

Cellular and Molecular Life Sciences

Regulation of AMPK activity by type 10 adenylyl cyclase: contribution to the mitochondrial biology, cellular redox and energy homeostasis --Manuscript Draft--

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Abstract:	The downregulation of AMP-activated protein kinase (AMPK) activity contributes to numerous pathologies. Recent reports suggest that the elevation of cellular cAMP promotes AMPK activity. However, the source of the cAMP pool that controls AMPK activity remains unknown. Mammalian cells possess two cAMP sources: membrane-bound adenylyl cyclase (tmAC) and intracellularly localized, type 10 soluble adenylyl cyclase (sAC). Due to the localization of sAC and AMPK in similar intracellular compartments, we hypothesized that sAC may control AMPK activity. In this study, sAC expression and activity were manipulated in H9C2 cells, adult rat cardiomyocytes or endothelial cells. sAC knockdown depleted the cellular cAMP content and decreased AMPK activity in an EPAC-dependent manner. Functionally, sAC knockdown reduced cellular ATP content, increased mitochondrial ROS formation and led to mitochondrial depolarization. Furthermore, sAC downregulation led to EPAC-dependent mitophagy disturbance, indicated by an increased mitochondrial mass and unaffected mitochondrial biogenesis. Consistently, sAC overexpression or stimulation with bicarbonate significantly increased AMPK activity and cellular ATP content. In contrast, tmAC inhibition or stimulation produced no effect on AMPK activity. Therefore, the sAC–EPAC axis may regulate basal and induced AMPK activity and supports mitophagy, cellular energy and redox homeostasis. The study argues for sAC as a potential target in treating pathologies associated with AMPK downregulation.							

Response to Reviewers:

First of all, we would like to thank the Editors and Reviewers for the thorough evaluation of our manuscript and for their valuable comments that helped us to improve the overall quality of the article. We hope that all comments have been addressed adequately after the revision of the manuscript.

Reviewer #1:

The authors data demonstrating that sAC-generated cAMP activates AMPK via EPAC might provide a mechanism linking the two findings; unfortunate, the major problem of this paper is that the data shown do not connect the two data sets.

The authors need to demonstrate the AMPK is between sAC and the mitochondrial biology shown; they should at least show that downregulation of AMPK prevents the postulated sAC-mediated effects.

Response:

As requested by reviewer we performed additional experiments to prove the causal role of AMPK in sAC knockdown-induced disturbance of mitophagy and mitochondrial ROS formation. For this purpose, a selective AMPK activator A769662 was used. The data presented in the new figure 6 suggest that artificial activation of AMPK does not significantly affect the sAC knockdown effects on mitochondrial mass and ROS formation. In opposite, the sAC knockdown-induced downregulation of TFAM and mitochondrial protein Sirt3 was prevented by AMPK activation. Thus, sAC knockdown leads to the disturbance of mitophagy and mitochondrial ROS homeostasis independently of AMPK activity. On the other hand, sAC depletion affects expression of some mitochondrial proteins in AMPK-dependent manner. The potential mechanism leading to disturbance of mitophagy under sAC knockdown is discussed in the revised version of the manuscript (page. no. 13, line 10 – 20)

Other minor and major problems:

Fig. 1d Please show a more representative example. The 2nd sAC shRNA lane did not transfer properly.

Response:

In the revised version a new representative image for pACC/ACC (Fig.1d) is presented.

Fig 3b does not make sense. They show that a PKA agonist downregulates AMPK in untreated cells, but why is the PKA agonist not down regulating pAMPK in the sAC shRNA lane? If the author's postulated mechanism is correct, with sAC knockdown there should be less intrinsic cAMP and, therefore, a stronger PKA agonist effect.

Response:

PKA is known to negatively affect AMPK activity, either directly via phosphorylation of AMPK at Ser-173 (Djouder et al 2010), or indirectly by inhibition of calcium/calmodulin-dependent kinase kinase (Davare et al 2004; Ichimura et al 2008), a key AMPK activator. Under sAC knockdown AMPK activity is reduced, which may induce feedback mechanism(s) leading to downregulation of the inhibitory PKA pathways.

References:

Djouder N et al. (2010). PKA phosphorylates and inactivates AMPK α to promote efficient lipolysis. EMBO J. 29(2), 469–481.

Davare MA et al. (2004). Inhibition of calcium/calmodulin-dependent protein kinase kinase by protein 14-3-3. J. Biol. Chem. 279, 52191–52199.

Ichimura T. et al. (2008). 14-3-3 Proteins directly regulate Ca(2+)/calmodulin-dependent protein kinase kinase α through phosphorylation-dependent multisite binding. FEBS Lett. 582, 661–665.

In Fig 3d, it makes sense that the control is lower with sAC shRNA, but why does EPAC agonist not lead to same pAMPK in both scrambled AND sAC shRNA?

Response:

This is often observed phenomenon under knockdown of enzymes, which may have

several causes. In our case it may be due to the difference of the endogenous cAMP content between scramble and sAC-depleted cells, which is essential for the AMPK activity. Thus, in shRNA group treatment with EPAC agonist may be insufficient to reach the similar AMPK activation level as in scramble group since the basal cAMP level in shRNA group is lower than in scramble group. Nevertheless, compared to the own vehicle-treated controls, the elevation of pAMPK/AMPK ratio, i.e., difference between EPAC agonist and control, is similar in scramble and sAC knockdown groups.

Fig 4. The observation that blocking sAC (with RNAi or inhibitors) decreases cellular ATP and increases ROS has been shown in multiple papers. Manfredi's lab has shown a PKA-mediated mechanism directly on OXPHOS, while others demonstrated EPAC mediates the sAC-dependent effects on mitochondrial ATP generation. The authors data might add to this story by implicating AMPK in the process, but they need to demonstrate AMPK dependence in the sAC pathway.

Response:

Indeed, our data confirm previous reports demonstrated sAC contribution to the mitochondrial function and ROS generation. Regarding the sAC role in mitochondrial biology, the novelty of the present study is that the sAC-dependent cAMP pool controls mitochondrial clearance, which was demonstrated by mitochondrial mass elevation without alteration in mitochondrial biogenesis. The role of AMPK in sAC knockdown effects on mitochondrial biology has been now analysed and is presented in the new Figure 6, as described above.

Fig 5. These data were recently observed in fibroblasts derived from sAC KO mice. The authors should acknowledge, compare and discuss Valsecci et al.

Response:

The data presented by Valsecci et al (2017) are discussed in the revised manuscript (page 11, line 20 – 27).

Fig 6 Is missing panel C.

Response:

We are sorry for the error in the Fig 6 legend. In the revised version the error (now in in the Fig. 7) has been corrected.

Reviewer #2:

Few points need to be clarify:

first of all, the AMPK phosphorylation appears to be not significant after EPAC agonist treatment (see fig. 3, panel d, the asterisk is absent). This is a very important point for that the authors claim.

Response:

We are sorry for unclear presentation of the data. In the submitted version the data have been compared only to the scrambled control. In the revised version we also compared EPAC effects to the own control. The analysis shows a significant elevation of pAMPK/AMPK ratio in shRNA+EPAC agonist group compared to the shRNA control group.

The absence of mitochondrial biogenesis has been observed, as well as the increased of mitochondrial mass. A marker of increased mitophagy should be also evaluated.

Response:

To our knowledge there are no reliable marker of mitophagy. The standard way to analyse the mitochondrial clearance is the evaluation of the mitochondrial mass (see refs 1,2 below). However, because alterations in mitogenesis may also affect mitochondrial mass independently of mitophagy, mitogenesis rate should be tested. In

the present study increased mitochondrial mass in combination with unchanged mitogenesis is a strong indicator for the disturbed mitophagy.
(1)Kundu M, et al. (2008). Ulk1 plays a critical role in the autophagic clearance of mitochondria and ribosomes during reticulocyte maturation. Blood 112, 1493–1502.
(2)Valentin-Vega YA, et al. (2012). Mitochondrial dysfunction in ataxia-telangiectasia. Blood 119, 1490–1500.

