

AMP-Activated Protein Kinase $\alpha 2$ Deficiency Affects Cardiac Cardiolipin Homeostasis and Mitochondrial Function

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AMP-activated protein kinase (AMPK) plays an important role in controlling energy homeostasis and is envisioned as a promising target to treat metabolic disorders. In the heart, AMPK is involved in short-term regulation and in transcriptional control of proteins involved in energy metabolism. Here, we investigated whether deletion of AMPK $\alpha 2$, the main cardiac catalytic isoform, alters mitochondrial function and biogenesis. Body weight, heart weight, and AMPK $\alpha 1$ expression were similar in control littermate and AMPK $\alpha 2^{-/-}$ mice. Despite normal oxygen consumption in perfused hearts, maximal oxidative capacity, measured using saponin permeabilized cardiac fibers, was $\sim 30\%$ lower in AMPK $\alpha 2^{-/-}$ mice with octanoate, pyruvate, or glutamate plus malate but not with succinate as substrates, showing an impairment at complex I of the respiratory chain. This effect was associated with a 25% decrease in mitochondrial cardiolipin content, the main mitochondrial membrane phospholipid that is crucial for complex I activity, and with a 13% decrease in mitochondrial content of linoleic acid, the main fatty acid of cardiolipins. The decrease in cardiolipin content could be explained by mRNA downregulation of rate-limiting enzymes of both cardiolipin synthesis (CTP:PA cytidyltransferase) and remodeling (acyl-CoA:lysocardiolipin acyltransferase 1). These data reveal a new role for AMPK $\alpha 2$ subunit in the regulation of cardiac muscle oxidative capacity via cardiolipin homeostasis. *Diabetes* 56: 786–794, 2007

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ALCAT1, acyl-CoA:lysocardiolipin acyltransferase 1; AMPK, AMP-activated protein kinase; CDS2, CTP:PA cytidyltransferase; CK, creatine kinase; COX, cytochrome c oxidase; CPT-1, carnitine palmitoyl transferase 1; FADH₂, reduced flavine adenine dinucleotide; G3P, glycerol-3-phosphate; MDH, malate dehydrogenase; mi-CK, mitochondrial creatine kinase; NAO, 10-N-nonyl acridine orange; OMC, oxoglutarate/malate carrier; PGC-1 α , peroxisome proliferator-activated receptor- γ coactivator-1 α .

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AMP-activated protein kinase (AMPK) signaling pathway plays an important role in controlling energy homeostasis at the whole-body level by responding to hormonal or nutrient signals in the central nervous system and peripheral tissues that modulate food intake and energy expenditure (1,2). AMPK is an ubiquitous serine/threonine protein kinase activated by pathological stimuli, such as oxidative damage, osmotic shock, hypoxia, and glucose deprivation, and by physiological stimuli, such as exercise, muscle contraction, and hormones, including leptin and adiponectin (3). It exists in cells as a heterotrimeric complex composed of a catalytic subunit (α) and two regulatory subunits (β and γ). Two α -subunit isoforms exist, $\alpha 1$ and $\alpha 2$; $\alpha 2$ is the chief isoform expressed in striated muscle, accounting for 70–80% of the total AMPK catalytic activity in this tissue (4,5). Activation of AMPK causes upregulation of ATP-producing catabolic pathways and downregulation of ATP-consuming processes (3,6). It is activated in response to decreased cellular energy charge (high AMP-to-ATP ratio) and is involved in regulating carbohydrate and fat metabolism (3,5). AMPK acutely modulates mitochondrial oxidative flux via phosphorylation of acetyl CoA carboxylase, decreasing malonyl-CoA levels, and increasing oxidative flux through the mitochondrial carnitine palmitoyl transferase 1 (CPT-1) (1–3,5). It also increases glucose transport (7) and stimulates glycolysis by activating phosphofructokinase 2 (8). As such, it is envisioned as a promising target to treat metabolic disorders such as metabolic syndrome, obesity, and type 2 diabetes.

Such patients have increased susceptibility to cardiovascular disorders. The role of AMPK in the heart is not fully understood. Whereas AMPK is activated during pressure overload and exercise-induced hypertrophy (9,10), it mediates the antihypertrophic effects of adiponectin (11). AMPK is involved in regulating carbohydrate and fatty acid transport notably during cardiac ischemia and reperfusion (5,8,12,13). Because the final steps of carbohydrate and lipid oxidation take place in mitochondria, it is very probable that this enzyme also plays a role in mitochondrial substrate oxidation pathways.

AMPK can also affect energy metabolism through changes in gene expression. It is involved in skeletal muscle adaptation to exercise by increasing the expression of the peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC-1 α) and activating mitochondrial biogenesis,

