). Inorganic phosphate (1mM) was added to the perfusate to prevent phosphorus depletion occurring in this protocol. After 25 min of control perfusion, 2DG was infused and the rate of apparition of 2-Deoxy-D-glucose-6-phosphate (2DG6P) was assessed during 15min (7 spectra)

Low flow Ischemia protocols

After 10 min of equilibration in isovolumic condition of work, the stability of metabolite content was checked for 15 min and CK flux assessed by TDST followed by 2 control spectra. Low flow ischemia was induced by reducing flow to 10% of its control (LFI_{10}) for 30 min to assess the time change of phosphorus metabolites and pH_i . Metabolic steady state was reached after 12min of LFI_{10} and CK flux measured by SSST followed by 2 control spectra. Some of the hearts were freeze clamped to estimate AMPK phosphorylation. Flow was further reduced to 5% of control (LFI_5) for 15 min and restored to its control value for 35 min (R: reperfusion) before freeze clamping (KO, n=6; WT, n=6). For the determination of ischemic glucose transport and phosphorylation, the same hearts used to

determined control 2DG6P accumulation were submitted to LFI₁₀ for 25 min (10 spectra) to assess ischemic stimulation in each individual heart.

Respiration and energetic yield

Oxygen consumption (QO₂) was measured in parallel experiments out of the magnet using the same LFI reperfusion protocol. QO₂ was measured on line from the difference in oxygen content in incoming (aortic PaO₂) and outgoing (pulmonary artery, PvO₂) perfusate using oxymeters (Stratkhelvin Inst., Glasgow) and Clark electrodes equipped with fast O₂-permeable membranes in thermoregulated chambers. QO₂ was expressed in $\mu\text{moleO}_2 \cdot \text{min}^{-1} \cdot \text{g WW}^{-1}$. The energetic yield was estimated by analyzing the relationship of steady state QO₂ to RPP in the same protocol of ischemia-reperfusion (WT, n=6; KO, n=6). To assess a wider range of contractile performance, a group of 6 WT and 6 KO hearts was submitted to beta stimulation by stepwise increases in isoprenaline concentrations (namely [ISO] = 10⁻¹⁰M, 10⁻⁹M, 3.16.10⁻⁹M, 10⁻⁸M, 3.16.10⁻⁸M and 10⁻⁷M). This group was perfused under constant pressure (75mmHg) at a lower external Ca concentration (Ca_o=1mM) to prevent calcium overload and fibrillation. Coronary flow was continuously measured by a T106 flowmeter equipped with a 1N probe (Transonic System Inc, Ithaca, NY USA). Steady state values of RPP and QO₂, obtained 10 min after the change in [ISO], were used to estimate the energetic yield by the linear regression between QO₂ and RPP

Intrinsic contractile properties of myofilaments

Mouse hearts were perfused under control condition (n= 4) or submitted to 10 minutes global ischemia after stabilization (n=4). Muscle fiber bundles were dissected from LV papillary muscles in a relaxing solution (pCa= -log 1/[Ca] = 9, see solutions below), incubated for 45 min at 4 °C with 1% Triton X-100 to solubilize the membranes and transferred to

relaxing solution at 4 °C until use. For ischemic fibers preparation and experiments, the solutions contained okadaic acid (100 nM) to inhibit phosphatases. After connection to a force transducer (model AE 801, SensoNor Microelectronics), sarcomere length was adjusted to slack length and stretched by 20%. All experiments were performed at 22 °C. Solutions, prepared as previously described (52), contained (in mM): EGTA: 10; imidazole: 30; Na⁺: 30.6; Mg²⁺, 3.16; dithiothreitol: 0.3. Ionic strength was adjusted to 0.16 M with potassium acetate, and pH to 7.1 with acetic acid. Relaxing solutions contained MgATP: 3.16 mM, PCr: 12 mM pCa: 9. Activating solutions contained same metabolites and pCa ranged from 9 to 4: active force and sensibility to Ca was determined under isometric conditions by stepwise decrease in pCa until maximum tension was reached. Rigor solutions contained ATP ranging from pMgATP 6 to 2.5 at pCa 9. Rigor force and sensitivity to ATP were assessed by stepwise decrease in pMgATP while MM CK functional activity was assessed by the same protocol in the presence of PCr 12mM to activate myofibrillar bound CK.

Tension was expressed in mN.mm⁻² and data were fitted using a nonlinear fit of the Hill equation (Microcal Origin software, version 6.0).

Expression and phosphorylation of AMPK

Freeze-clamped hearts were kept in liquid nitrogen until their homogenization in a lysate buffer containing TRITON X-100: 0-1 %, and in mM HEPES: 50, KCl: 50, EDTA: 1, EGTA: 1, beta-glycerol-phosphate: 5, orthovanadate: 1, DTT: 1, NaPPi: 5, PMFS: 2, and a cocktail of protease inhibitors (Calbiochem Set V EDTA free). Fifty µg of protein were used for western blotting detection of AMPK isoforms using the polyclonal Phospho-AMPK-α (Thr172) and AMPK-αpan, antibodies (Cell Signaling) diluted at 1:500 and 1:1000 respectively. Samples were resolved by 8% SDS-PAGE, transferred to PVDF membranes which were blocked with 3% skimmed milk and then probed overnight at 4°C with the

antibody. HRP conjugated-donkey anti-rabbit secondary antibody (Santa Cruz) was used for further detection by enhanced chemiluminescence (AMS). Sample loading was confirmed by Coomassie dyeing of the gel after the transfer process. Quantification of signals was performed using the Quantity-One software from Bio-Rad and arbitrary units were standardized by loading a standard sample in every separated gel. For Glut1 and Glut4 detection, 20 μg of protein of total conventional extracts were probed with polyclonal anti Glut1 and Glut4 antibodies (Chemicon) diluted at 1:1000.

Statistical analysis

All results are shown as mean \pm sem. T test was used to compare WT and $\alpha 2$ -KO. Variance analysis followed by a Student Newman Keuls test was used to compare control, LFI and reperfusion in each group. A value of $p < 0.05$ was considered as significant.

RESULTS

Cardiac phenotype function and energetics of the $\alpha 2$ -KO hearts

As also found in younger mice (58) deletion of the $\alpha 2$ subunit did not modify the expression of $\alpha 1$ protein, the $\alpha 2$ subunit was not detectable and the total AMPK protein level was reduced by about 60% (not shown) in agreement with the known cardiac distribution of α -isoforms. The characteristics of the animals were similar and no sign of cardiac hyper- or hypotrophy was found in the $\alpha 2$ -KO old mice (Table 1A) Thus, aging did not reveal morphological consequences of the $\alpha 2$ AMPK deletion in contrast with other transgenic models (36),.

The contractile parameters, left ventricular developed pressure (LVP), heart rate (HR), rate pressure product (RPP) and the coronary pressure were similar in AMPK KO and WT

hearts (Table 1 B). The energetic status of the $\alpha 2$ -KO did not differ from that of the WT heart (Table 2), although a slight non significant reduction in PCr could be observed in the former. ATP, Pi, pHi, as well as the calculated indexes: free ADP and AMP concentrations and the free energy of ATP hydrolysis ($-A_{ATP}$) were similar in both strains. Oxygen consumption (QO₂) was also similar both under constant flow or constant pressure perfusion conditions (see further). However glycogen content was about two fold lower in the $\alpha 2$ -KO than in the WT. This shows that, apart from decreasing the glycogen reserve, the deletion of the $\alpha 2$ catalytic subunit had no major effect on the normoxic contractile respiratory and energetic status.