To improve the article, ROS production, ATP level, and mitochondrial membrane potential should be evaluated after EPAC agonist treatment in sAC silenced cells.

Response:

As suggested by reviewer, the effects of EPAC agonist under sAC knockdown have been investigated. The data presented in the supplementary figure 4 demonstrate that EPAC agonist significantly reduces mitochondrial ROS formation and prevents mitochondrial depolarization induced by sAC knockdown. These results are in agreement with attenuation of the sAC knockdown-induced mitochondrial mass elevation by treatment with EPAC agonist (supplementary figure 3). In contrast, no rescue effect of EPAC agonist was found on the total ATP content. Since the total cellular ATP content is a result of ATP synthesis and ATP consumption, one may suppose that EPAC agonist may affect both of these processes.

[Click here to view linked References](#)

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Title: Regulation of AMPK activity by type 10 adenylyl cyclase: contribution to the mitochondrial biology, cellular redox and energy homeostasis

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Abstract

The downregulation of AMP-activated protein kinase (AMPK) activity contributes to numerous pathologies. Recent reports suggest that the elevation of cellular cAMP promotes AMPK activity. However, the source of the cAMP pool that controls AMPK activity remains unknown. Mammalian cells possess two cAMP sources: membrane-bound adenylyl cyclase (tmAC) and intracellularly localized, type 10 soluble adenylyl cyclase (sAC). Due to the localization of sAC and AMPK in similar intracellular compartments, we hypothesized that sAC may control AMPK activity.

In this study, sAC expression and activity were manipulated in H9C2 cells, adult rat cardiomyocytes or endothelial cells. sAC knockdown depleted the cellular cAMP content and decreased AMPK activity in an EPAC-dependent manner. Functionally, sAC knockdown reduced cellular ATP content, increased mitochondrial ROS formation and led to mitochondrial depolarization. Furthermore, sAC downregulation led to EPAC-dependent mitophagy disturbance, indicated by an increased mitochondrial mass and unaffected mitochondrial biogenesis. Consistently, sAC overexpression or stimulation with bicarbonate significantly increased AMPK activity and cellular ATP content. In contrast, tmAC inhibition or stimulation produced no effect on AMPK activity. Therefore, the sAC–EPAC axis may regulate basal and induced AMPK activity and supports mitophagy, cellular energy and redox homeostasis. The study argues for sAC as a potential target in treating pathologies associated with AMPK downregulation.

Keywords: ADCY10, cAMP, AMPK, ROS, ATP, mitophagy

Abbreviations:

ACC, acetyl-CoA carboxylase; AMP, adenosine mono-phosphate; ADP, adenosine di-phosphate; AMPK, AMP-activated protein kinase; ATP, adenosine tri-phosphate; CAMKK2, calmodulin kinase kinase 2; cAMP, 3'-5'-cyclic adenosine monophosphate; CFP, cyan fluorescence protein; Drp1, dynamin-like protein 1; EPAC, exchange protein activated by cAMP; FRET, föster resonance energy transfer; GFP, green fluorescence protein; LKB1, liver kinase B1; MFF, mitochondrial fission factor; PDE, phosphodiesterase; PGC1 α , peroxisome proliferator-activated receptor- γ co-activator 1 α ; PKA, protein kinase A; ROS, reactive oxygen cycle; sAC, soluble adenylyl cyclase; TFAM, mitochondrial transcription factor A; tmAC, transmembrane adenylyl cyclase; ULK1, unc-51 like kinase 1; YFP, yellow fluorescence protein.

Introduction

AMP-activated protein kinase (AMPK) regulates diverse metabolic and physiological processes, such as lipid and glucose metabolism, protein synthesis, autophagy and redox homeostasis [1]. Emerging evidence highlights the role of AMPK in controlling cellular energy and redox homeostasis, particularly by supporting mitochondrial health [2].

Due to the association of dysregulated AMPK activity with aging and major chronic diseases, e.g., obesity, inflammation and diabetes [3-5], efforts have been made to understand the molecular mechanisms that control AMPK activity. AMPK activity regulation is a complex process. Two well-defined mechanisms of AMPK activation include the binding of AMP/ADP to the γ -subunit and the phosphorylation of the α -subunit at T172. Several signaling pathways, such as ROS, Akt, PKD1 and S6K [6-9], regulate AMPK activity. An emerging role of cAMP signaling has also been suggested. cAMP signaling promotes AMPK activity in endothelial cells [10], muscle cells [11] and adipocytes [12]. These effects have been partially attributed to the activation of EPAC, an exchange protein directly activated by cAMP [11]. In contrast, the inhibitory effect of the PKA-dependent phosphorylation of the AMPK α -subunit at S173, S485/491 or S497 has been observed [13,14], although a stimulatory effect of PKA on AMPK by the phosphorylation and activation of the upstream kinase LKB1 has also been identified [15]. The discrepancy in the cAMP signaling effects on AMPK activity may be due to the unspecific elevation of cellular cAMP, e.g., PDE inhibition [11]. PDE inhibition or treatment with cAMP analogous leads to the uncontrolled diffusion of cAMP throughout the cytosol, followed by the unselective activation of numerous cAMP targets within diverse compartments. Another widely used approach, the activation of tmAC [16,14], while specific, leads to cAMP elevation primarily within the sub-plasmalemmal compartment due to PDE activity [17], i.e., far from AMPK compartments within the cytosol or nucleus. Apart from tmAC, a second source of cAMP, type 10 soluble adenylyl cyclase (sAC), has been demonstrated for mammalian cells [18]. sAC has been shown to localize in various intracellular compartments [18,19] and provides both specificity and selectivity by allowing cAMP generation in close proximity to its intracellular targets. Of note, cytosolic and nuclear sAC localization allows the building of the cAMP pool in proximity to the main AMPK compartments. Whether the sAC-generated cAMP pool may modulate AMPK activity and, thereby, mitochondrial biology remains unknown and was the aim of the present study.

Using cardiac and endothelial cells, we demonstrated that sAC downregulation diminished AMPK activity, whereas sAC upregulation or activation promoted it. Reduced sAC activity was associated with disturbed mitochondrial clearance that culminated in oxidative stress and energy imbalance. Contrary to sAC, elevation of

cellular cAMP via tmAC activation did not affect AMPK activity, which suggests an exclusive role of the sAC-dependent cAMP pool in regulating AMPK activity.

Material and Methods

Cell culture and treatments

Cardiac rat embryonic myoblasts (H9C2, ATCC CRL-1446) was purchased from American Type Culture Collection. Cells were expanded and frozen in aliquots within 4 weeks of purchase. For the experiments, cells were thawed and cultured for no more than 4 further passages and were not allowed to grow more than 60% confluence. Cells were cultured in phenol red-free Dulbecco's modified Eagle's medium (DMEM, Sigma, #D2902), supplemented with 10% fetal bovine serum (FBS), 21 mmol/l sodium bicarbonate and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) in a 5% CO₂ incubator at 37°C.

In the bicarbonate stimulation experiments, cells were incubated in DMEM containing no bicarbonate (0% CO₂), 21 mmol/l (5% CO₂) or 42 mmol/l bicarbonate (10% CO₂) and supplemented with 15 mmol/l HEPES and 3% FBS. NaCl was used to adjust osmolarity and pH was set to 7.4 with sodium hydroxide and hydrochloric acid.

Treatments with 1 and 10 µmol/l forskolin (Sigma, Germany), 200 µmol/l 2',3'-dideoxyadenosine (Sigma, Germany), 200 µmol/l IBMX (Sigma, Germany), 200 µmol/l 6-Bnz-cAMP (BioLog, Germany), 200 µmol/l 8-CPT-2Me-cAMP (BioLog, Germany), 10 µmol/l antimycin A (Sigma, Germany), 1 mmol/l H₂O₂ (Sigma, Germany), 1 mmol/l NaCN (Sigma, Germany), 5 mmol/l 2-deoxy-D-glucose (Sigma, Germany), 50 µmol/l resveratrol (Sigma, Germany), 10 µmol/l A769662 (Tocris, Germany) or 10 µmol/l compound C (Sigma, Germany) were performed as indicated.