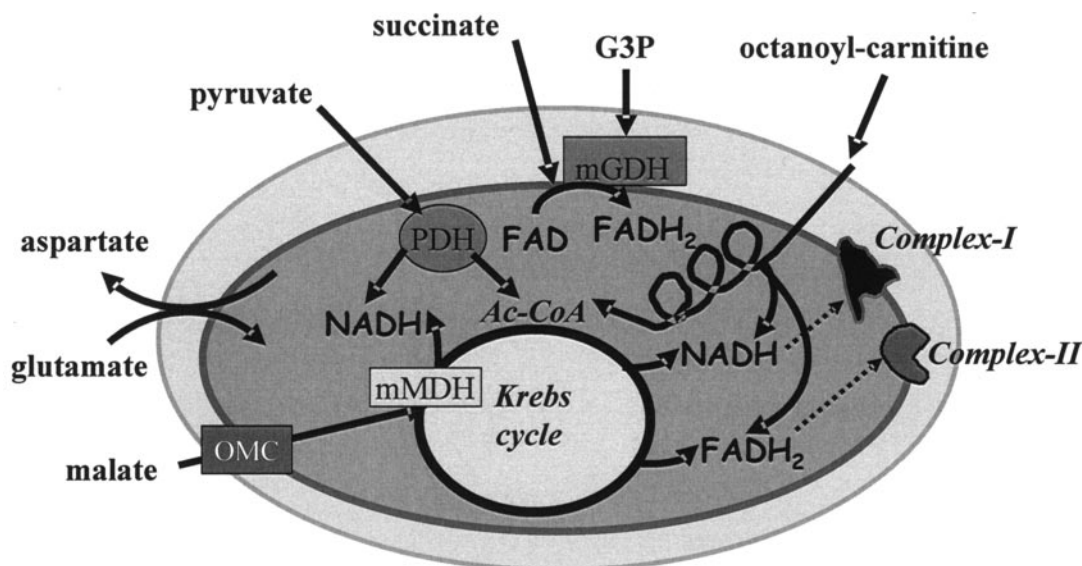


FIG. 1. Substrate utilization by cardiac mitochondria. Pyruvate enters the Krebs cycle by pyruvate dehydrogenase (PDH), producing acetyl-CoA (Ac-CoA). Octanoyl-carnitine undergoes β -oxidation and produces acetyl-CoA, NADH, and FADH₂. Malate enters mitochondria through OMC and produces mainly NADH by mitochondrial MDH (mMDH), whereas G3P produces FADH₂ by the mitochondrial glycerol-phosphate dehydrogenase (mGDH). Succinate produces mainly FADH₂ that enters the respiratory chain through complex II. NADH is reoxidized in the respiratory chain at complex I and FADH₂ at complex II.

but again, nothing is known concerning cardiac muscle (14). The aim of the present study was to investigate the possible involvement of AMPK in the control of cardiac mitochondrial function and biogenesis using specific AMPK α 2-deficient mice (15). These mice exhibit normal echocardiographic and hemodynamic characteristics but have altered glucose metabolism and a worse metabolic adaptation to ischemia (16).

RESEARCH DESIGN AND METHODS

The generation of AMPK α 2^{-/-} mice has been described elsewhere (15). Ten-month-old male AMPK α 2^{-/-} ($n = 27$) and control ($n = 25$) littermate mice were used. Animals were housed under temperature-controlled conditions (21°C) and had free access to water and to a standard mouse chow. All procedures were performed in accordance with the principles and guidelines established by the European Convention for the Protection of Laboratory Animals. Mice were killed by lethal intraperitoneal injection of pentothal (150 mg/kg) for mitochondrial respiration experiments and tissue storage. Left ventricular tissue was isolated, part of which was immediately used for mitochondrial function measurement and part of which was rapidly frozen and kept at -80°C.

Perfused hearts. Additional mice were anesthetized with urethane (2 g/kg). Hearts were quickly removed and retrogradely perfused at a constant flow of 2.5 ml/min in the isovolumic Langendorff perfused mode without pacing. They were first equilibrated with 11 mmol/l glucose for 30 min and then with 5 mmol/l glucose and 0.4 mmol/l oleate prebound to 1% BSA as substrates for 20 more min. Left ventricular pressure was monitored from a water-filled balloon introduced in the left ventricle. Oxygen consumption (QO₂) was calculated from the difference in oxygen content between incoming (aortic) and outgoing (pulmonary artery) perfusates (17).

Electron microscopy. Left ventricular wall and papillary muscles were quickly isolated in oxygenated buffered Krebs solution without calcium to avoid ischemia and fixed with 2% glutaraldehyde as previously described (18). For stereological analysis, papillary muscles were used to ensure longitudinal sections, and three randomly selected levels separated by >50 μ m were used. From randomly selected cardiomyocytes (12–14 myocytes from each animal), the volume density of organelles was estimated by the point counting method (18). The volume density V_v of the organelles was estimated as $V_v = p/P$ (p is the number of the test points hitting the image of the cellular components, P is the number of all points falling on the cardiomyocytes).

Study of in situ mitochondrial respiration. Oxygen consumption measurements of saponin-skinned fibers from left ventricle have been described previously (19,20). Rates of respiration are given in micromoles O₂ per minute per gram dry weight.

Two different experimental protocols were used based on substrate utilization pathways (Fig. 1). The first protocol determined the sensitivity of mitochondrial respiration to various substrates in the presence of 2 mmol/l ADP, by cumulative substrate addition as described previously (21). The second protocol was aimed at determining the dependency of respiration on external [ADP] and [creatine] (22), with glutamate plus malate as substrates. Respiration through complex III was designed according to Ray et al. (23).

Biochemical studies. Frozen tissue samples were weighed and homogenized in ice-cold buffer, and enzyme activities were determined as described previously (24). Complex I activity was measured in heart homogenized in ice-cold buffer containing 10 mmol/l Tris base (pH 7.2), 75 mmol/l sucrose, 225 mmol/l mannitol, 100 μ mol/l EDTA, and 0.1% Triton-X 100. To measure NADH-coenzyme Q reductase activity with decyl-ubiquinone as electron acceptor, samples were incubated in 25 mmol/l phosphate buffer (pH 7.5), 2.5 mg/ml BSA, and 100 μ mol/l decyl-ubiquinone at 30°C. Activity was reported as rotenone-insensitive decrease in NADH absorbance at 340 nm. NADH-ferricyanide reductase activity was measured with ferricyanide as electron acceptor, and the decrease in absorbance was followed at 410 nm.

Real-time quantitative RT-PCR analysis. Total muscle RNA was extracted using standard procedures. Real-time RT-PCR was performed using the SYBR Green method on a LightCycler rapid thermal cycler (Roche Diagnostics) as previously described (25). Values of each gene were normalized to cyclophilin A mRNA content and then corrected for the amount of RNA relative to muscle weight. Primers are listed in Table 1.

Western blot analysis. Specific antibodies were used to measure the protein content of the oxoglutarate/malate carrier (OMC) (26) (gift from Thomas Scholz and Stacia Koppenhafer, University of Iowa), AMPK α 1, and AMPK α 2 (Upstate Biotechnology, Lake Placid, NY) in control and AMPK α 2^{-/-} mice.

Mitochondrial isolation and cardiolipin quantification. Mitochondria were isolated from cardiac ventricles of control and AMPK α 2^{-/-} mice according to Moreno-Sanchez et al. (27). Cardiolipin was quantified by the spectrophotometric method of Petit et al. (28), using the high affinity of 10N-nonyl acridine orange (NAO) for cardiolipin of freshly isolated mitochondria.

Fatty acid composition. Lipids were extracted from heart, isolated cardiac mitochondria, liver, and plasma in 2:1 chloroform:methanol. Phospholipids were separated from nonphosphorous lipids on silica acid cartridges, and fatty acids were *trans*-methylated with BF₃-methanol. Methyl esters were analyzed by gas chromatography on an Econo-cap EC-WAX capillary column (0.32 \times 30 m; Alltech Associates) coupled to a flame ionization detector using C17:0 as the internal standard as previously described (29).