Contractile and metabolite changes in low flow ischemia and reperfusion

The acceleration of the global ischemic contracture reported in the accompanying paper in the $\alpha 2$ -KO could result from a defect in the anaerobic ATP synthesis pathways (glycolytic or glycogenolytic), and/or in the energy transfer or buffering by creatine kinase. Indeed both events inhibit the myofibrillar ATPase activity by increasing ADP concentration (52). To understand the factors contributing to the acceleration of the ischemic contracture we used a model of partial ischemia, reducing flow to 10% (LFI₁₀) and then to 5% (LFI₅) of its control value, and followed the time course changes in phosphorylated compounds and pHi in WT and KO hearts. The immediate drop in RPP induced by LFI₁₀ (resulting from both decreased LVP and HR, not shown) was similar in WT and $\alpha 2$ -KO (Fig. 1). The end diastolic pressure rose in 4 out of 9 $\alpha 2$ -KO heart but in none of the WT heart. Due to the variability of EDP rise in the $\alpha 2$ -KO heart, the average EDP during moderate LFI₁₀ was not significantly different in both groups.

In terms of phosphorus compounds, the onset of LFI₁₀ resulted in an immediate drop in [PCr] followed by the establishment of a new steady state after about 10 min (allowing to

measure CK flux, see further). Steady state [PCr] was not significantly different in a2-KO and WT (Fig. 1), although its percent decrease was less pronounced in the α 2-KO (down to $38 \pm 5\%$ and $55 \pm 4\%$, $p < 0.05$ respectively, Fig. 2). [ATP] decreased monotonously to about 70% of its control value in both strains and a similar moderate acidosis occurred (pH_i was 6.87 ± 0.05 in KO and 6.91 ± 0.09 in WT, ns). Despite these discrete changes, the PCr to ATP ratio was less affected by ischemia in KO than in WT (1.38 ± 0.05 and 0.92 ± 0.09 , respectively $p < 0.001$) and thus the free [ADP] increased largely in WT ($146 \pm 21 \mu\text{M}$) compared to a2-KO ($82 \pm 6 \mu\text{M}$, $p < 0.01$, Fig. 2). Free AMP rose about 2 fold in a2-KO compared to 12 fold in WT (to $1.4 \pm 2 \mu\text{M}$ and 4.4 ± 0.9 respectively $p < 0.01$) The free AMP to ATP ratio increased by 3 fold and 17 fold, respectively. The glycogen content measured at the end of LFI₁₀, was significant lower in a2-KO than in WT (3.4 ± 0.8 and $9.7 \pm 1.5 \mu\text{mol glucosyl unit.gFW}^{-1}$, $p < 0.01$). Due to remnant pyruvate oxidation, glycogen depletion was modest and the rate of its utilization appears similar in both groups (about $0.12 \mu\text{mol glucosyl unit.gFW}^{-1} \cdot \text{min}^{-1}$).

To increase the metabolic challenge, flow was further reduced to 5% of control (LFI₅). A significant ischemic contracture developed in all a2-KO hearts but only in one of the WT: the average EDP rose by 28 ± 8 and 5 ± 2 mmHg respectively at the end of the 15min LFI₅ period, ($p < 0.05$, Fig. 1). Metabolic changes were exacerbated in both a2-KO and WT: lower [ATP] and pH_i occurred in a2-KO (Fig. 1 and 2). As for moderate LFI₁₀ ischemia, both the accumulation of ADP and AMP as the increase in AMP to ATP ratio were modest in the α 2 KO compared to the WT heart (Fig 2). For example free [AMP] rose to $1.8 \pm 0.2 \mu\text{M}$ in a2-KO vs $6.7 \pm 1.6 \mu\text{M}$ in WT ($p < 0.01$). The lower global adenylate content observed in the a2-KO, suggests the existence of a larger cellular efflux of adenylate moieties probably contributing to the lower ADP and AMP concentrations of the a2-KO heart.

Upon reperfusion EDP further rose in both strains. Despite the exacerbation of ischemic contracture, post ischemic systolic activity recovered to similar levels in both a2-KO

and WT (to about 60% of control, Fig. 1). ATP recovery was insignificant, as expected from the leakage of purine bases occurring during ischemia and from the slow rate of purine biosynthesis in the myocardium. The time course of PCr and pHi recovery appeared slower in $\alpha 2$ -KO than in WT. At the end of reperfusion period, the ATP concentration, the sum of adenylates and pHi were significantly lower in $\alpha 2$ -KO than in WT and did not recover to their control value in $\alpha 2$ -KO. AMP recovery was complete in both strains. The ischemia-recovery protocol (I-R) induced similar leakage of cytosolic enzymes (LDH and AK) in both strains while the mitochondrial citrate synthase activity was unchanged (Table 4).

Creatine kinase activity and flux

To understand if alterations of creatine kinase fluxes participate in the early development of contracture, CK function was evaluated. The specific activity of the enzymes involved in energy transfer (CK, and adenylate kinase, AK) was unaltered by the $\alpha 2$ deletion (Table 4). The unidirectional forward CK flux (PCr \rightarrow ATP) was measured by ^{31}P NMR saturation transfer in control and LFI₁₀ (Fig. 3A). In control, the apparent rate constant k_f and the CK forward flux were similar in $\alpha 2$ -KO and WT (average CK flux ca. 8.5mMole.s⁻¹). LFI₁₀ induced a similar 50% impairment of CK flux in both strains. The decrease in CK specific activity was expected from the well known leakage of cytosolic CK at reperfusion (Table 4). Thus the deletion of the $\alpha 2$ -AMPK did not alter global control and ischemic CK fluxes, suggesting that the regulation of CK is not critically dependent on the presence of $\alpha 2$ AMPK isoform or on the ischemic stimulation of AMPK activity.

However, the preservation of global CK flux does not imply unaltered subcellular CK function (24). Indeed the function of the MM-CK isoform bound to the myofilaments controls the local ADP concentration responsible of the formation of rigor bonds in ischemia (53). Because AMPK co-localizes with MM-CK in myofibrils and was early suggested to control

its activity by phosphorylation (39), the absence of the $\alpha 2$ -isoform could directly affect the rigor properties of the myofibrils. The consequence of the $\alpha 2$ deletion on the activity of MM-bound CK and the rigor properties of the myofibrils were assessed in triton-skinned fibers isolated from normoxic or ischemic perfused hearts. Figure 3B shows the rigor force induced by decreasing MgATP concentration and the sensitivity to ATP, (characterized by $pMgATP_{50}$: the $pMgATP$ inducing half rigor force development). The sensitivity to ATP of the rigor force was unaffected by $\alpha 2$ -deletion ($pMgATP_{50}$ was similar in WT and KO, Table 4). An index of the functional activity of MM CK activity bound in the vicinity of the ATPase is the shift in the apparent $pMgATP_{50}$, induced by addition of PCr (54). The addition of PCr induced a similar shift of $pMgATP_{50}$ in WT and $\alpha 2$ -KO (fig 3B, Table 4) showing that the absence of $\alpha 2$ AMPK did not modify the functional activity of myofibrillar bound CK. Contractile properties of fibers isolated from ischemic WT and $\alpha 2$ -KO were similar to those of their normoxic control (not shown). In conclusion our results show that the acceleration of the ischemic contracture in the $\alpha 2$ -KO is not due to an inhibition of the energy transfer and/or buffering by CK or to an alteration of the function of MM-CK bound in the vicinity of myofibrils.