Isolation of adult rat coronary endothelial cells

Treatment of rats to isolate endothelial cells were performed in consent with the directive of the EU (2010/63/EU). Coronary endothelial cells were obtained and cultured as described previously [20]. For this purpose, adult male rats (200–300 g) were euthanized using deep isoflurane (5%) anesthesia, hearts were rapidly excised, washed with ice-cold 0.9% NaCl and connected to the perfusion system for perfusion with collagenase type II (300 U/ml). Anesthesia depth was monitored by limb withdrawal using toe pinching. Myocytes-free cell suspension was treated with trypsin for 10 min and seeded in cell culture dishes for 1 h in DMEM supplemented with 15% FBS followed by vigorous washing. This protocol excludes all non-endothelial cells, while leaving endothelial cells in sufficient quantity. The purity of the cell culture (>98%) was confirmed by immunochemical staining with antibodies against

von Willebrand factor (Sigma, Germany). Experiments were performed with monolayers reaching 90% confluence.

sAC overexpression and knockdown

For sAC overexpression experiments, cells were transfected with GFP or sAC (50 kDa) encoding plasmids (pTurbo-CMV) kindly provided by Dr. R. Acin-Perez (Centro Nacional de Investigaciones Cardiovasculares, Madrid). Briefly, 400,000 cells resuspended in 100 µl electroporation buffer (5 mmol/l KCl, 15 mmol/l MgCl₂, 50 mmol/l mannitol, 86.4 mmol/l Na₂HPO₄, 33.6 mmol/l NaH₂PO₄, pH=7.2) containing 8 µg plasmids and were electroporated using T-020 program for H9C2 cells and S-005 for coronary endothelial cells in Amaxa Nucleofector II Device (Lonza). The protocol was optimized by series of experiments with GFP-targeted plasmids and shows 81.5 ± 3.8% (n=5) GFP-positive cells 24 h after transfection determined by fluorescence microscopy (Online Resource, Supplementary Fig. 1). All downstream analyses were carried out 24 h after transfection.

For sAC knockdown experiments, cells were transfected either with scramble or with sAC-shRNA encoding plasmids. Electroporation procedure was the same as for sAC overexpression. All downstream analyses were carried out 3 days after transfection. sAC shRNA (5'-GGGGTACCAAAAAAGTGGAAAGTGGAAACGAAAGCATCTCTTGAATGCTTTTCGTTCCACTTTCCACAACAAGGCTTTTCTCCAAG-3') was expressed by the U6 promoter and a randomized sequence (scramble) based on sAC shRNA sequence was used as a control. Hairpin loop in the sequence is underlined.

Western blotting

Cells were lysed in Laemmli buffer and proteins were quantified using Pierce 660 nm Assay Kit. Equal amounts of total proteins were separated by SDS-polyacrylamide gels and transferred onto nitrocellulose membrane. The blots were blocked with 5% BSA in TBST for 1 h at room temperature and incubated overnight with the primary antibodies: ACC (Cell Signaling, Germany), AMPK (Cell Signaling, Germany), GAPDH (MD Millipore, Germany), pACC (Cell Signaling, Germany), pAMPK (Cell Signaling, Germany), PGC1α (Abcam, Germany), sAC (R21, CEP Biotech, NY), β-actin (Santa Cruz, Germany), TFAM (Acris, Germany), α-Tubulin (Sigma, Germany). After incubation with the HRP-conjugated secondary antibodies, specific bands were visualized by chemiluminescence using an ECL kit (Promega, Germany). To confirm equal protein loading, membranes were stripped and immunostained for actin, tubulin or GAPDH.

FRET-based assay of cytosolic cAMP

For FRET-based live cell imaging, cells were transfected with plasmids encoding EPAC-based cAMP sensor, H187 [21] for cAMP analysis. Transfection was performed by electroporation as mentioned above and cells were seeded on 24 mm glass coverslips and cultured for 48 h, followed by 24 h starvation in DMEM supplemented with 3% FBS.

The FRET-based cytosolic cAMP assay was performed as previously described [22]. cAMP probes were excited at 430 nm and emission light was acquired at 470 nm for CFP and 530 nm for YFP channel. Images were acquired with an inverted microscope (oil immersion objective 40x, Zeiss, Germany) and an imaging system (Visitron, Germany) every 6 s. The analysis of the FRET signal was performed by VisiView software (Visitron, Germany). Emission signals obtained in the cell-free region (background) were subtracted from corresponding emission signals obtained within the region of interest and presented as ratio of CFP/YFP.

Analysis of mitochondrial membrane potential

H9C2 cells were loaded with TMRM (50 nmol/l; Thermo Scientific, Germany) and Mitogreen (100 nmol/l; Thermo Scientific, Germany) at 37°C for 20 min. Cells were then washed, trypsinized and resuspended in ice-cold PBS and were subjected to the flow cytometry analysis applying MACSQuant Analyzer 10 (Miltenyi Biotec, Germany). Cells were excited at 488 nm and the emission fluorescence was collected at 535 nm for MitoGreen and 590 nm for TMRM. Mitochondrial membrane potential is expressed as the fluorescence ratio of TMRM to Mitogreen fluorescence. At least 10,000 events were recorded per sample.

In some experiments TMRM and MitoGreen fluorescence was analyzed applying imaging system. Images were acquired with an inverted microscope (oil immersion objective 40x, Zeiss, Germany) and an imaging system (Visitron, Germany) every 10 s. The analysis of the emission signal was performed by VisiView software (Visitron, Germany). Emission signals obtained in the cell-free region (background) were subtracted from corresponding emission signals obtained within the region of interest and presented as ratio of TMRM/MitoGreen.

ROS analyses

To examine total cellular reactive oxygen species (ROS), cells were loaded with DCF (2',7'-dichlorodihydrofluorescein diacetate, succinimidyl ester, 10 µmol/l, Invitrogen, Germany), a nonfluorescent dye, which is converted into the highly fluorescent DCF in the presence of free radicals. DCF fluorescence was analyzed by excitation at 488 nm and emission at 530 nm applying ELISA reader. Values are presented as a ratio of DCF fluorescence to total protein amount per well.

Mitochondrial ROS was examined by loading cells with MitoSox (10 µmol/l, Invitrogen, Germany), a fluorescent dye that accumulates in mitochondria. It is rapidly oxidized by superoxide anion and produces red fluorescence. MitoSox fluorescence was analyzed by excitation at 510 nm and emission at 580 nm with ELISA reader. Values are presented as a ratio of DCF fluorescence or MitoSox fluorescence to total protein amount per well.

Analysis of mitochondrial mass

Amounts of nuclear DNA and mitochondrial DNA (mtDNA) were determined by quantitative real-time PCR. The ratio of mtDNA to nuclear DNA was used as a measure the mitochondrial content. Total genomic DNA was isolated using Quick-gDNA Miniprep Kit (Zymo Research, Germany). Quantitative PCR was performed by using the following primers (mtDNA specific cytochrome b primers: forward 5'-CCACATCTGCCGAGACGTAA-3' and reverse 5'-TAGTCCTCGTCCCACATGGA-3'; nuclear DNA specific β-globin primers: forward 5'-AAGTACCACTAAGCCCCCTTTC-3' and reverse 5'-GGGAACACAAAAGACCTCTTCTGG-3') and SYBR Green PCR Kit in a StepOnePlus real-time PCR system (Applied Biosystem, USA).

Mitogreen dye (Invitrogen, Germany) accumulates in mitochondria irrespectively of mitochondrial membrane potential [23] and, thus widely used for determination of mitochondrial mass. Briefly, cells were loaded with Mitogreen (100 nmol/l) at 37°C for 20 min. Cells were then washed, trypsinized and resuspended in ice-cold PBS and were subjected to flow cytometry analysis applying MACSQuant Analyzer 10 (Miltenyi Biotec, Germany). Cells were excited at 490 nm and the emission fluorescence was collected at 535 nm. At least 10,000 events were analyzed per sample.