Statistical analysis. Data are expressed as means \pm SE. Student's *t* test was used to determine the statistical significance of differences between group means. Statistical significance was defined as $P < 0.05$.

TABLE 1
Primers used for real-time PCR amplification

Gene	GenBank accession no.	Primer (5'-3')	PCR product size (bp)
PGC-1 α	NM_008904	Forward: CACCAAACCCACAGAGAACAG Reverse: GCAGTTCCAGAGAGTTCCACA	210
CDS2	NM_138651	Forward: CGGTTTCATCTCCTTTGCC Reverse: GATGACGCACGAGATGGGA	201
CPT-1	NM_009948	Forward: TCACCTGGGCTACACGGAGA Reverse: TCGGGGCTGGTCCTACTT	219
TAZ	NM_181516	Forward: TTGGACGGCTGATTGCTGAG Reverse: TGTGAGGGCTTTCCGCATCT	215
ALCAT1	NT_039658	Forward: GGAAGTGAAGGATGATAAG Reverse: GTTTGAGGGATGTTGTAAGG	286
CycA	NM_017101	Forward: GAGCACTGGGAGAAAGGAT Reverse: CTTGCCATCCAGCCACTCAG	259

CycA, cyclophilin A; TAZ, tafazzin.

RESULTS

General characteristics and baseline cardiac function. AMPK $\alpha 2^{-/-}$ mice had normal body (32 ± 1 in control vs. 37 ± 3 g) and heart (187 ± 8 in control vs. 197 ± 16 mg) weights, indicating that cardiac atrophy or hypertrophy were not present. Neither left ventricular pressure or heart rate nor oxygen consumption of isolated hearts differed between control and AMPK $\alpha 2^{-/-}$ mice whether glucose or glucose plus oleate were used as substrates, suggesting that AMPK $\alpha 2$ deficiency does not alter cardiac work during normal perfusion (Table 2).

AMPK $\alpha 1$ protein content (as measured by Western blotting; not shown) was the same in control and AMPK $\alpha 2^{-/-}$ mice (1.24 ± 0.12 in control vs. 1.49 ± 0.21 arbitrary units [AU]). Thus the deficiency in AMPK $\alpha 2$ was not compensated for by AMPK $\alpha 1$ overexpression.

Ultrastructure of the cardiomyocytes. In control cardiomyocytes, mitochondria formed longitudinal rows under sarcolemma and between myofibrils (Fig. 2A and B). In contrast, in AMPK $\alpha 2^{-/-}$ cardiomyocytes from both papillary and ventricular muscles, splitting of myofibrils was frequently observed (Fig. 2C–F). No change in total mitochondrial volume was observed (Table 3), but mitochondria lost their ellipsoid shape and became irregular in size (Fig. 2C–F). They lost their arrangement in longitudinal rows and formed large and spread clusters especially under the sarcolemma (Fig. 2D and F), so that the volume density of subsarcolemmal mitochondria was increased while that of intermyofibrillar mitochondria was decreased (Table 3). No change in the volume of lipid

droplets was observed (0.97 ± 0.22 in control vs. $0.79 \pm 0.61\%$).

Cardiac mitochondrial function. Respiration rates for almost all substrates were significantly lower in cardiac fibers of AMPK $\alpha 2^{-/-}$ mice (Fig. 3A). Compared with controls, respiration was decreased by 35% with malate, 34% after addition of octanoyl-carnitine, 38% after addition of pyruvate, and 31% after final addition of glutamate in AMPK $\alpha 2^{-/-}$ mice, but it did not change with glycerol-3-phosphate (G3P).

Respiration in the presence of phosphate acceptors ADP and creatine was studied with glutamate plus malate ($V_{glu+mal}$) as substrates (Table 3). Basal respiration rate (V_0) and acceptor control ratio (ACR = $V_{glu+mal}/V_0$) were similar in control and AMPK $\alpha 2^{-/-}$ cardiac fibers. No difference was observed between the two groups for the affinity for ADP with or without creatine. The maximal oxygen consumption rate was again 24% lower with glutamate plus malate in the AMPK $\alpha 2^{-/-}$ mice (Table 3; Fig. 3). To determine at which point the respiratory chain was altered in AMPK $\alpha 2^{-/-}$ mice, complex I was inhibited by amobarbital, and respiration through complex II was measured with succinate (V_{suc}). Under succinate, no significant difference between the two groups was observed, whereas the ratio between complex I- and complex II-stimulated respiration ($V_{suc}/V_{glu+mal}$) was increased by 42% in AMPK $\alpha 2^{-/-}$ mice (Table 3; Fig. 3B). No difference was observed for complex III-activated respiration with duroquinol (V_{duroq}). Thus, AMPK $\alpha 2$ deficiency induces a

TABLE 2
Functional data

	Wild type ($n = 5$)		AMPK $\alpha 2^{-/-}$ ($n = 5$)	
	Glucose	Glucose+oleate	Glucose	Glucose+oleate
Ventricular pressure (mmHg)	87 ± 7	109 ± 7	85 ± 5	118 ± 6
Heart rate (beats/min)	358 ± 18	317 ± 17	365 ± 9	337 ± 16
RPP (10^4 mmHg · beats $^{-1}$ · min $^{-1}$)	2.9 ± 0.1	3.2 ± 0.1	2.9 ± 0.2	3.6 ± 0.2
+DP/dt (mmHg/s)	$3,818 \pm 229$	$4,088 \pm 264$	$3,778 \pm 356$	$4,533 \pm 465$
-DP/dt (mmHg/s)	$-2,719 \pm 187$	$-3,053 \pm 131$	$-2,513 \pm 153$	$-3,167 \pm 141$
QO $_2$ (μ mol O $_2$ · min $^{-1}$ · g $^{-1}$ wet wt)	6.4 ± 0.7	$8.6 \pm 0.7^*$	6.0 ± 0.6	8.0 ± 1.0
QO $_2$ /RPP	2.3 ± 0.3	2.7 ± 0.3	2.1 ± 0.3	2.3 ± 0.3

Data are means \pm SE. n , number of mice. Hearts were first perfused with glucose 11 mmol/l and then with glucose 5 and 0.4 mmol/l oleate. DP/dt, developed pressure per unit time; RPP, rate pressure product; QO $_2$, oxygen consumption rate. No statistical difference between control and AMPK $\alpha 2^{-/-}$. * $P < 0.05$, glucose vs. glucose + oleate.