Glucose uptake and phosphorylation

Because AMPK, as a metabolic switch, is involved in the control of glycolytic activation under stress, we assessed the consequence of $\alpha 2$ deletion on glucose transport and phosphorylation by following the phosphorylation rate of 2-deoxy-D-glucose by ^{31}P NMR spectroscopy in control and under LFI₁₀. Figure 4A presents a series of spectra obtained from representative WT and $\alpha 2$ -KO hearts perfused at normal flow before (spectrum a) and various time after the addition of 2DG 5mM (spectra b, c, and d). In control flow, the accumulation of 2DG6P was 3 fold slower in $\alpha 2$ -KO than in WT (0.2 ± 0.1 and 0.6 ± 0.1 nmol.min⁻¹.mg prot⁻¹

respectively $p < 0.05$, Fig. 4B). Each individual heart was then subjected to 15 min of LFI₁₀. The rate of 2DG6P accumulation increased in both strains albeit to a lower level in a2-KO than in WT (0.6 ± 0.2 and $1.5 \pm 0.2 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg prot}^{-1}$, respectively $p < 0.01$). Notice however that the 3 fold ischemic stimulation of 2DG transport and phosphorylation was similar in both mice (Fig. 4B). The defect in glucose transport and/or phosphorylation in the control $\alpha 2$ -KO is not due to a reduction in the expression of glucose transporters Glut 1 and Glut4 (Fig. 4C). Thus, the a2 AMPK isoform is required for a normal glucose uptake and glycogen content under normoxic condition although the stimulation of glucose uptake occurring during ischemia seems to be independent on the presence of the a2 isoform. In conclusion both the impaired glucose transport and phosphorylation and the low content in glycogen could account for the early rise in ischemic contracture of the heart deleted in a2-AMPK.

Phosphorylation of AMPK

Figure 5A shows western blot of total AMPK and AMPK phosphorylated in WT and a2-KO hearts under control condition as well as the stimulation of phosphorylation by LFI₁₀ (both in the presence or in the absence of 2DG). In control condition, a very low basal phosphorylation of total AMPK was found in a2-KO compared to WT. Low flow ischemia (LFI₁₀) significantly increased AMPK phosphorylation in both a2-KO and WT (Fig. 5C). However phosphorylation of AMPK by LFI₁₀ was 3 fold lower in a2-KO than in WT and 6 fold lower in LFI in the presence of 2DG. Upon reperfusion, AMPK phosphorylation decreased in both strains, remaining slightly higher than control in the a2-KO.

Energetic cost of contraction

To understand how the contractility of the $\alpha 2$ -KO heart can be similar to that of a WT, we assessed their energetic cost of contraction. Indeed one of the strategies of the myocardium submitted to a chronic stress is to improve the economy of its contraction (44). The oxygen consumption (QO_2) was continuously measured in a protocol of LFI-reperfusion performed out of the magnet (Fig. 6A). Contractile response, RPP and rise in EDP, were similar to those described in Fig. 1. QO_2 in basal condition although slightly lower in $\alpha 2$ -KO was not significantly different from WT (5.1 ± 0.4 and $6.6 \pm 0.6 \mu\text{molO}_2 \cdot \text{min}^{-1} \cdot \text{gWW}^{-1}$, respectively). QO_2 during LFI₁₀, LFI₅ and reperfusion were also similar in both strains. The energetic cost of contractility (estimated by the slope of the relationship between QO_2 and contractility) tended to be lower in $\alpha 2$ -KO than in WT, but the difference did not reach significance. To analyze more precisely this relationship, higher contractile performance was induced by β -stimulation. Because perturbation of the urinary excretion of catecholamines was previously described in this model (55), we first verified that the $\alpha 2$ deletion did not alter the response of the myocardium to catecholamines. The sensitivity to isoprenaline (ISO) was similar in both strains as shown in Fig. 6 B (pooled value of $EC_{50} = 4.9 \pm 0.5 \cdot 10^{-9} \text{M ISO}$). Maximal stimulation, obtained in presence of $3 \cdot 10^{-8} \text{M ISO}$, resulted in non statistically different values of RPP (10.3 ± 0.7 and $8.3 \pm 0.8 \cdot 10^4 \text{ mmHg} \cdot \text{beat} \cdot \text{min}^{-1}$ in $\alpha 2$ -KO and WT respectively) and QO_2 (18.5 ± 2.9 and $20.5 \pm 2.5 \mu\text{molO}_2 \cdot \text{min}^{-1} \cdot \text{gWW}^{-1}$). Thus, the $\alpha 2$ -KO perfused in presence of glucose and pyruvate was not limited in its energy production. However, the energetic cost of contraction, estimated from the slope of the linear relationship between oxygen consumption and RPP was significantly lower in $\alpha 2$ -KO than in WT (Fig. 6 C, see legend for details). This favors the hypothesis that the $\alpha 2$ -AMPK deletion induced an improvement in the economy of contraction

In order to improve the economy of its contraction, a rodent heart under chronic stress expresses slow isoform of myosin-ATPase, which consumes less ATP per force unit, or might

change the activation properties of its ATPase. The isomyosin profile of the WT, shows a pure V1 phenotype (100% fast type, WT n=9) as expected in a mouse heart, and this profile was unaltered by the $\alpha 2$ -AMPK deletion ($\alpha 2$ -KO n= 8). The active tension and the calcium sensitivity of the ATPase studied in triton skinned fibers are shown in Table 4. Both the maximal active tension of myofibrils (at pCa 4.5) and the sensitivity of myofibrillar ATPase to calcium activation were similar in $\alpha 2$ -KO and WT (the half activation, pCa₅₀, was 5.64 ± 0.03 in $\alpha 2$ -KO and 5.55 ± 0.03 in WT, ns). In conclusion the improvement in the economy of contraction observed in $\alpha 2$ -KO did not result from adaptations of the contractile machinery.

DISCUSSION

The basal cardiac energetic status, contractility and respiration are unaffected by $\alpha 2$ -AMPK deletion. We found a dual effect of the $\alpha 2$ AMPK deletion on the contractile and metabolic response of the myocardium submitted to a low flow ischemia-reperfusion episode. We confirm that the $\alpha 2$ AMPK is more susceptible to the development of an ischemic contracture. We show that this acceleration of contracture can be explained by a deficit in energy synthesis resulting from both a constitutive impairment of glycogen stores and a decreased glucose uptake and phosphorylation and not by an impairment of energy transfer. The unaltered energy transport by creatine kinase and function of the MM bound CK, suggest that the $\alpha 2$ AMPK does not control creatine kinase in the myocardium. A novel finding is that the $\alpha 2$ -AMPK deletion does not alter the post ischemic contractile function of an isolated heart perfused in presence of glucose and pyruvate.

Contractility and energetics of the normoxic heart

Despite the 3 fold decrease in glucose uptake and phosphorylation observed in presence of pyruvate (Fig. 4), the $\alpha 2$ AMPK deletion did not induce contractility defect as also observed *ex vivo* and *in vivo* in the same transgenic model (58). This shows that the presence of $\alpha 2$ -AMPK isoform is needed for optimal normoxic glucose transport and phosphorylation, although it is not required for the expression of glut transporters (Fig. 4). The similarity of the energy status of the $\alpha 2$ -KO and WT heart indeed shows that the balance of energy utilization and synthesis is unaffected by $\alpha 2$ deletion: thus the $\alpha 2$ -KO heart is not an energetically starved myocardium. One could argue that the contractility of the isolated isovolumic perfused heart is low compared to the condition prevailing *in vivo*. However steady state high levels of contractility (similar to that of a WT heart) can be supported even

at maximal level of β -stimulation when pyruvate and glucose are available (Fig. 5). A novel interesting observation is that the $\alpha 2$ -deletion induced an increase in the economy of contraction. We showed that this does not result from the usual strategies developed by the pathological rodent heart which tends to reduce the cost of its myofibrillar ATPase by expression of a slow type myosin. Thus the $\alpha 2$ -AMPK is not involved in the expression of myosin or regulatory contractile proteins (as suggested by the similarity of Ca sensitivity of the myofibrillar ATPase). Further investigation is needed to understand if the economical contraction of the $\alpha 2$ -KO results from a modification in Ca homeostasis, in excitation contraction coupling and/or in mitochondrial efficiency.