Quantitative real-time PCR

Total RNA was isolated by using the RNA-Bee reagent (AMS Biotechnology, Germany), according to the manufacturer's instructions. RNA was subsequently reverse transcribed to cDNA by using the high capacity cDNA reverse transcription kit (Applied Biosystems, Germany). The mRNA levels were measured by real time PCR using iTaq Universal SYBR Green Supermix and the CFX96 qPCR system (Bio Rad, Germany). Quantitative PCR was performed by using the following primers for mitochondria-encoded genes (COX1: forward primer 5'-ATCGCAATTCTCTACAGGCGT-3'; reverse primer 5'-GGCTCATAATATGGCGGGGG-3'; ATP6: forward primer 5'-CGCCTAATCAGCAACCGACT-3'; reverse primer 5'-TTAGGGCTCAGGTTCTGTCCT-3'; ND4: forward primer 5'-ACCCCCTACCCTCAACATGA-3'; reverse primer 5'-GCTTCTACGTGGGCTTTTGG-3'; Cytb: forward primer 5'-TGCCGAGACGTAAACTACGG-3'; reverse primer 5'-

1 TAGTCCTCGTCCCACATGGA-3') and nuclear-encoded mitochondrial genes (Atp5b: forward primer 5'-
2 AGCAGACTGGTTTTGGAGGT-3'; reverse primer 5'-GGCCTCTAACCAAGCCTTCA-3'; COX5B: forward
3 primer 5'-GATAGCAGCACAGAGGGGAC-3'; reverse primer 5'-CGGATGGGACTAGATTGGGG-3';
4 COX7B: forward primer 5'-TAGTCACCGCAGTTCCATCC-3'; reverse primer 5'-
5 CTTGCCACCACTTGCTGAATG-3'; NDUF51: forward primer 5'-GGCAGGACTTGCCAAAGGAT-3';
6 reverse primer 5'-GCAGCCCCTGGGAGAATAAC-3'; NDUF57: forward primer 5'-
7 CTACTCGGTTGTTTCGTGGCT-3'; reverse primer 5'-AGCTGCAAGATGCCGTAGAG-3').

16 **Analyses of total cellular ATP and cAMP**

17 Total cellular ATP was measured using ATPLite Luminescence Assay System (PerkinElmer, USA). Briefly, cells
18 were washed with PBS and lysed using the ice-cold lysis buffer provided by the manufacturer. Cell lysates were
19 transferred to a white-walled 96-well plate alongside the series of ATP standards used for calibration.
20 Bioluminescence produced after the addition of ATPLite substrate was measured using the Victor Luminometer
21 (PerkinElmer, USA) and the amount of ATP in the lysates was calculated using the calibration curve. Values were
22 presented as a ratio of ATP to total protein content.

23 The analysis of the total cellular cAMP content was performed using a Direct cAMP ELISA Kit (Enzo, Germany).
24 Shortly, cells were washed with PBS and lysed using the ice-cold lysis buffer provided by manufacturer. The
25 measured absorbance at 405 nm was used to calculate the concentration of the cAMP by applying a calibration
26 curve. Values were presented as a ratio of cAMP to total protein content.

42 **Data and statistical analysis**

43 The data are given as mean $\pm$ SEM, where mean indicates biological replicates. Comparisons of the mean between
44 two groups were performed using unpaired two-tailed t-tests assuming equal variance. For more than two groups,
45 two-way ANOVA followed by Bonferroni post-test was used. Statistical significance was accepted when $P < 0.05$.

52 **Results**

56 **sAC-EPAC axis controls basal AMPK activity**

57 To test the role of sAC in basal AMPK activity in cardiac cells, sAC knockdown was performed in H9C2 cells.
58 The downregulation of sAC expression significantly reduced cellular cAMP and the phosphorylation of AMPK

and its direct target ACC, a marker of AMPK activity (Fig. 1). In addition, by applying the protein samples from a study performed with a primary culture of adult rat cardiomyocytes [24], we found that sAC knockdown significantly reduced AMPK phosphorylation (Fig. 2a, b). A similar effect of sAC knockdown was found in rat coronary endothelial cells (Fig. 2c, d). Therefore, sAC supports the basal activity of AMPK independently of cell type.

Because sAC knockdown was accompanied by a significant reduction in cellular cAMP (Fig. 1b), we aimed to determine which downstream target of cAMP, PKA and/or EPAC, may be involved in the regulation of AMPK activity by sAC. Treatment with specific PKA agonist had no effect on AMPK phosphorylation in sAC knockdown cells but reduced AMPK phosphorylation in control cells (Fig. 3a, b). In contrast, EPAC agonist rescued the effect of sAC knockdown on AMPK phosphorylation (Fig. 3c, d). Thus, the sAC–EPAC axis is involved in regulating basal AMPK activity.

sAC downregulation jeopardizes mitophagy, as well as cellular energy and redox balances

Because AMPK activity is a key regulator of cellular redox and energy homeostasis as well as mitochondrial biology [2], we examined the functional effects of sAC knockdown. The downregulation of sAC expression led to the elevation of total cellular (DCF fluorescence) and mitochondria-specific (MitoSox fluorescence) ROS formation (Fig. 4a, b). These effects were accompanied by mitochondrial depolarization and a significant reduction in the total cellular ATP level (Fig. 4c, d).

AMPK plays a substantial role in regulating mitochondrial quality control, or mitophagy [2]. In line with these data, downregulation of sAC expression in H9C2 cells significantly increased mitochondrial mass, as demonstrated by an elevation in Mitogreen staining, a mitochondrial membrane potential-independent mitochondrial dye [23], and by the mtDNA/nDNA ratio (Fig. 4e, f). Similarly, the knockdown of sAC in rat coronary endothelial cells significantly increased mitochondrial ROS formation and mitochondrial mass (Fig. 4g, h).

To exclude the potential effect of mitochondrial biogenesis on mitochondrial mass, expression analyses of nuclear (PGC-1 α) and mitochondrial (TFAM) transcription factors (western blot) as well as nuclear- and mitochondria-encoded mitochondrial proteins (qPCR) were performed. We found significant downregulation of both transcription factors and no effect on mitochondrial gene expression (Fig. 5). Thus, sAC knockdown likely does not affect mitochondrial mass via upregulating mitochondrial biogenesis but rather due to disturbed mitochondrial clearance.

To strengthen these findings, a direct pharmacological suppression of AMPK activity, i.e., treatment with AMPK inhibitor compound C [25], was applied. Compound C markedly reduced AMPK phosphorylation, which was accompanied by significant mitochondrial depolarization and the elevation of mitochondrial mass (Online Resource, Supplementary Fig. 2).

To prove the causal role of AMPK downregulation in disturbed mitochondrial clearance and redox balance, treatment with AMPK agonist A769662 was performed in control and sAC depleted cells. Though, AMPK agonist significantly elevated AMPK and ACC phosphorylation (Fig. 6a, b), it did not prevent elevation of mitochondrial mass and ROS formation induced by sAC knockdown (Fig. 6c, d). In contrast, treatment with AMPK agonist rescued the sAC knockdown-induced downregulation of TFAM and Sirt3 (Fig. 6e, f), two key mitochondrial proteins involved in expression of mitochondria-encoded genes and activation of numerous mitochondrial enzymes, respectively.

Because EPAC in our study is involved in regulating AMPK activity, we were also interested in its role in mitochondrial homeostasis regulation. Consistently, the sAC knockdown-induced elevation of mitochondrial mass was significantly attenuated by treatment with EPAC but not PKA agonist (Online Resource, Supplementary Fig. 3). Similarly, EPAC agonist partly reversed the elevation of mitochondrial ROS formation and prevented mitochondrial depolarization, though it did not affect reduction of cellular ATP content in sAC depleted cells (Online Resource, Supplementary Fig. 4)

These results suggest that sAC knockdown leads to the impaired mitophagy that is accompanied by disturbed cellular energy and redox balances.