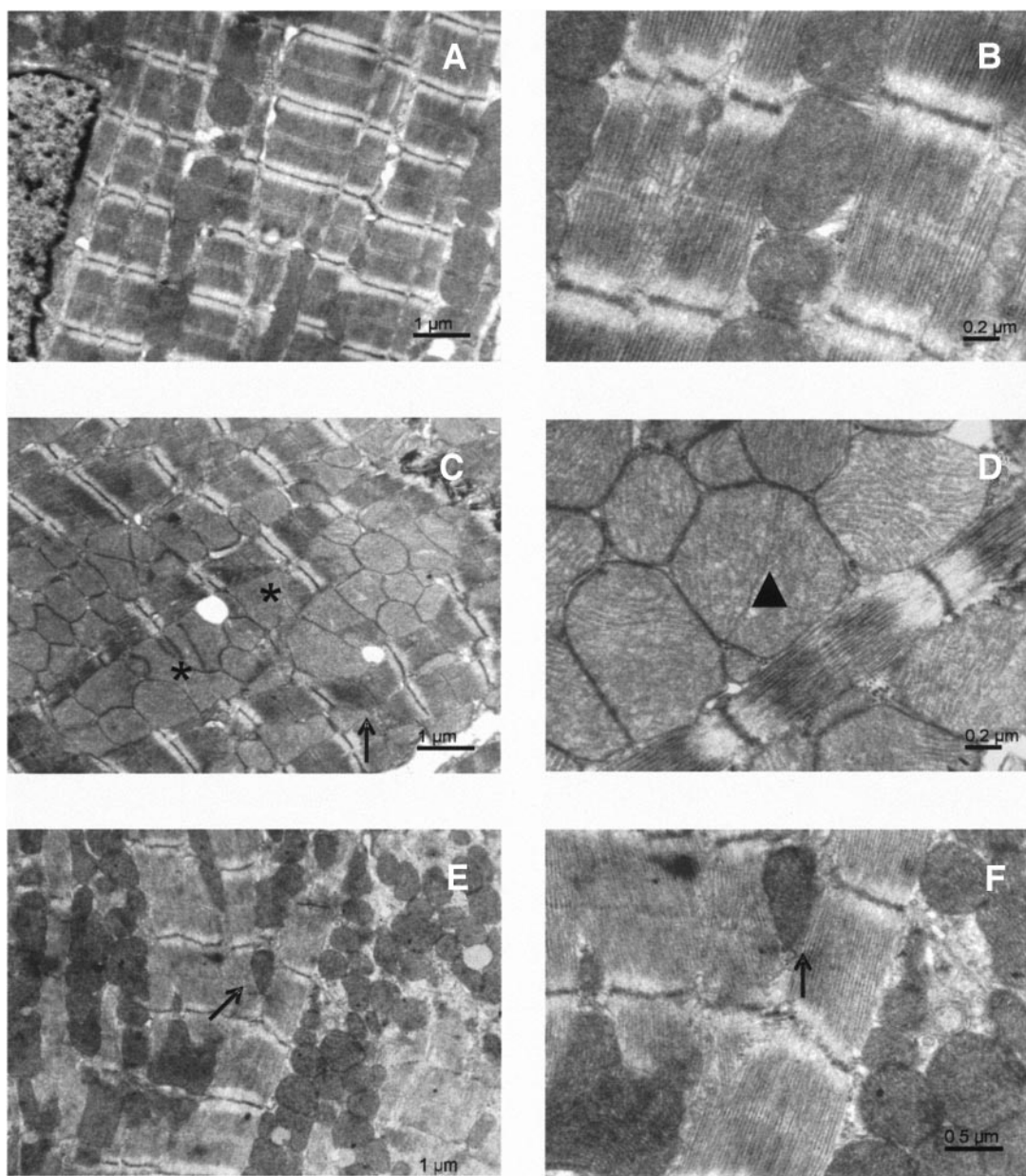


FIG. 2. Electron microscopic images of left ventricle and papillary muscles from control (*A* and *B*) and AMPK α 2^{-/-} (*C-F*) mice. *A*: Overview of a control myocyte in longitudinal section, with mitochondria and myofibrils arranged in regular longitudinal columns. *C* and *E*: Longitudinal section of cardiomyocytes from papillary (*C*) and ventricular (*E*) muscles from AMPK α 2^{-/-} mice showing myofibrillar disorganization and irregular arrangement of intermyofibrillar mitochondria with clusters of mitochondria of variable size. *B*: Detail of sarcomeres in a control myocyte, showing mitochondria tightly packed along sarcomeres. *D* and *F*: Details of sarcomeres in AMPK α 2^{-/-} myocytes from papillary (*D*) and ventricular (*F*) muscles, showing dense packing of mitochondria of irregular size. Asterisk, large mitochondria having irregular shape. Arrows, splitting of myofibrils. Arrowhead, dividing mitochondrion.

defect in mitochondrial respiration that appears limited to complex I.

Energy metabolism enzymes. To understand the origin of mitochondrial defects, activities of key enzymes of energy metabolism were assessed (Table 3). Activity of citrate synthase, an index of mitochondrial mass, was similar in the two groups of mice. Other enzymes of the energy metabolism, total creatine kinase (CK), mitochondrial CK (mi-CK), adenylate kinase, total lactate dehydrogenase, and cytochrome *c* oxidase (COX) activities also remained unchanged in AMPK α 2^{-/-}. Despite decreased respiration through complex I, maximal complex I and complex II activities determined in tissue extracts were

the same in both groups. To ensure that maximal complex I activity was preserved in AMPK α 2^{-/-}, we measured its activity using different electron acceptors, but no difference between the two groups was observed, suggesting that AMPK deficiency did not affect mitochondrial enzyme content.