Alpha2 AMPK protects the heart from the ischemic contracture

LFI differs from global ischemia in two major ways. Degradation products (mainly lactate, protons, adenosine...) are washed out of the cell enabling continuation of glycolytic ATP production. Besides, due to the remnant oxygen supply, mitochondrial substrates oxidation still occurs: even at very low flow (down to 3% of control), as much as a third of the total ATP production originates from oxidative metabolism (33). Thus, both glycolytic and oxidative ATP supplies concur to less severe bioenergetics defect in LFI than in global ischemia which results in cessation of contractility, huge rise in end diastolic pressure and drastic changes in high energy phosphates. A 30 minutes period of flow restriction down to 10% induced a moderate decrease in PCr, ATP, and pHi (as previously reported in the rat heart (9) and rapidly resulted in a new metabolic and contractile steady state in both WT and $\alpha 2$ -KO. The increased susceptibility for the development of an ischemic contracture observed in global ischemia (see (58)) was also evidenced in the low flow model (Fig. 1A): EDP rose in the $\alpha 2$ -KO hearts and hardly in WT. The development of the ischemic contracture results mostly from an imbalance of the energy homeostasis leading to accumulation of ADP at the

level of myofibrils, inhibition of ATPase and development of a rigor state (52). Neither the sensitivity of myofilament to ATP nor the efficacy of bound creatine kinase was altered in $\alpha 2$ -KO. In this model calcium overload may contribute to the development of the contracture (47), the Ca homeostasis was not assessed in the $\alpha 2$ -KO hearts, thus a perturbation in the activity of the Na, Ca and proton sarcolemmal exchanges or of the sarcoplasmic reticulum (SR) Ca ATPase, can not be excluded. Indeed the abundance of glycogen in the vicinity of SR, and the requirement of glycogenolytic and glycolytic activities for optimal SR function (7) suggest that the consequence of the glycogen deficit on the SR function in the $\alpha 2$ -KO is an interesting field to be studied.

The imbalance in energy homeostasis can result from an increased energy demand, an impairment of energy transfer by creatine kinase and/or a deficit in the anaerobic ATP synthesis pathways. The hypothesis of an increased energy demand is eliminated by the similarity of LFI contractility in $\alpha 2$ -KO and WT (Fig. 1 and 6 A) and the absence of change in the intrinsic contractile properties of the myofibrils (Table 4). The similarity of ischemic creatine kinase fluxes (Fig. 3A) eliminates the hypothesis of a deficit in energy transfer by CK. On the contrary, because AMPK has earlier been suggested to inhibit CK activity (39), lower phosphorylation of AMPK in the ischemic $\alpha 2$ -KO could have been expected to prevent the ischemic decrease in CK flux. This was not observed (Fig. 3A). Thus, provided the $\alpha 1$ isoform is not specifically implied in the regulation of CK flux, our results showed that the inhibition of CK by AMPK in the ischemic cardiac cell is of a minor physiological importance compared to other factors known to decrease global CK flux as for example acidosis or impairment of mitochondrial CK flux (18), (24).

Surprisingly, LFI induced in the $\alpha 2$ KO a high steady state PCr to ATP ratio which prevented a major free ADP (and AMP) rise in LFI₁₀. This could have resulted from an unbalanced subcellular CK fluxes. However this hypothesis is not supported. Neither the

expression of MM, MB, BB or mitochondrial isoform of CK, nor the coupling of MM-CK to myofibrillar ATPase (Fig. 3) or that of mito-CK to adenine nucleotide translocase (3) were altered by the $\alpha 2$ AMPK deletion. The ischemic free ADP and AMP concentrations, besides being controlled by CK and AK activities, also depend upon the activity of the enzymes of the AMP degradation pathways (AMP deaminase and 5'-nucleotidase). Except in global ischemia, in which the degradation products can not be extruded from the cell, an inhibition of respiration (by hypoxia, chemical inhibition or LFI) rapidly releases purine bases in the cardiac effluent. This results from AMP hydrolysis and rapidly follows the initiation of ATP depletion (10), (19). This mechanism is thought to be beneficial in short term hypoxia (28). The sum of adenylates (ATP+ADP+AMP) was indeed significantly lower in $\alpha 2$ -KO than in WT at the end of LFI (4.4 ± 0.4 vs 6.4 ± 0.4 mM, $p < 0.01$). This suggests that the low ADP and AMP concentrations found in the $\alpha 2$ -KO (Fig. 2) might result from an increase in the degradation pathways. It is thus an interesting possibility, which to our knowledge has not been explored yet, that the $\alpha 2$ AMPK subunit might interfere with the regulation or the expression of enzymes involved in the AMP degradation pathways.

Thus in the absence of alteration in energy utilization and transfer, the energetic imbalance mostly results from a deficit in energy synthesis. Indeed both a decrease in glycolytic and in glycogenolytic ATP production accelerate the low flow ischemic contracture in the rat heart (12). A major deficit in the basal glycogen content of the $\alpha 2$ -KO was observed (Table 2 and by (58)). As discussed in the accompanying paper, because the AMPK glycogen binding domain plays a critical role in glycogen storage, the deficit in $\beta 2$ -subunit of AMPK, together with decreased glucose uptake most probably contribute to the impaired glycogen storage of the $\alpha 2$ -KO. Indeed the deficit in glycogen storage, rather than in its rate of LFI utilization, is involved in the early development of contracture (Fig. 4). Similar results were

observed in the KD mutant while unaltered glycogen stores but a reduced ischemic mobilization was found in the DN mutation (42), (56). The increase in glucose transport being a primary component of the glycolysis stimulation by ischemia, the slower glucose transport and phosphorylation in LFI (Fig. 4) as well as the marked decrease in lactate production found in global ischemia (58) shows that the $\alpha 2$ deletion impairs anaerobic glycolysis. As ischemia was still able to stimulate glucose uptake in $\alpha 2$ -KO (Fig. 4B), the $\alpha 2$ isoform is not required for the ischemic stimulation of glucose transport and phosphorylation and the $\alpha 1$ isoform is responsible for this remaining stimulation. This again differs from the model of DN over expression model, where the inactivation of $\alpha 2$ did not affect basal 2DG but blunted its ischemic stimulation (56). In conclusion, the decreased glycogen stores, the impaired glucose transport and/or phosphorylation both concurring to impaired anaerobic ATP synthesis in the $\alpha 2$ -KO myocardium, show that the presence of $\alpha 2$ AMPK is essential for the prevention of ischemic contracture.

$\alpha 2$ deletion does not impair ischemic and post ischemic contractility

We deliberately choose to use pyruvate as substrate because its oxidation is efficient in LFI, it enhances cardiac efficiency and also acts as an antioxidant (33), (34). In term of fuel utilization, pyruvate increases the activity of complex II and that of complex I by anaplerotic flux via oxaloacetate. Moreover, through its conversion to lactate, pyruvate could relieve glycolytic constraint due to NADH accumulation under ischemia (5). The antioxidant effect of pyruvate is well documented: exogenous pyruvate carboxylation produces citrate, citrate generating NADPH, maintains the glutathione system redox state in LFI (33),(34). Indeed pyruvate was shown to prevent cardiac dysfunction and AMPK activation induced by hydrogen peroxide in the isolated rat heart (32). Thus, pyruvate, by reducing the consequences of free radical production occurring massively at reperfusion, allowed the study

of the role of $\alpha 2$ -AMPK isoform in conditions that minimize the complex interference of oxygen and nitrosyl radicals with AMPK activity.