Stimulation of sAC, but not tmAC, promotes AMPK activity

Several studies have demonstrated that the upregulation of cAMP signaling affects AMPK activity, although the results are controversial, and AMPK activation and inhibition have been reported [26,11]. Thus, to prove whether the upregulation of sAC signaling may affect AMPK activity, two models were applied.

First, the overexpression of active 50 kDa sAC isoform in H9C2 cells was performed. sAC overexpression significantly elevated cellular cAMP concentration and increased AMPK phosphorylation (Fig. 7). sAC overexpression was accompanied by a significant increase in cellular ATP content and had no effect on mitochondrial mass (Fig. 7).

Second, stimulation of sAC with bicarbonate, a natural sAC activator [27,28], was applied. Bicarbonate led to the stable (until 24 h) and dose-dependent elevation of AMPK activity (Fig. 8a and Online Resource, Supplementary Fig. 5). Because this effect could be prevented by sAC knockdown (Fig. 8b), we suggest a causal role of sAC in

bicarbonate-induced AMPK activation. Similarly to sAC overexpression, treatment with bicarbonate increased the cellular ATP content (Fig. 8c).

To determine whether tmAC stimulation may also promote AMPK activity, treatment with forskolin was performed. A western blot assay demonstrated that tmAC stimulation had no effect on cellular AMPK phosphorylation (Fig. 8d), although in the present study (Online Resource, Supplementary Fig. 6a) and in previous study applying a similar model [22] it significantly elevated cytosolic cAMP content. Consistently, tmAC suppression with 2',5'-dideoxyadenosine had no effect on AMPK phosphorylation, although it significantly inhibited tmAC activity (Online Resource, Supplementary Fig. 6). Altogether, these findings demonstrate that AMPK activity is supported by sAC but not tmAC.

Discussion

The aim of this study was to prove the role of sAC in AMPK activity regulation and its functional significance in mitochondrial biology. The main findings are as follows: the downregulation of sAC expression (i) significantly reduced basal AMPK activity in an EPAC-dependent manner and (ii) disturbed mitochondrial clearance, cellular energy and redox balances. (iii) The upregulation of sAC expression or activity promoted AMPK activity and improved cellular energy balance. (iv) In contrast to sAC, the stimulation or suppression of tmAC had no effect on AMPK activity.

Although several studies suggest the regulation of AMPK activity by cAMP, the results are controversial [26,14,11], and only a tmAC-dependent source of cAMP was suggested. Of note, cAMP synthesized by tmAC is mainly restricted within the subplasmalemmal compartment as long as PDEs are active. In contrast, numerous functional compartments distributed throughout the cell can be built by sAC [29]. Particularly, sAC is localized within the nucleus and in the cytosol near the main AMPK compartments [30-32]. Therefore, sAC is an ideal candidate in regulating AMPK activity by cAMP, particularly under basal conditions. Indeed, applying sAC knockdown in H9C2 cells a significant downregulation of AMPK activity was observed in the present study. This effect appears to not be restricted to a particular cell type and was also observed in primary cardiomyocytes and coronary endothelial cells. Altogether, sAC plays an essential role in supporting basal AMPK activity independently of cell type.

In contrast to our findings, Valsecci et al [33] have shown an increased AMPK activity in mouse embryonic fibroblast derived from sAC knockout mice compared to cells from wild type mice. The elevated AMPK activity might be due to the metabolic stress, a primary stimulus for AMPK activation, as mitochondrial sAC plays a key

role in ATP synthesis [34]. Indeed, ATP synthesis rate was significantly reduced in the study. The discrepancy to our results, i.e., reduction of AMPK activity, may be due to the partial suppression of sAC expression in our study, which is not sufficient to reach a threshold for AMPK activation due to metabolic stress. Thus, the present study uncovers an unique mechanism of the AMPK activity regulation, which was masked in the study of Valsecchi et al [33] under sAC knockout due to metabolic stress.

By analyzing the potential downstream target of sAC involved in basal AMPK activity regulation, we examined the contribution of PKA and EPAC. Previous studies suggested their controversial roles in regulating AMPK activity, i.e., a stimulating effect of EPAC [11] and an inhibitory effect of PKA [26,14]. In agreement with these reports, we determined that the activation of EPAC, not PKA, rescued AMPK activity in sAC knockdown cells. Additionally, control cells treated with EPAC agonist promoted AMPK activity, whereas PKA agonist suppressed it. Therefore, the sAC–EPAC axis supports AMPK activity under basal conditions. Though we did not further investigate the signaling link between EPAC and AMPK, previous studies clearly demonstrated the contribution of Rap1/2 - phospholipase C - calcium/calmodulin-dependent kinase kinase β in various cell types, including cardiomyocytes [35,11].

Due to the significance of AMPK in regulating cell metabolism and the association of its downregulation with aging and metabolic diseases [2], intensive efforts have been made to develop tools that promote AMPK activity, such as AMPK agonists and resveratrol [11,36-38]. Therefore, we were interested in whether sAC upregulation or stimulation promotes AMPK activity. Since bicarbonate is a natural sAC stimulator [27], cells were treated with bicarbonate and a stable, at least within 24 h, and, dose-dependent upregulation of AMPK activity was observed. The effect of bicarbonate is likely due to the activation of sAC because (i) in a similar model, bicarbonate leads to cAMP elevation in a sAC-dependent manner [22] and (ii) sAC knockdown prevented a bicarbonate effect on AMPK (Fig. 8b). Similarly to the sAC stimulation using bicarbonate, the overexpression of sAC significantly upregulated AMPK activity. In agreement with these findings, a previous study by Jaitovich et al. [39] demonstrated that hypercapnia, a state of increased intra-cellular bicarbonate concentration due to the activity of carbonic anhydrases [40], leads to AMPK activation in skeletal muscle, although no mechanistic analyses have been performed.

In contrast to sAC, the activation of tmAC with forskolin had no significant effect on AMPK phosphorylation or activity. In line with our finding, negative effects of tmAC stimulation on AMPK activity have also been identified in several cell types by other studies and were attributed to the PKA-dependent phosphorylation of the AMPK α -subunit at S173, S485/491 or S497 [13,14]. Thus, the two cAMP sources in the cell, tmAC and sAC, play controversial roles in AMPK regulation.

1 AMPK is a well-known key regulator of mitochondrial biogenesis, dynamics and clearance, and hence the cellular
2 energy and redox homeostasis [2]. Therefore, we were interested in whether the sAC-dependent regulation of
3 AMPK activity may be translated into mitochondrial biology and cellular ATP content. In agreement with previous
4 reports [34,33,41], we discovered that sAC knockdown led to mitochondrial depolarization, increased
5 mitochondrial ROS formation and reduced cellular ATP content, which are typical fingerprints of mitochondrial
6 dysfunction. Surprisingly, we also observed a significant increase in mitochondrial mass, whereas no effect on
7 mitochondrial biogenesis was observed. These observations argue for a disturbed mitochondrial clearance, or
8 mitophagy, in sAC-depleted cells. Similar mitochondrial depolarization and mitochondrial mass elevation have
9 been found with pharmacological approaches, such as the suppression of AMPK activity with compound C.
10 The role of AMPK in regulating mitophagy has only recently been appreciated. A recent study by Toyama et al.
11 [42] demonstrated that the AMPK-dependent phosphorylation of MFF resulted in the mitochondrial recruitment
12 of Drp1 and consequent mitochondrial fission, a key step in generating mitochondrial particles that are sufficiently
13 sized for autophagosome engulfment. Another recent study demonstrated that in ULK1-knockout murine
14 embryonic fibroblasts, neither metabolic stress nor starvation was able to induce autophagy [43]. The study also
15 reported that the increased accumulation of defective mitochondria was due to a lack of mitophagy in cells
16 expressing a mutated form of ULK1 that cannot be phosphorylated by AMPK.