In an attempt to understand the origin of the decreased respiration through complex I, malate-aspartate shuttle enzymes were measured (Table 3). Total malate dehydrogenase (MDH), mitochondrial MDH, and the amount of OMC (6.8 ± 1.2 [$n = 6$] in control vs. 5.1 ± 0.5 AU in AMPK α 2^{-/-} [$n = 6$]; not shown) were unchanged in AMPK α 2-deficient mice. Moreover, the mRNA levels of

TABLE 3
Energy metabolism of cardiac fibers

	Control	AMPK $\alpha 2^{-/-}$
Morphometric data		
Hearts (<i>n</i>)	3	3
Total mitochondria	36.1 ± 1.8	37.6 ± 0.5
SS mitochondria	3.9 ± 0.4	11.3 ± 1.1*
IM mitochondria	32.2 ± 1.4	26.3 ± 1.4*
Mitochondrial function		
Fibers (<i>n</i>)	20	18
V_0 ($\mu\text{mol O}_2 \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ dry wt)	3.0 ± 0.3	2.7 ± 0.2
$V_{\text{glu+mal}}$ ($\mu\text{mol O}_2 \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ dry wt)	18 ± 1	13 ± 1*
V_{suc} ($\mu\text{mol O}_2 \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ dry wt)	19 ± 2	23 ± 2
$V_{\text{suc}}/V_{\text{glu+mal}}$	1.2 ± 0.1	1.7 ± 0.2*
V_{duroq} ($\mu\text{mol O}_2 \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ dry wt)*	50 ± 10	54 ± 8
K_{mADP}	253 ± 54	289 ± 49
K_{mCr}	73 ± 14	52 ± 11
ACR	6.2 ± 0.9	4.9 ± 0.4
Enzymatic activity (IU/mg protein)		
Animals (<i>n</i>)	9	8
Citrate synthase	836 ± 58	742 ± 48
Cytochrome oxidase	1,207 ± 120	1,114 ± 114
CK	2,637 ± 182	2,860 ± 171
mi CK	653 ± 96	716 ± 67
Adenylate kinase	2,107 ± 150	2,414 ± 219
Lactate dehydrogenase	932 ± 75	999 ± 70
Complex I (deacyl-ubiquinone)	450 ± 64	413 ± 51
Complex I (ferricyanure)	990 ± 86	914 ± 77
Complex II	264 ± 27	226 ± 21
MDH	5,906 ± 500	5,843 ± 573
Mitochondrial MDH	1,913 ± 250	1,818 ± 112

Data are means ± SE. Morphological data are expressed in percent cell volume. Oxygen consumption rates in the absence (V_0) and presence of 2 mmol/l ADP ($V_{\text{glu+mal}}$, respiration through complex I with glutamate+malate; V_{suc} , respiration through complex II with succinate; V_{duroq} , respiration through complex III with reduced duroquinol). **n* = 10 for each. Michaelis-Menten constant of respiration rate for ADP ($\mu\text{mol/l}$) without (K_{m}) or with (K_{mCr}) 20 mmol/l creatine. ACR, acceptor control ratio ($V_{\text{glu+mal}}/V_0$). IM, intermyofibrillar; SS, subsarcolemmal. **P* < 0.05 vs. control mice.

PGC-1 α , the main regulator of mitochondrial biogenesis, were similar in AMPK $\alpha 2^{-/-}$ and control hearts (Fig. 4B), consistent with the preserved mitochondrial enzyme activities.

Cardiolipins and fatty acid composition. Complex I activity in situ is critically dependent on the cardiolipin environment (30,31). In AMPK $\alpha 2^{-/-}$ mice, the amount of cardiolipin was 25% lower than in controls (Fig. 4A). In addition, as evidenced in Table 4, the fatty acid composition of whole-heart phospholipids and of isolated cardiac mitochondria in the AMPK $\alpha 2^{-/-}$ mice showed a significant decrease in linoleic acid proportion (~80% of cardiolipin fatty acids). Conversely, linoleic acid was significantly increased in the nonphosphorous lipid fraction (which mainly contains storage triacylglycerol) of the whole-heart homogenate. This decrease in linoleic acid content was specific to the heart because it decreased neither in liver phospholipids (19.4 ± 0.8 and 19.6 ± 1.1%, control vs. AMPK $\alpha 2^{-/-}$) nor in plasma lipids (31.3 ± 0.4 and 27.8 ± 2.2%, respectively).

The level of expression of enzymes involved in cardiolipin homeostasis was determined (Fig. 4B). Levels of CTP:PA cytidyltransferase (CDS2), the enzyme that catalyzes the initial key step of cardiolipin synthesis, i.e., the conversion of phosphatidic acid to CDP-diacylglycerol, and of acyl-CoA:lysocardiolipin acyltransferase 1 (ALCAT1), which is implicated in rapid cardiolipin remodeling