A major novel observation is that, despite the acceleration of the ischemic contracture, the $\alpha 2$ -AMPK deletion did not exacerbate the contractile consequences of an ischemia reperfusion episode in the heart perfused with glucose and pyruvate. At first sight surprising, such dichotomic effect has already been reported. Indeed, although the concept has been on debate for a long time, the ischemic contracture is not a good predictor of the postischemic recovery. For example, post ischemic contractility is improved, despite an acceleration of the development of contracture by preconditioning and depletion of glycogen stores (25), (26), (12). Our results are in the same line: the $\alpha 2$ AMPK deletion decreased glycogen, accelerated the contracture, while it did not affect postischemic contractile recovery albeit the depletion in ATP content. It must be mentioned that, although ATP depletion has been used as a predictor index of heart insult *in vivo* (38), there is no causal relationship between ATP depletion and contractile activity (37), (23), (4). Such dissociation is also shown here: the contractile recovery of the $\alpha 2$ -KO was normal despite its lower ATP content. In fact, the ATP concentration (around 4 mM) is sufficient to fully activate the ATPases because it is still 10^3 fold higher than their K_m for ATP. Indeed contractility depends on the turnover fluxes of ATP and not on its concentration (37), (4), (49). Thus despite a lower energetic status at reperfusion, the similarity of post ischemic contractility in $\alpha 2$ -KO and WT suggests similar recovery of ATP turnover. This is most probably related to the low cost of contraction in the $\alpha 2$ -KO. Because the isomyosin profile and the regulation by calcium of the active tension were similar in $\alpha 2$ -KO and WT hearts this economical contraction does not originate from a lower ATP requirement of the myofibrillar ATPase but rather from an improvement in the ATP generating steps. Several mechanisms could contribute to the higher efficiency of the mitochondria in $\alpha 2$ -KO. A shift in endogenous substrate utilization could increase the P/O

ratio or the intrinsic properties of mitochondrial respiration could be altered (3). Besides, because AMPK activation upregulates the uncoupling protein (UCP) in skeletal muscle (59), one can not currently exclude that the chronic impairment of AMPK activity could decrease UCP expression in the $\alpha 2$ -KO myocardium. Such mechanism would promote ATP synthesis because the activation of UCP at the onset of reperfusion increases QO_2 at the expense of ATP synthesis (22). Finally, because high adenosine levels improve the economy of contraction (20), the depletion in adenylate in the ischemic $\alpha 2$ -KO could increase coronary adenosine and contribute to its low cost contractility. Further work is required to test the origin of the mitochondrial efficiency of the $\alpha 2$ -KO myocardium.

Activators of AMPK

As discussed in detail by (58) the AMPK kinases-AMPK signaling pathway is sensitive to alteration in AMP concentration at various levels including AMPK activity and phosphorylation and AMPKK phosphatase. Moreover the activation of AMPK by AMP is antagonized by high concentrations of ATP explaining the sensitivity of the system to both AMP and ATP. The sensitivity to AMP and ATP depends on the type of α and γ isoform composing the heterotrimer (13); (48), (1). The estimation of the AMP (and ADP) concentration relevant for enzyme activation in a whole cell is not easy because their low concentrations prevent their NMR detection and the majority of the nucleotides are bound to intracellular compartments. The majority of the total AMP measured from a muscle extract, is bound in mitochondria and metabolically inactive (46). Alternatively free ADP and AMP concentrations are calculated from the NMR measured PCr ATP and H^+ concentrations in the hypothesis of CK and AK equilibrium. Quasi metabolic steady state is acceptable in our protocol, first because both CK flux, about 8mM/s and AK flux (40) are at least 10^3 higher than the higher rate of ATP degradation (estimated from Fig 1 in the ischemic $\alpha 2$ -KO to 1.5

$\mu\text{M/s}$) and the V_{max} of enzymes involved in AMP degradation (45), (21) and second because CK and AK had similar specific activities in both WT and KO (Table 3). Indeed the free [AMP] increase observed in our mouse heart perfused in low flow ischemia (from 1.5 to $7\mu\text{M}$) is in the relevant range of AMPK activation found in vitro and in the perfused mouse heart (48), (16). Obviously lower level of AMPK phosphorylation in ischemia is expected from the 60% decrease in total AMPK induced by $\alpha 2$ deletion. But in all our experimental conditions, in both $\alpha 2$ -KO and WT, the phosphorylation of AMPK was related to the change in AMP to ATP ratio (compare Fig. 2 and Fig. 6 C). This holds true for LFI₁₀. ILF₅ exacerbated the AMP/ATP changes and further increased phosphorylation in both strains and after reperfusion. Under global ischemia, lower level of AMPK activation is also found in the same $\alpha 2$ -KO heart but the accumulation of AMP in $\alpha 2$ -KO was higher than in WT (58). Both results, might appear at first sight contradictory, but this discrepancy is due to the difference between global ischemia, a close system where degradation products accumulate in the cell, and low flow ischemia, an open system resulting in metabolic steady state where AMP hydrolysis and washout of degradation products can occur.

Both PCr and the PCr to Cr ratio were early suspected to modulate AMPK activity (39), however more recent data evidence no direct regulation of AMPK or LKB1 by PCr (50). Thus the slightly higher PCr or PCr to ATP ratio of the $\alpha 2$ -KO is most probably insignificant in the control of AMPK phosphorylation. However dual mechanisms operate in the ischemic heart that regulates AMPKK mediated phosphorylation and activation of AMPK. The AMP independent increase in AMPK activity in hypoxia (17) and the activation of AMPKK independent of an AMP increase or a change in LKB1 activity in the mildly ischemic rat heart (2) could contribute to the phosphorylation of the $\alpha 1$ subunit in the $\alpha 2$ KO.

Is AMPK involved in cardiac protection or in cardiac injury?

The preservation of the ischemic and reperfusion function in the $\alpha 2$ -KO, showing that $\alpha 2$ AMP isoform is not needed for the postischemic contractile recovery, is at major discrepancy with the results obtained in the $\alpha 2$ KD model (42). The $\alpha 2$ -KD heart exhibits poor contractile function both during moderate low flow ischemia and reperfusion, associated with a major leakage of cytosolic enzymes and significant apoptotic response to I-R. A major difference between the two studies is the choice of substrates. We presented above our rationale for choosing pyruvate and glucose as substrate. The availability of long chain fatty acids in Russels' study (42) most probably contributes to the severe ATP depletion and massive cytosolic enzyme leakage associated with a mild reduction in perfusion flow (to 30% compared to 5% in our protocol). Although a physiological substrate, long chain fatty acids in normoxia decrease cardiac efficiency, activate AMPK, and increase phosphorylation of acetyl-CoA carboxylase (8), (27), (11). It has been proposed that during reperfusion, sustained activation of AMPK and inactivation of ACC mediates the acceleration of fatty acid oxidation and the uncoupling of glucose oxidation and glycolysis (29), (30). Besides the difference in substrate, two crucial discrepancies between the studies are puzzling. Ischemia, as expected, phosphorylated $\alpha 1$ -AMPK and stimulates glucose uptake in our model of $\alpha 2$ -deletion (Fig. 6 and (58)), as well as in the DN mutation (56) but surprisingly failed to do so in the KD mutation. The reasons for this lack of activation, which might participate in the severity of the ischemic insult, are not obvious but it is possible that the overexpression of inactive α subunit perturbs the heterotrimer α - β - γ association and the activation of AMPK. Before a definite conclusion about the beneficial or adverse role of $\alpha 2$ AMPK in ischemia can be drawn, it is indeed very important to understand if these opposite results are related to the available substrate, to the model of transgene insertion which might exert non specific effects, or to different adaptations often occurring in the various transgenic models.