17 Applying AMPK activator A769662 to prove the causal role of AMPK in mitophagy disturbance induced by sAC
18 knockdown, we found no effect of AMPK agonist. This finding suggests that sAC knockdown may affect
19 mitophagy independently of AMPK. Interestingly, a recent report of Rahman et al [19] demonstrated an essential
20 role of sAC in lysosomal function via supporting lysosomal acidification. Since lysosomes play a key role at the
21 mitophagy process, one may suppose that sAC knockdown-induced failure in lysosomal acidification may cause
22 mitophagy disturbance.

23 Nevertheless, AMPK activity seems to play a causal role in mitochondrial homeostasis disturbed by sAC
24 knockdown. Particularly sAC knockdown-induced downregulation of two mitochondrial proteins playing key
25 roles in expression of mitochondria-encoding genes and regulation of enzyme activity in mitochondria, i.e., TFAM
26 and Sirt3, was rescued by treatment with AMPK agonist (Fig. 6e, f). Altogether, the study suggests that sAC
27 knockdown affect mitochondrial biology in AMPK-dependent as well as AMPK-independent manner.

28 Although no data are available regarding the role of sAC in mitochondrial dynamics, the PKA localized on the
29 outer mitochondrial membrane suppresses mitochondrial fission by the phosphorylation of Drp1 at Ser637 [44,45]
30 or Ser656 [46] and by the destabilization of PINK1 [47]. Because mitochondrial fission is a key step in mitophagy
31 [48], PKA may suppress mitophagy. Thus, PKA is unlikely to be involved in disturbed mitophagy under sAC

knockdown. Indeed, treatment with PKA-agonist in our study failed to rescue mitochondrial mass elevation in sAC-depleted cells (Online Resource, Supplementary Fig. 3).

The role of cAMP target EPAC in regulating the mitochondrial clearance process remains unknown. A recent report argues for the supporting role of EPAC1 in mitochondrial fission [49]. The findings of the present study that treatment with EPAC agonist, not PKA agonist, rescued both (i) the sAC knockdown-induced mitophagy disturbance and (ii) AMPK inactivation provide the first clue for the role of the sAC–EPAC-AMPK axis in regulating mitochondrial clearance.

Accumulating data suggest the involvement of sAC-dependent cAMP pool in numerous pathologies accompanied by excessive cell death [24,50,51] or growth [52,53]. In a recent study, Wang et al [54] showed that advanced heart failure in rats is accompanied by a significant downregulation of sAC that leads to a reduced mitochondrial resistance to Ca^{2+} , which demonstrates a potential role of sAC in cardiac pathology. Whether this sAC downregulation may contribute to AMPK activity and mitochondrial biology is yet to be investigated.

In conclusion, this study suggests that a sAC–EPAC axis controls basal AMPK activity and supports mitochondrial clearance, cellular energy and redox homeostasis. The overexpression or activation of sAC promotes AMPK activity and improves the cellular energy balance. In contrast to sAC, tmAC does not affect AMPK activity. This study suggests sAC as a potential target in diseases accompanied by mitochondrial dysregulation such as heart failure [54].

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figure Legends

Fig. 1

sAC knockdown suppressed AMPK activity. Transfection of H9C2 cells with plasmids encoding either sAC-targeted (sAC shRNA) or scramble shRNA significantly reduced sAC expression (**a**), cellular cAMP content (**b**) and the phosphorylation of AMPK (**c**) and its direct target ACC (**d**). Data are means $\pm$ SEM. n = 10 for **a**, **c**, **d** and n = 6 for **b**. *P<0.05 vs. scramble

Fig. 2

Downregulation of sAC expression reduced AMPK activity in adult rat cardiomyocytes and coronary endothelial cells. Statistical expression analyses of sAC and phosphorylated AMPK performed in adult rat cardiomyocytes (**a,b**) and rat coronary endothelial cells (**c,d**) transfected with plasmids encoding either sAC-targeted (sAC shRNA) or scramble shRNA (for methodological details see [24]). Data are means $\pm$ SEM. n = 3 for **a**, n = 8 for **b** and n = 4 for **c**, **d**. *P<0.05 vs. scramble

Fig. 3

Activation of EPAC, not PKA, rescued AMPK phosphorylation. Effect of 1 h treatment with 200 μ mol/l PKA agonist 6-Bnz-cAMP (**a–b**) or with 200 μ mol/l EPAC agonist 8-CPT-2Me-cAMP (**c–d**). Treatments were performed in H9C2 cells transfected with plasmids encoding either sAC-targeted (sAC shRNA) or scramble shRNA. Data are means $\pm$ SEM. n = 4 for **a–c** and n = 6 for **d**. *P<0.05 vs. scramble control, #P<0.05 vs. sAC shRNA control.

Fig. 4

sAC knockdown disturbed cellular redox and energy homeostasis and increased mitochondrial mass. Analyses of total cellular ROS formation (DCF fluorescence, **a**), mitochondrial ROS formation (MitoSox fluorescence, **b**), mitochondrial membrane potential (**c**), total cellular ATP (**d**), and mitochondrial mass (**e–f**) performed with H9C2 cells. Analyses of mitochondrial ROS formation (**g**) and mitochondrial mass (**h**) performed with adult rat coronary endothelial cells (RCEC). Cells were transfected with plasmids encoding either sAC-targeted (sAC shRNA) or scramble shRNA. Data are means $\pm$ SEM. n = 6–8. *P<0.05 vs. scramble. Treatments with H₂O₂ (1 mmol/l, 1 h), antimycin A (10 μ mol/l, 1 h), 1 mmol/l NaCN + 5 mmol/l 2DOG (40 min) and resveratrol (50 μ mol/l, 24 h) were used as positive controls

Fig. 5

sAC knockdown reduced the expression of mitochondria-related transcription factors but did not affect the expression of mitochondrial genes. Expression analysis of transcription factors PGC1 α and TFAM (western blot, **a–b**), and mitochondria- and nucleus-encoded mitochondrial genes (**c**) performed in H9C2 cells transfected with plasmids encoding either sAC-targeted (sAC shRNA) or scramble shRNA. Data are means $\pm$ SEM. n = 5. *P<0.05 vs. scramble

Fig. 6

AMPK activator rescued the expression of mitochondrial proteins under sAC knockdown in H9C2 cells. Treatment with A769662 (A76, 10 μ mol/l) for 24 hours significantly increased phosphorylation of AMPK (**a**) and its direct target ACC (**b**). Analyses of mitochondrial ROS formation (MitoSox fluorescence, **c**), mitochondrial mass (Mitogreen, **d**) and expression of mitochondrial proteins TFAM and Sirt3 (**e, f**) under sAC knockdown with or without treatment with A76 10 μ M for 24 hours. Data are means $\pm$ SEM. n=3–4 for **a, b, e, f** and n=6–8 for **c** and **d**. *P<0.05 vs. control or scramble and #P<0.05 vs. sAC shRNA

Fig. 7

sAC overexpression increased AMPK phosphorylation and the cellular ATP content. Analyses of sAC expression (**a**), AMPK phosphorylation (**b**) and mitochondrial mass (**d**) and total cellular ATP content (**d**) performed in H9C2 cells transfected with plasmids encoding either 50-kDa sAC isoform (sAC) or GFP. Data are means $\pm$ SEM. n = 4. *P<0.05 vs. GFP. Treatment with 1 mmol/l NaCN + 5 mmol/l 2DOG (40 min) was used as a positive control