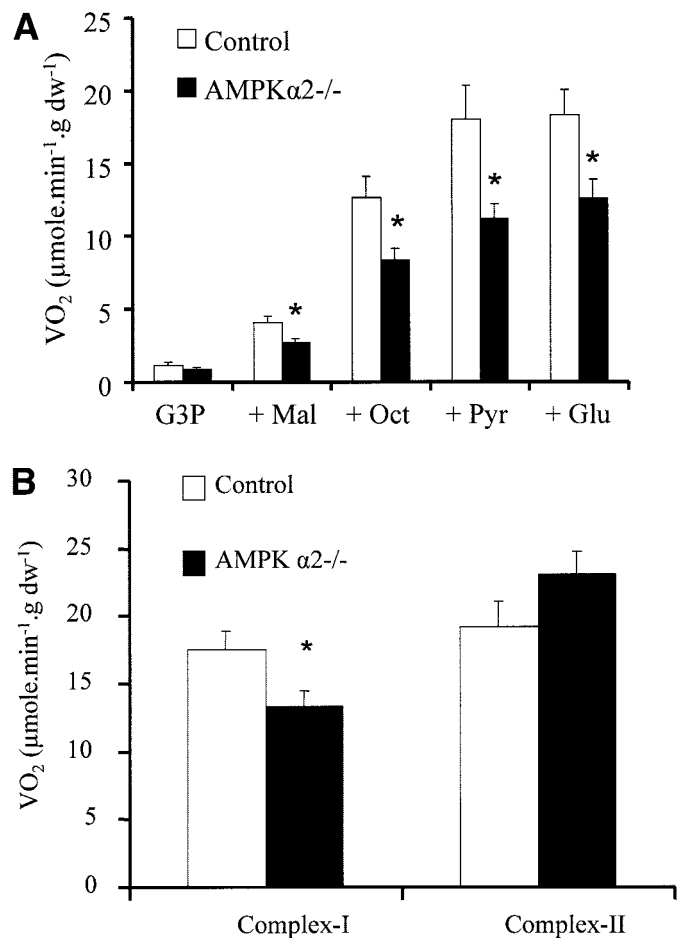


FIG. 3. A: Decreased substrate utilization by cardiac mitochondria in AMPK $\alpha 2^{-/-}$ mice. Respiration rates were measured during the cumulative addition of substrates in saponin-skinned cardiac fibers of control and AMPK $\alpha 2^{-/-}$ mice. V_{O_2} , rate of O_2 consumption in micromoles per minute per gram dry weight. Glu, glutamate; Mal, malate; Oct, octanoyl-carnitine; Pyr, pyruvate. **P* < 0.05 vs. control. B: Respiration rate through complex I is specifically inhibited in AMPK $\alpha 2^{-/-}$ mouse heart. V_{O_2} , rate of O_2 consumption in micromoles per minute per gram dry weight in saponin-permeabilized cardiac fibers. Complex I, respiration with 2 mmol/l malate and 5 mmol/l glutamate. Complex II, respiration with 10 mmol/l succinate. **P* < 0.05 vs. control.

(32,33), were significantly decreased in AMPK $\alpha 2^{-/-}$ mice, whereas mRNA content of taffazin, another phospholipid acyltransferase, was unchanged. Interestingly, expression of CPT-1, the mitochondrial middle- and long-chain fatty acid transporter, was also significantly decreased in AMPK-deficient mice.

DISCUSSION

The main results of this study can be summarized as follows. 1) Specific deficiency in AMPK $\alpha 2$ catalytic subunit did not induce cardiac atrophy or hypertrophy. 2) Oxygen consumption of isolated perfused heart was normal, but mitochondrial ultrastructure was altered. 3) AMPK deficiency induced a decrease in maximal oxidative capacity of the cardiac muscle whether lipids, pyruvate, or glutamate plus malate were used as substrates. 4) This was not accompanied by changes in mitochondrial enzyme activities, the malate/aspartate shuttle, or isolated complex I or II in vitro activities. 5) When succinate was used as substrate, mitochondrial respiration was normal in AMPK $\alpha 2^{-/-}$ cardiac fibers, suggesting a defect in complex

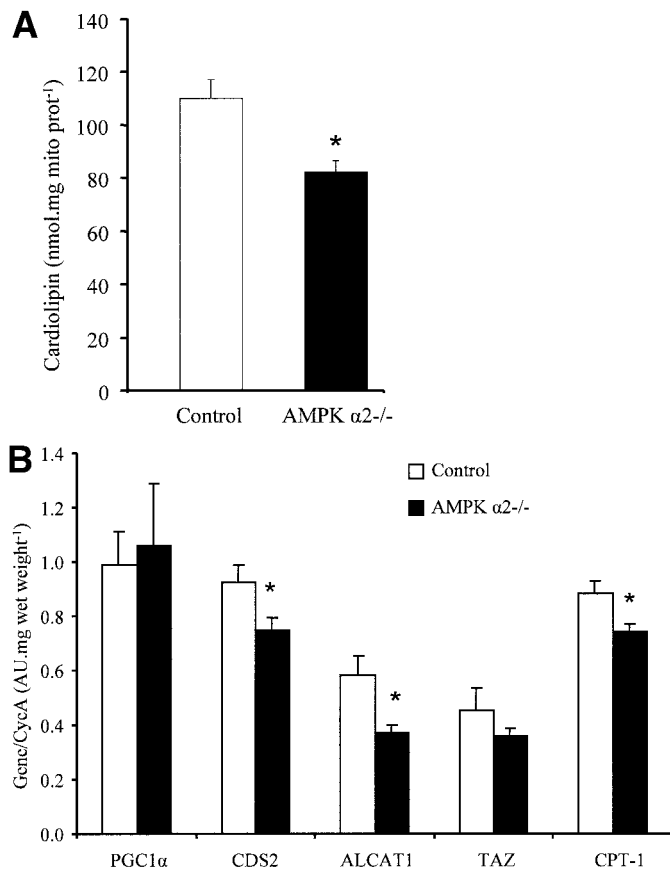


FIG. 4. A: Cardiolipin content is decreased in AMPK $\alpha 2^{-/-}$ mice mitochondria. Data obtained by colorimetric assay with the NAO in cardiac muscle of AMPK $\alpha 2^{-/-}$ ($n = 6$) and littermate ($n = 6$) mice in nanomoles per milligram mitochondrial proteins. * $P < 0.05$ vs. control. **B:** Gene expression. Real-time quantitative RT-PCR analysis of mRNA expression of PGC-1 α , CDS2, CPT-1, tafazzin (TAZ), and ALCAT1 in cardiac muscle of AMPK $\alpha 2^{-/-}$ ($n = 6$) and littermate ($n = 6$) mice. Results are given as means \pm SE in AU normalized to Cyclophilin A transcription and corrected for the amount of total RNA relative to muscle weight.

I function in situ. 6) Cardiolipin content of AMPK $\alpha 2^{-/-}$ cardiac mitochondria was decreased by 25%, consistent with the altered ultrastructure and the functional defect of

mitochondrial respiration by complex I. 7) This defect was accompanied by a significant decrease in linoleic acid content of mitochondrial phospholipids and 8) could be explained by the decrease in the expression of key enzymes of cardiolipin biosynthesis and remodeling. Altogether, these results suggest that AMPK $\alpha 2$ is involved in the control of mitochondrial respiration through cardiolipin homeostasis.

AMPK $\alpha 2^{-/-}$ mice exhibit perturbation in whole-body insulin sensitivity, probably modulated by the sympathetic nervous system (15). These mice show normal cardiac content in AMPK $\alpha 1$ isoform, suggesting that remnant AMPK $\alpha 1$ is unable to compensate for the lack of AMPK $\alpha 2$ (16).

A role for AMPK in controlling cardiac weight is still controversial. Although AMPK was suggested to play a role in pressure overload hypertrophy (9), it was recently reported that adiponectin blocks cardiac hypertrophy by an AMPK-dependent mechanism (11). Hearts of mice expressing a dominant-negative mutant of AMPK $\alpha 2$ exhibiting a residual AMPK $\alpha 2$ activity have preserved weight and baseline function (13). In another model overexpressing a kinase-dead rat $\alpha 2$ isoform, the $\alpha 2$ protein content and activity were absent, and $\alpha 1$ content and activity were also decreased, with the remnant $\alpha 1$ activity being attributed to endothelial cells (12). These mice exhibit a slightly decreased cardiac weight and contractility. However, we show here that AMPK $\alpha 2^{-/-}$ mice had normal cardiac weight. This suggests that the $\alpha 2$ subunit by itself is not essential for cardiac growth.