Acknowledgment

Grants

This work is supported by Institut National de la Santé et de la Recherche Médicale, France, by a grant of the Fondation de France, by a grant from the Fonds National de la Recherche Scientifique et Médicale, Belgium and by the European Commission (grant QLG1-CT-2001-01488), E. Z. by the Fonds Spéciaux de Recherche, UCL, Belgium. LB is Research Associate of the Fonds National de la Recherche Scientifique, Belgium.

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FIGURE LEGENDS

Figure 1: Time dependent response to a low flow ischemia and reperfusion protocol: contractility and NMR measured phosphorus compounds

Top panels: NMR measured parameters .Concentrations of PCr and ATP (in mM), ratio of PCr to ATP and pH_i .

Bottom panel: mechanical activity. End diastolic pressure, EDP in mmHg; Rate pressure product, RPP in 10^4 .mmHg.beats.min⁻¹

C: control flow (WT, n=12, a2-KO, n=11); LFI₁₀ for 30 min. (n= 9); LFI₅ for 15 min, (n=6)

R: reperfusion in control flow (n=6) ? : WT ? : a2-KO. Significant difference between a2-KO and WT *: $p < 0.05$ (for clarity the difference with the control value is not shown, see result section)

Figure 2 steady state of some metabolites in low flow ischemia and reperfusion

Steady state: average of the last two spectra in each perfusion condition in hearts of Fig1, empty bars = WT, filled bar = a2-KO. Sum of adenylates = ATP + ADP + AMP.

Significant difference between WT and a2-KO: *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

Significantly different from the respective control group \$ $p < 0.05$; \$\$: $p < 0.01$; \$\$\$: $p < 0.001$.

Figure 3 Creatine kinase function

A- ³¹P NMR measured forward CK flux

CK forward flux during metabolic steady state under control flow (c: n=11) and low flow ischemia (LFI₁₀ n=9) in WT (empty bars) and a2-KO (filled bars). Significant difference between WT and a2-KO: *: $p < 0.05$. Significant difference with control group \$: $p < 0.05$; \$\$: $p < 0.01$; \$\$\$: $p < 0.001$.

B- Functional activity of myofibrillar MM-bound CK

Sensitivity of the rigor tension to ATP was similar in X100 triton fibers of a2-KO and WT hearts: half maximum rigor tension occurred at similar $pATP_{50}$. The efficiency of MM-bound CK (arrow), estimated from the shift in the apparent sensitivity to MgATP induced by the addition of 12mM PCr, was similar in both strains (see Table 4 for mean data)

Figure 4: ^{31}P NMR estimation of the glucose transport and phosphorylation and its stimulation by low flow ischemia

A- Stacked plot of NMR spectra showing the accumulation of 2DG6P as a function of time in a representative WT (left panel) and $\alpha 2$ -KO heart (right panel) perfused at control flow of 2.5 mL.min⁻¹. Spectrum a: control; spectrum b, c, d were acquired 2.5, 5, 7.5 min, respectively after infusion of 2-Deoxy-D-glucose (2DG= 5mM). Substrate: pyruvate 2mM in presence of external Pi 1mM, 5 min acquisition time for each spectrum

B- Rate of 2DG6P accumulation in control and LFI

Left panel: average rate of 2DG6P accumulation was slower in a2-KO than in WT both in control and under LFI₁₀. Significant difference between a2-KO and WT *: $p < 0.05$; **: $p < 0.01$. Significantly different from control: \$: $p < 0.05$

Right panel: stimulation of 2DG transport and phosphorylation by LFI₁₀ was similar in a2-KO and WT. expressed as the fold increase in 2DG6P rate of accumulation induced by LFI₁₀ for each individual heart

C- Expression of glut 1 and glut4 protein

Western Blot analysis. Empty bars WT n= 7, filled bar a2-KO, n= 7

Figure 5: AMPK and its phosphorylation in low flow ischemia in WT and KO

A- WB showing total AMPK protein and its phosphorylation at Thr172 in control condition and low flow ischemia (LFI₁₀) in absence (left) and in presence of 2DG (right)

B- Total AMPK protein in WT (empty bar) and a2-KO (filled bar)

C- Average level of phosphorylation of total AMPK in C: control (WT, n=4; a2-KO n=4), LFI₁₀: 30 min of low flow ischemia (WT n=7, a2-KO n=7), R: 35 min after reperfusion (WT n=4, a2-KO n=3) and LFI-2DG 15 min of 2DG + LFI₁₀ =15 min (WT n= 5, a2-KO n=6). Normalized unit: AMPK-P normalized to AMPK-P of a rat heart after 10 min of global ischemia.

Significantly different from control \$: p<0.05; \$\$ p<0.01; \$\$\$ p<0.001

Significant difference between a2-KO and WT *: p< 0.05; ** p<0.01; ***:p<0.001

Figure 6 oxygen consumption and the economy of contraction

A- Low flow ischemia, and reperfusion protocol. RPP (in 10^4 .mmHg.beats.min⁻¹) and oxygen consumption (in μmoleO_2 .min⁻¹.gWW⁻¹), EDP changes were similar to Fig. 1A (●: WT, n=6; ●: a2-KO, n=6)

B- Response of a2-KO and WT hearts to a β -stimulation induced by increasing concentrations of isoprenaline ranging from 10^{-10} M to 10^{-7} M. Steady state values obtained 10 minutes after the change in isoprenaline concentration (□: WT, n=6 ■: a2-KO, n=6).

C- Energy cost of contraction estimated from the linear relationship between QO₂ and RPP for both protocols. Data included both LFI and isoprenaline protocols Average linear regressions were described by $y = 2.26x + 0.91$, $r^2 = 0.9967$ in WT and by $y = 1.50x + 1.43$, $r^2 = 0.9907$ in a2- KO (where $y = \text{QO}_2$ and $x = \text{RPP}$). T test analysis (performed on the individual relationship of each heart) showed a significant difference between the slopes of WT and KO (n=12 for each WT and a2-KO, p<0.05) but no difference for the ordinate at origin .

Table 1 Comparison of the cardiac function of normoxic $\alpha 2$ -AMPK KO and WT hearts

Characteristics of the animals		
	WT, n= 56	$\alpha 2$ -KO, n= 60
Age month	8.9 \pm 0.5	8.1 \pm 0.5
Body weight g	36.5 \pm 0.9	35.2 \pm 0.7
Heart weight g	0.29 \pm 0.01	0.28 \pm 0.01
HW/BW mg. g ⁻¹	7.6 \pm 0.2	7.9 \pm 0.2

Cardiac function of the isolated heart perfused at constant flow		
	WT, n=24	$\alpha 2$ -KO, n=19
LVDP, mmHg	71 \pm 4	71 \pm 4
Heart rate, beats.min ⁻¹	319 \pm 17	292 \pm 16
RPP, 10 ⁴ .mmHg.beats.min ⁻¹	2.1 \pm 0.1	2.0 \pm 0.1
Coronary Pressure, mmHg.	103 \pm 8	112 \pm 9
EDP, mmHg	8 \pm 1	9 \pm 1

Hearts were perfused at a constant flow of 2.5mL.min⁻¹ in presence of pyruvate 2mM and glucose 11mM as substrate. Measured parameters were: heart rate, LVDP (left ventricular developed pressure), EDP (end diastolic pressure), coronary pressure and RPP (the rate pressure product). Significantly different from WT *: p<0.05.