Oxygen consumption and contractile function were not different under basal conditions either with glucose or glucose plus oleate as substrates, as already described with glucose and pyruvate (16) in this mouse line. In KD mutant mice, cardiac function is slightly depressed at baseline (12). This difference could be due to the decreased AMPK $\alpha 1$ activity in this line. However, the workload of hearts perfused in the Langendorff mode is significantly less than in the working mode or in vivo, and oxygen consumption does not reach maximal capacity.

To assess maximal mitochondrial function, respiration rates were measured in skinned fibers with saturating amounts of substrates, oxygen, and phosphate acceptor.

TABLE 4
Fatty acid composition of cardiac lipids

Fatty acid	Composition					
	Heart mitochondria		Heart phospholipids		Nonphosphorous lipids	
	Control	AMPK $\alpha 2^{-/-}$	Control	AMPK $\alpha 2^{-/-}$	Control	AMPK $\alpha 2^{-/-}$
16:0	13.4 \pm 0.6	14.6 \pm 0.7	14.2 \pm 0.9	16.4 \pm 0.5	19.8 \pm 0.5	18.1 \pm 0.4
16:1 ω 9	1.1 \pm 0.2	1.0 \pm 0.3	0.3 \pm 0.1	0.35 \pm 0.03	6.2 \pm 0.7	4.5 \pm 0.3
18:0	18 \pm 1	17 \pm 1	21 \pm 1	21 \pm 1	8.9 \pm 0.3	9 \pm 1
18:1 ω 9	6.7 \pm 0.3	7.0 \pm 0.2	6.3 \pm 0.3	6.3 \pm 0.3	19.0 \pm 0.4	22.0 \pm 0.4*
18:1 ω 7	2.51 \pm 0.03	2.7 \pm 0.1	2.26 \pm 0.04	2.5 \pm 0.1	1.4 \pm 0.1	1.5 \pm 0.3
18:2 ω 6 LA	19.2 \pm 0.6	16.8 \pm 0.4*	18.0 \pm 0.2	16.3 \pm 0.5*	20.1 \pm 1.5	24.3 \pm 0.3*
20:4 ω 6	4.5 \pm 0.2	4.3 \pm 0.2	5.2 \pm 0.2	4.9 \pm 0.3	3.0 \pm 0.1	2.6 \pm 0.4
22:5 ω 3	1.02 \pm 0.03	1.2 \pm 0.1	1.1 \pm 0.1	4.1 \pm 3.1	1.5 \pm 0.1	1.5 \pm 0.4
22:6 ω 3	30 \pm 2	31 \pm 2	28 \pm 2	24 \pm 1	5.1 \pm 0.4	5.4 \pm 0.6
SFA	32 \pm 1	33 \pm 1	36 \pm 2	34 \pm 5	36 \pm 1	39 \pm 5
MUFA	12 \pm 1	11 \pm 1	9.7 \pm 0.3	9 \pm 1	31 \pm 1	36 \pm 6
PUFA ω 6	25.1 \pm 0.5	22.5 \pm 0.6*	25.2 \pm 0.4	20 \pm 3	25 \pm 1	33 \pm 5
PUFA ω 3	31 \pm 2	33 \pm 2	29 \pm 2	24 \pm 4	8.1 \pm 0.3	9 \pm 1

Data are means \pm SE (%). LA, linoleic acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid. Three control and three AMPK $\alpha 2^{-/-}$ mice were used. * $P < 0.05$.

Depending on available substrates and cardiac demand, mitochondria are able to use acetyl-CoA produced from pyruvate by pyruvate dehydrogenase downward of glycolysis and/or acetyl-CoA produced by the β -oxidation of fatty acids, which enters the Krebs cycle (Fig. 1). Mitochondria can also slightly use G3P by mitochondrial glycerophosphate dehydrogenase, which produces reduced flavine adenine dinucleotide (FADH₂) that enters the respiratory chain directly at the level of complex II (34). We found a clear decrease in the respiration rate of AMPK α 2^{-/-} cardiac fibers, whether carbohydrate- or lipid-derived substrates were used. This suggests that AMPK α 2 is involved in the control of cardiac oxidative capacity and in mitochondrial substrate utilization. Decreased energy availability in AMPK α 2^{-/-} mice would probably occur only at maximal workloads, so that the mitochondrial alterations observed here might be more evident during heavy exercise or under pathologic stress.

To understand the decrease in respiration of cardiac fibers, we looked at indicators of mitochondrial activity. In AMPK α 2^{-/-} mouse heart, levels of citrate synthase, a marker of mitochondrial mass; of COX, an enzyme of the respiratory chain; of mi-CK, a phosphotransfer kinase; and of markers of the malate/aspartate shuttle, which are involved in glutamate plus malate utilization, were normal. Additionally, PGC-1 α , which induces mitochondrial biogenesis (35), and total mitochondrial volume density were unchanged as was also observed in skeletal muscles of mice with dominant-negative mutant of AMPK (36). All of this suggests that AMPK α 2 is not essential for determining and maintaining mitochondrial mass in skeletal and cardiac muscle. However, it may play a role in cardiac mitochondrial biogenesis induced by external stimulus, as was observed in skeletal muscle in response to exercise or chronic energy depletion (36).

Mitochondrial function also depends on the sensitivity to ADP and to creatine, the final acceptor of high-energy phosphates in cardiac mitochondria (37). No change in the sensitivity of respiration to ADP and creatine was observed, showing that the efficiency of cardiac mitochondria in phosphorylating ADP and producing phosphocreatine was not affected by AMPK α 2 loss.

To explain the decreased respiration with preserved enzyme activities, we compared the respiration rates during activation from complex I, II, or III (Figs. 1 and 3; Table 2). Respiration with glutamate plus malate mainly produces NADH, which activates respiration through complex I, whereas succinate oxidation mainly produces FADH₂, which is further oxidized by complex II. Compared with controls, respiration was lower in AMPK α 2^{-/-} mice with glutamate plus malate but was normal with succinate, revealing an inhibition of complex I in these animals. Interestingly, respiration from G3P, which produces only FADH₂, was normal in AMPK α 2^{-/-} mice, whereas it was decreased for NADH-producing substrates: pyruvate and octanoyl-carnitine. Moreover, respiration through complex III was unchanged in AMPK α 2^{-/-} mice.

Despite a 24% decrease in respiration rate through complex I, complex I activity measured in total heart extracts was normal in AMPK α 2^{-/-} mice, independent of the electron acceptor. This indicated that the activity of isolated complex I was not affected by AMPK α 2 deficiency, suggesting an alteration of the in situ regulation of complex I by cardiolipin. Activity of the complexes of the respiratory chain, and particularly of complex I, strongly depends on the surrounding phospholipid environment of

the inner mitochondrial membrane. Cardiolipin is the main functional phospholipid of the mitochondrial inner membrane, being present almost exclusively in mitochondria and representing 8–15% of the entire cardiac phospholipid mass. A 25% lower mitochondrial cardiolipin content was observed in AMPK α 2^{-/-} mice. Cardiolipin content is critical for the adaptation of energy metabolism to demand. It rises with increased metabolic rate or muscle performance (38) and plays a key role in the activity of several inner membrane proteins including complex I (rev. in 39). Both complex I and III were shown to require cardiolipin, but although necessary for full complex I activity (40), cardiolipin plays a structural rather than catalytic role for complex III (41). Accordingly, respiration through complex I but not complex III was decreased in AMPK α 2^{-/-} mice. Interestingly, in CHO cells, similar decrease in cardiolipin content induces similar alteration in complex I activity in the respiratory chain without changes in isolated, complex II, complex III and IV, and NADH reductase activity (31). mi-CK binding in the vicinity of translocase is dependent on cardiolipin environment (42). It is possible that the rather small decrease in cardiolipin observed here was not sufficient to affect mi-CK function and activity.

Examination of cardiac ultrastructure of AMPK α 2^{-/-} mice revealed abnormal structure of mitochondria of various sizes arranged in clusters under the sarcolemma and less in regular rows between myofilaments as in control hearts. Although unchanged total mitochondrial volume was in accordance with preserved mitochondrial enzyme activity and PGC-1 α expression, partial relocation of mitochondria from myofibrillar to subsarcolemmal space occurred. No evidence of ultrastructural changes was observed in AMPK KD mutant (12). However, in the present study, these morphological changes were observed at higher magnification and could be more pronounced in older animals. Similarly, in CHO cells, an alteration of mitochondrial ultrastructure is specifically associated with reduction in cardiolipin content (31). Moreover, although to a lower extent, this resembles the ultrastructural abnormalities of mitochondria in the Barth syndrome, an X-linked disease. This disease, due to mutations in the tafazzin gene that belongs to the superfamily of phospholipid acyltransferase, is characterized by a dramatic reduction in cardiolipin content and by abnormal mitochondrial ultrastructure (43).

Cardiolipin is the only phospholipid with four acyl chains, ~80% of which are composed of linoleic acid (18:2 ω 6), a polyunsaturated fatty acid of the ω 6 series. The decrease in cardiolipin content in AMPK α 2^{-/-} heart was accompanied by a significant decrease in linoleic acid content of mitochondrial and whole-heart phospholipids. The decrease in mitochondrial membrane linoleic acid observed in this study is due to neither nutritional differences, because animals received the same chow diet, nor a systemic effect because the plasma and liver lipid composition was unchanged. The fact that 1) the linoleic acid decrease parallels the cardiolipin decrease, 2) cardiolipin contains ~80% linoleic acid, and 3) the linoleic acid content in storage lipids was higher suggests that the decrease in linoleic acid is a consequence rather than a cause of the cardiolipin decrease in AMPK α 2-deficient mice. The lower linoleic acid content in heart compared with liver of AMPK α 2^{-/-} mice could be related to the higher proportion of AMPK α 1 in liver (4). These animals are thus characterized by a dysfunction of cardiac cardiolipin homeostasis that affects the inner mitochondrial

membrane and that could be the key effector of mitochondrial dysfunction.

Similar decreases in cardiolipin content and complex I activity were reported after partial inhibition of cardiolipin synthesis in CHO cells (31). One important rate-limiting step of de novo cardiolipin synthesis is the initial reaction catalyzed by CDS (33). In AMPK $\alpha 2^{-/-}$ mice, expression of CDS2, the only cardiac isoform (44), is decreased, possibly explaining the decrease in cardiolipin content. A second mechanism of cardiolipin biosynthesis is through deacylation-reacylation, which is catalyzed by phospholipases and acyltransferases, which are regarded as the principal enzymes involved in phospholipid remodeling in mammalian tissues (33). The acyltransferase ALCAT1 is regulated in concert with the level of cardiolipin and cardiolipin biosynthesis in mammalian heart (32,45). ALCAT1 mRNA expression was decreased in AMPK $\alpha 2^{-/-}$ mice like both CDS2 mRNA and cardiolipin content. All of this suggests that AMPK is involved in cardiolipin homeostasis at least at the transcriptional level. Finally, expression of the mitochondrial fatty acid transporter CPT-1 was also decreased in AMPK $\alpha 2$ -deficient mice. Decreased activation of CPT-1 because of the lack of AMPK-modulated regulation of malonyl CoA content, together with the downregulation of CPT-1, would have an additive inhibitory effect on fatty acid oxidation in AMPK $\alpha 2^{-/-}$ mice.

In summary, selective deficiency of AMPK $\alpha 2$ causes a significant decrease in maximal mitochondrial respiration, whether carbohydrates or lipids were used as substrates. This is due to a defect in the function of complex I of the respiratory chain within the inner mitochondrial membrane, probably because of the decrease in cardiolipin content. Interestingly, it has been recently reported that the diabetic heart, characterized by altered lipid homeostasis and mitochondrial dysfunction, exhibits a dramatic decrease in cardiolipin content (46). These findings suggest that AMPK plays a critical role in the control of cardiac phospholipid homeostasis, possibly by modulating their metabolism through transcriptional machinery. More work is needed to elucidate the precise mechanisms by which AMPK may enhance energy production by favoring cardiolipin synthesis and thus improving the efficiency of the respiratory chain.

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