Table 2 Energetic parameters of the basal control WT and $\alpha 2$ -AMPK KO heart

	WT , n=12	$\alpha 2$ -KO, n=11
ATP, mMoles.l ⁻¹	8.6± 0. 3	8.4 ± 0. 2
PCr, mMoles.l ⁻¹	16.2 ± 0.7	14.4 ± 0.6
Pi, mMoles.l ⁻¹	4.0 ± 0.5	3.6 ± 0.4
PCr/ATP	1.9 ± 0.1	1.7 ± 0.1
pHi	7.09 ±0.02	7.08±0.01
ADP, μ Moles.l ⁻¹	59 ± 7	68 ± 6
Δ ATP, kJ.M ⁻¹	-56.2 ± 0.4	-56.0 ± 0.5
AMP, μ Moles.l ⁻¹	0.48 ± 0.10	0.76 ± 0.22
AMP/ATP * 10 ⁵	0.56 ± 0.11	0.64 ± 0.08
Glycogen,		
μ mol glucosyl unit.gFW ⁻¹	12.2 ± 0.8	5.5 ± 0.7 ***

ATP, PCr, Pi and pHi were measured from ³¹P NMR spectroscopy after 30 min equilibration in normoxic perfusate containing pyruvate and glucose using the ATP measured biochemically as standard. Correction from partial saturation of NMR signal due to incomplete magnetization relaxation was applied. Free ADP, Free AMP and ΔA_{ATP} the free energy of ATP hydrolysis were calculated using the equilibrium constant of CK and AK. No significant difference was found in any of the energetic parameters except for the glycogen content (measured in WT, n=4 and $\alpha 2$ -KO n=5).

Table 3 Enzymatic activities

	Control		Reperfusion	
	<i>WT, n=3</i>	<i>a2-KO, n=3</i>	<i>WT, n=7</i>	<i>a2-KO, n=5</i>
Creatine kinase	507 ± 39	669 ± 47	273 ± 47 ^s	258 ± 39 ^s
Adenylate kinase	370 ± 12	389 ± 9	116 ± 16 ^s	104 ± 9 ^s
Citrate synthase	150 ± 17	133 ± 6	137 ± 10	140 ± 15
LDH	203 ± 19	211 ± 15	118 ± 8 ^s	104 ± 9 ^s

Data from freeze clamped hearts. Control obtained after 20 min of basal perfusion; Reperfusion: 35 min after LFI_{10 and 5} (hearts of Fig. 1). Activities are expressed in UI. g FW⁻¹.
^s: significantly different from control p < 0.05. No significant difference between WT and a2-KO for any variable.

Table 4 *Intrinsic contractile properties of the myofilaments*

	WT n=9	a2-KO n=9
Fiber diameter, μm	239 \pm 17	215 \pm 10
<i>Ca activated force</i>		
Resting force, $\text{mN}\cdot\text{mm}^{-2}$	3.8 \pm 0.6	4.0 \pm 0.7
Active force, $\text{mN}\cdot\text{mm}^{-2}$	34.2 \pm 2.4	32.3 \pm 1.7
pCa ₅₀	5.55 \pm 0.03	5.64 \pm 0.03
n _H	2.90 \pm 0.10	2.67 \pm 0.08
<i>Rigor force</i>		
pMgATP ₅₀	3.28 \pm 0.05	3.30 \pm 0.05
pMgATP ₅₀ + PCr	5.03 \pm 0.03	4.98 \pm 0.05
MM-CK efficacy	1.73 \pm 0.05	1.68 \pm 0.06

pCa₅₀ = -log 1/[Ca] is the concentration in Ca leading to half of the maximum active force. n_H Hill coefficient. pMgATP₅₀ and pMgATP₅₀+PCr are the concentrations of MgATP needed to develop half of the rigor force (respectively in the absence and in the presence of PCr 12mM). MM-CK efficacy = ((pMgATP₅₀ +PCr)-pMgATP₅₀) is the shift in the apparent sensitivity to MgATP induced by CK activation

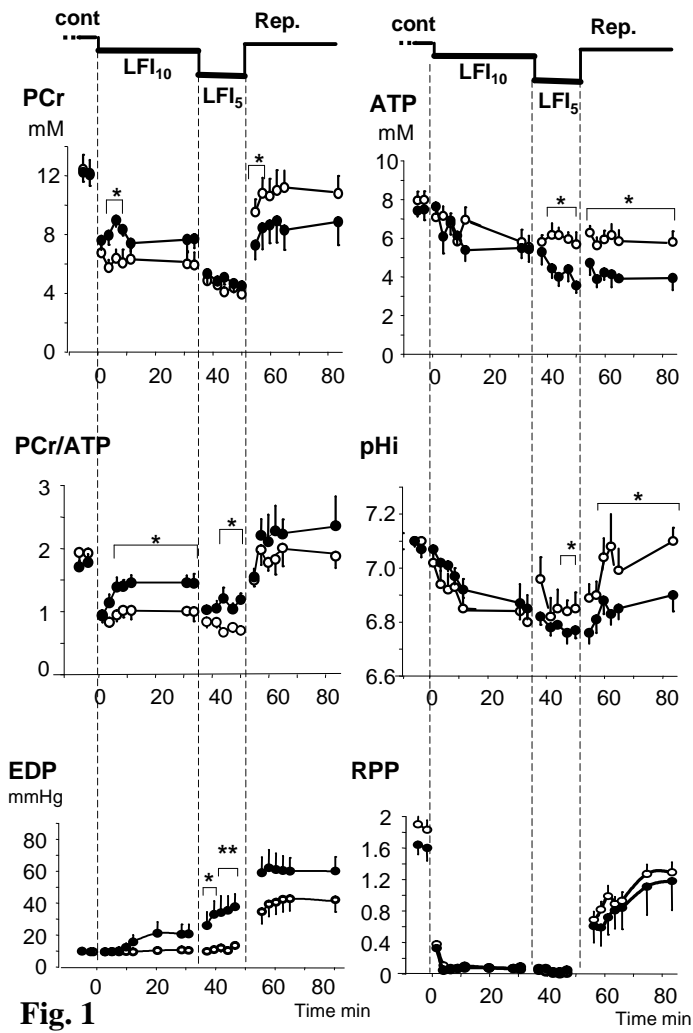
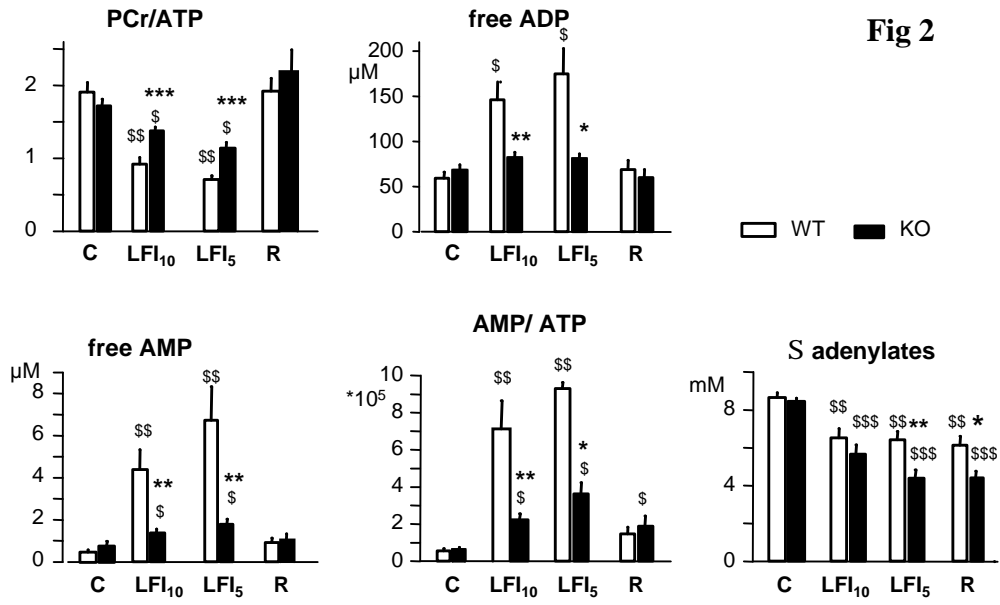
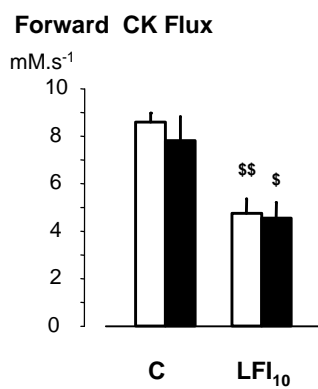


Fig. 1



A



B

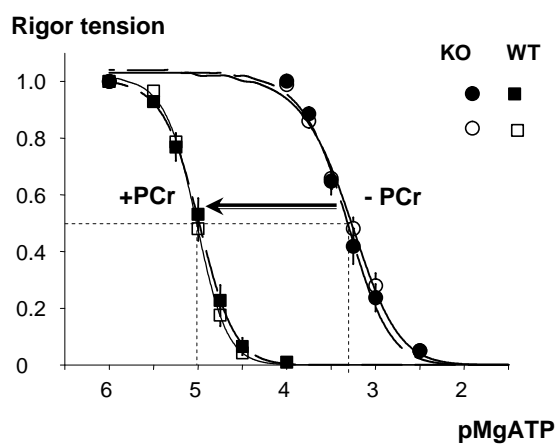
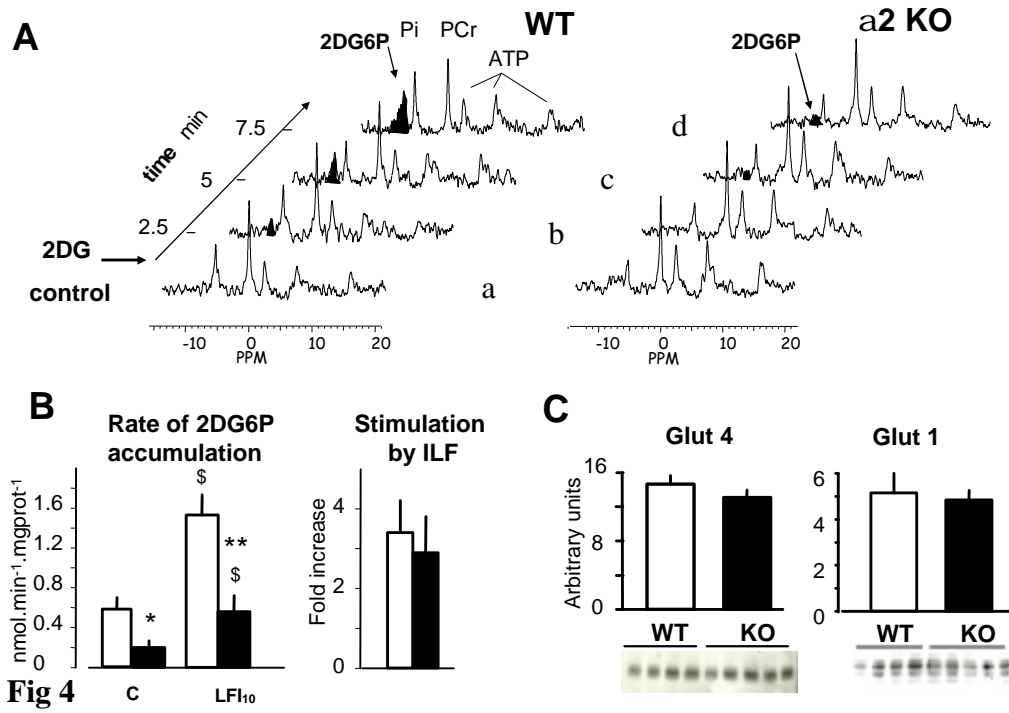


Fig. 3



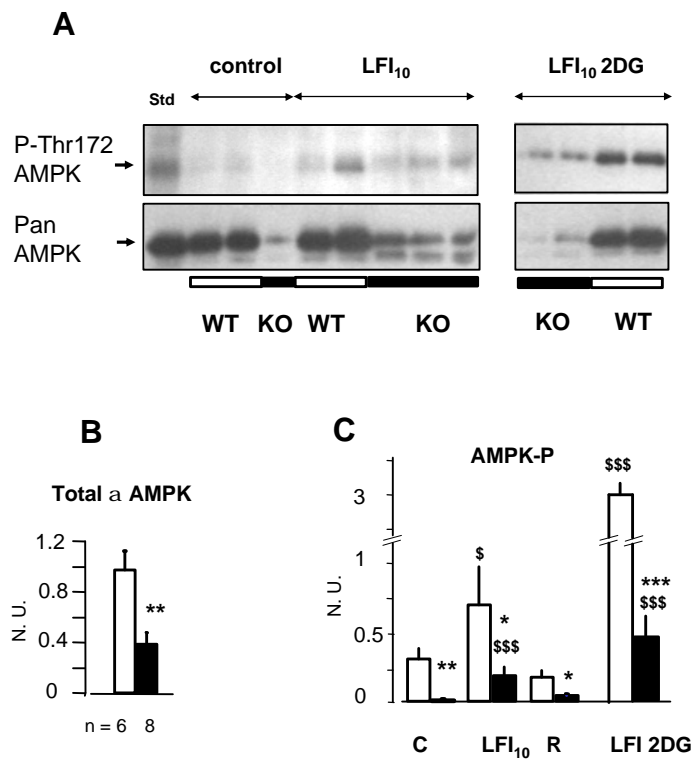


Fig 5

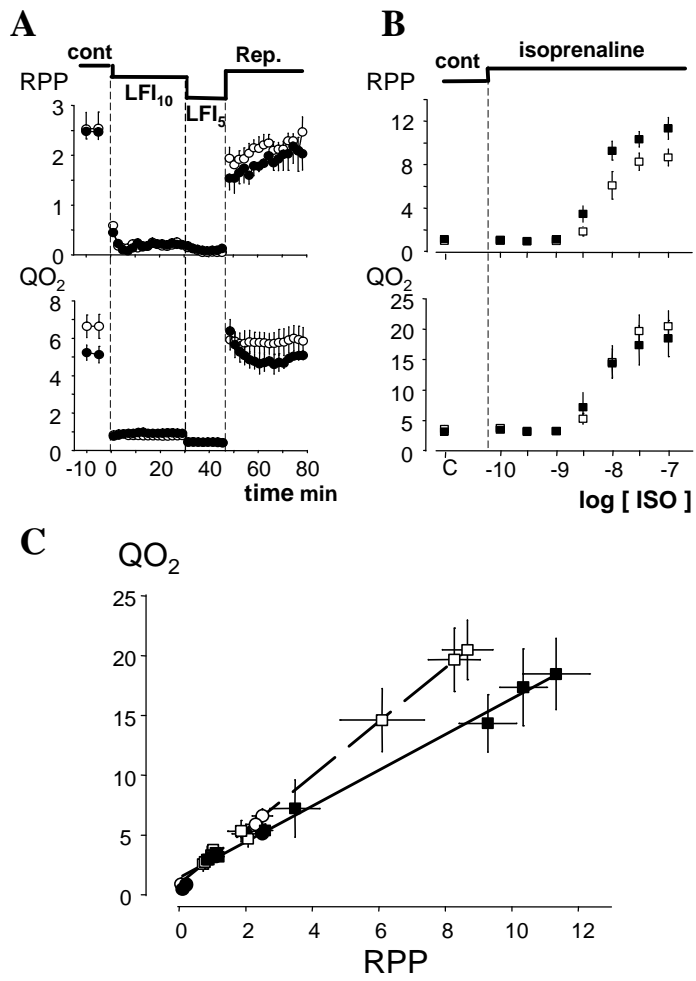


Fig. 6