Fig. 8

Stimulation of sAC but not tmAC increased AMPK phosphorylation and cellular ATP content. Analyses of AMPK phosphorylation (**a, b, d**) and total cellular ATP content (**c**) performed in control, untransfected H9C2 cells, or in cells transfected with plasmids encoding either sAC-targeted (sAC shRNA) or scramble shRNA. Cells were incubated in medium containing 0, 21 or 42 mmol/l bicarbonate for 1 h in (**a, b, d**) and for 4 h in (**c**). In (**d**), cells were treated with 1 or 10 μ mol/l forskolin, or vehicles (DMSO) in medium containing 21 mmol/l bicarbonate for 1 h. Data are means $\pm$ SEM. n = 16 for (**a, d**), n = 6 for (**b**) and n = 4 for (**c**). *P<0.05 vs. 0 mmol/l HCO₃⁻ in (**a**). *P<0.05 vs. scramble at 21 mmol/l HCO₃⁻ in (**b**). *P<0.05 vs. 21 mmol/l HCO₃⁻ in (**c**). *P<0.05 vs. DMSO in (**d**). #P<0.05 vs. 21 mmol/l HCO₃⁻ in (**a**)

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Figure 1

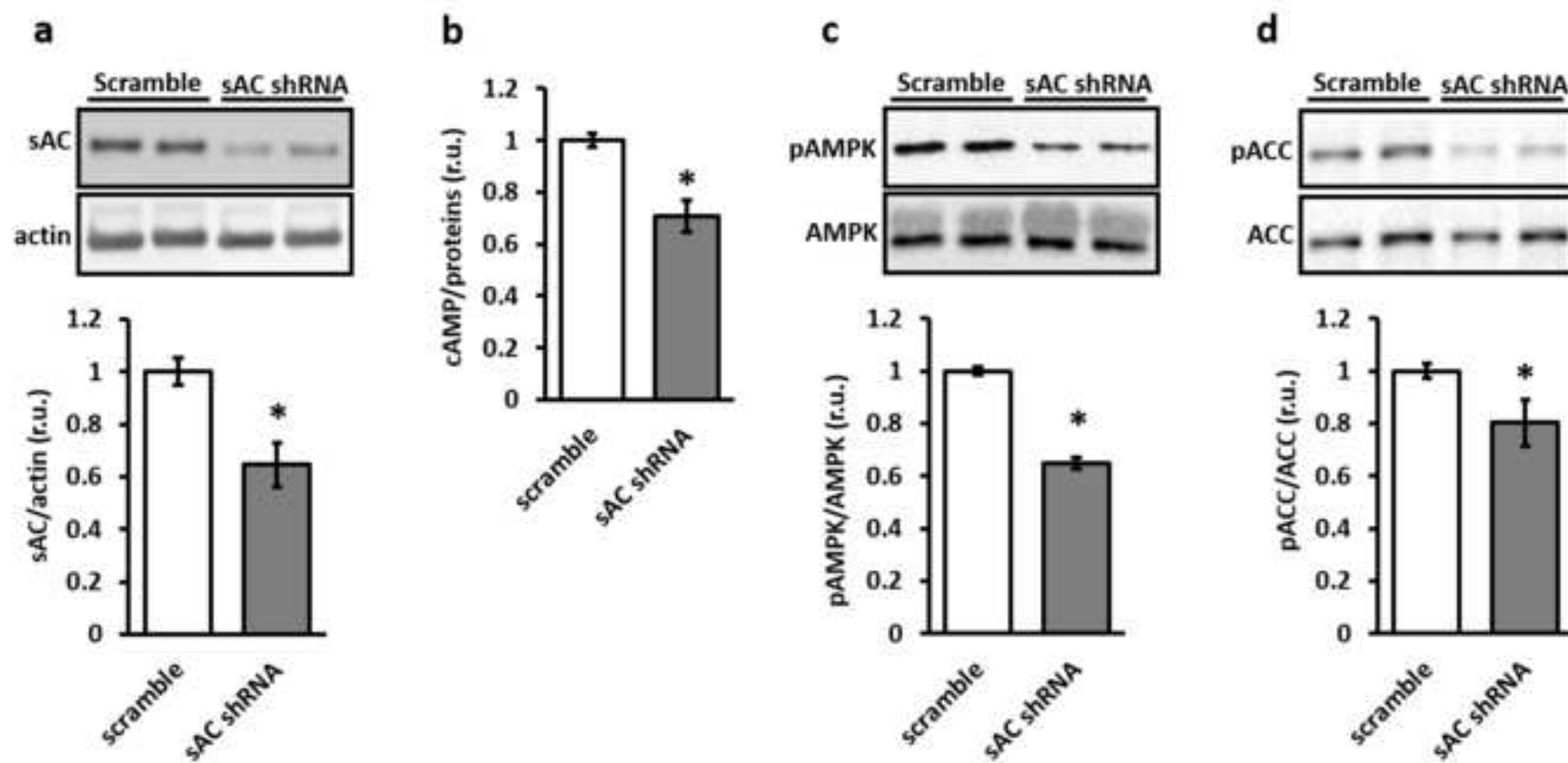


Figure 2

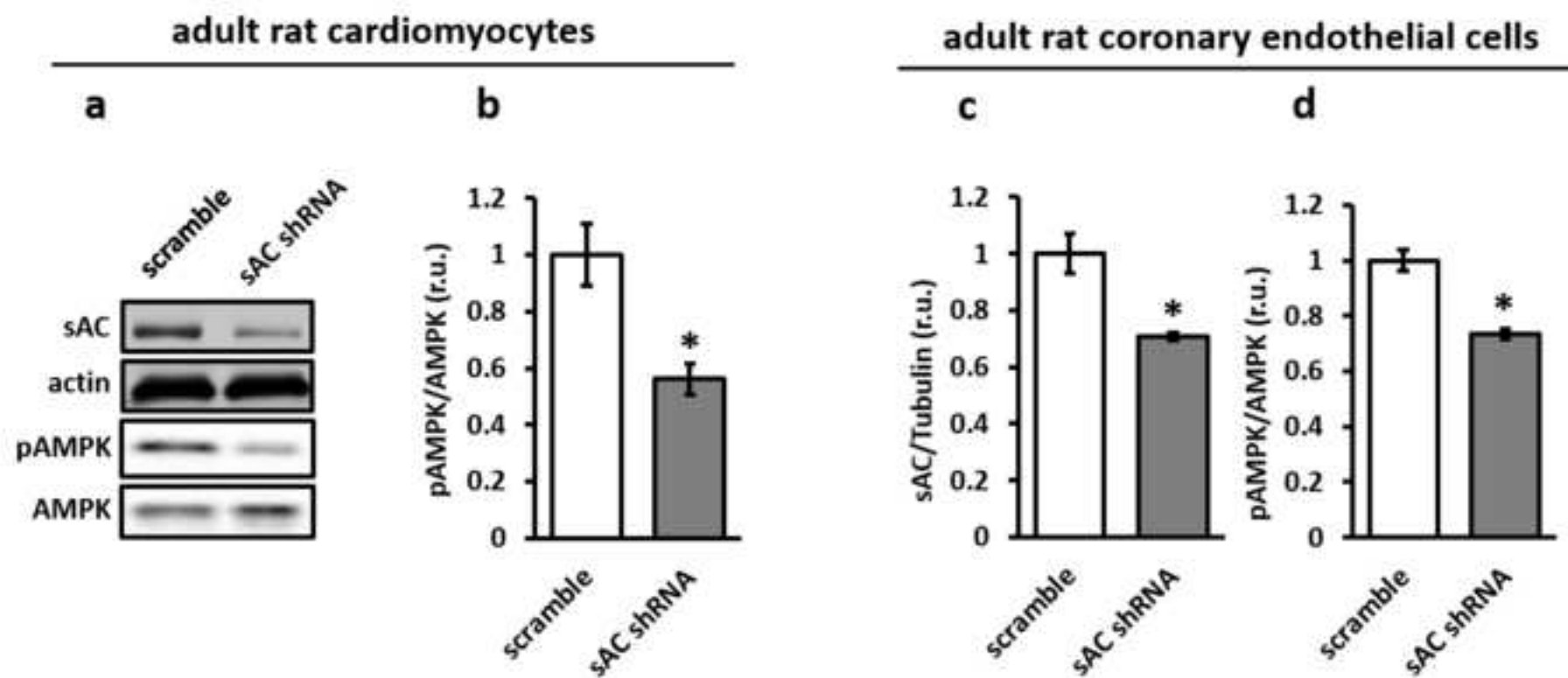


Figure 3

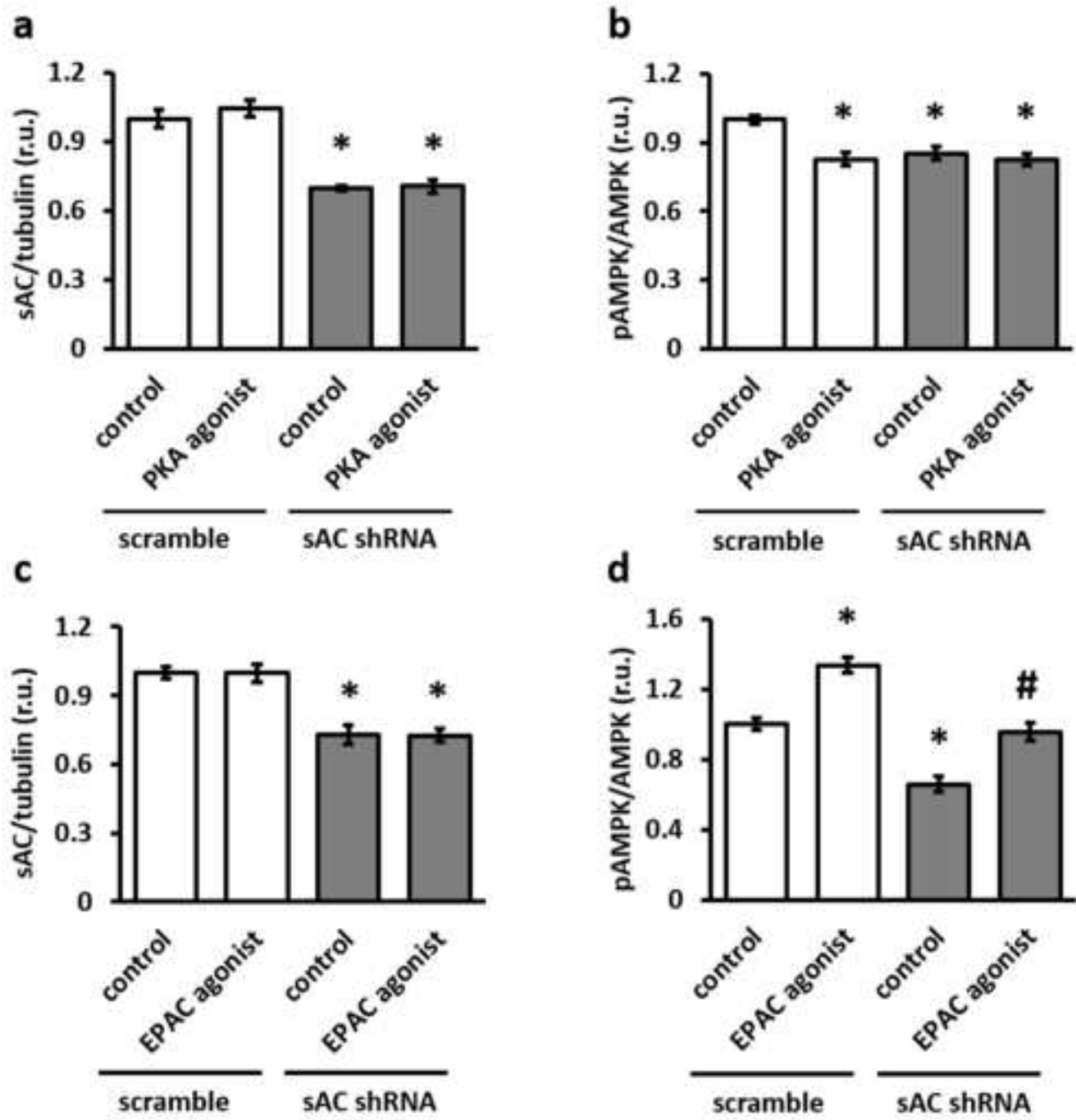


Figure 4

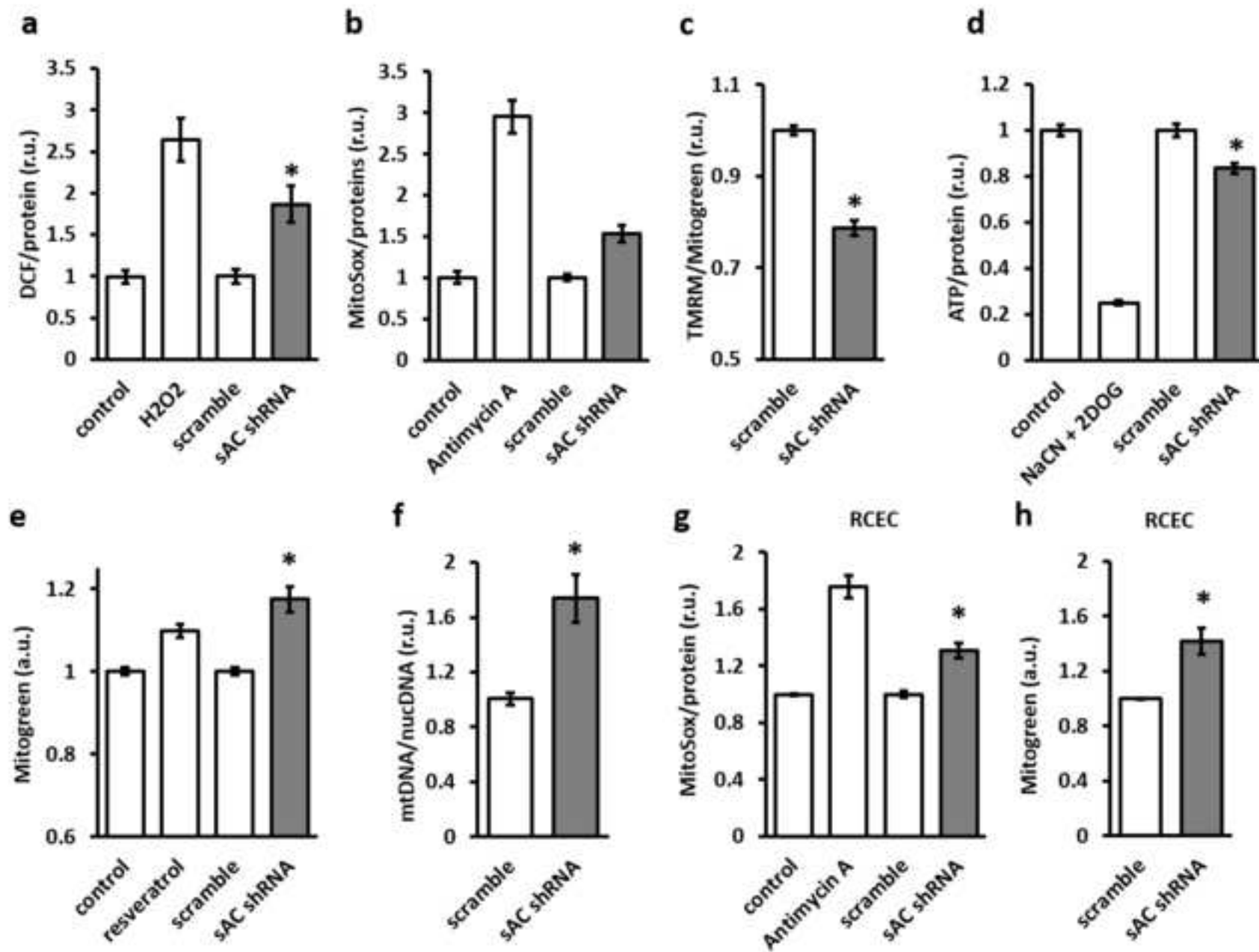


Figure 5

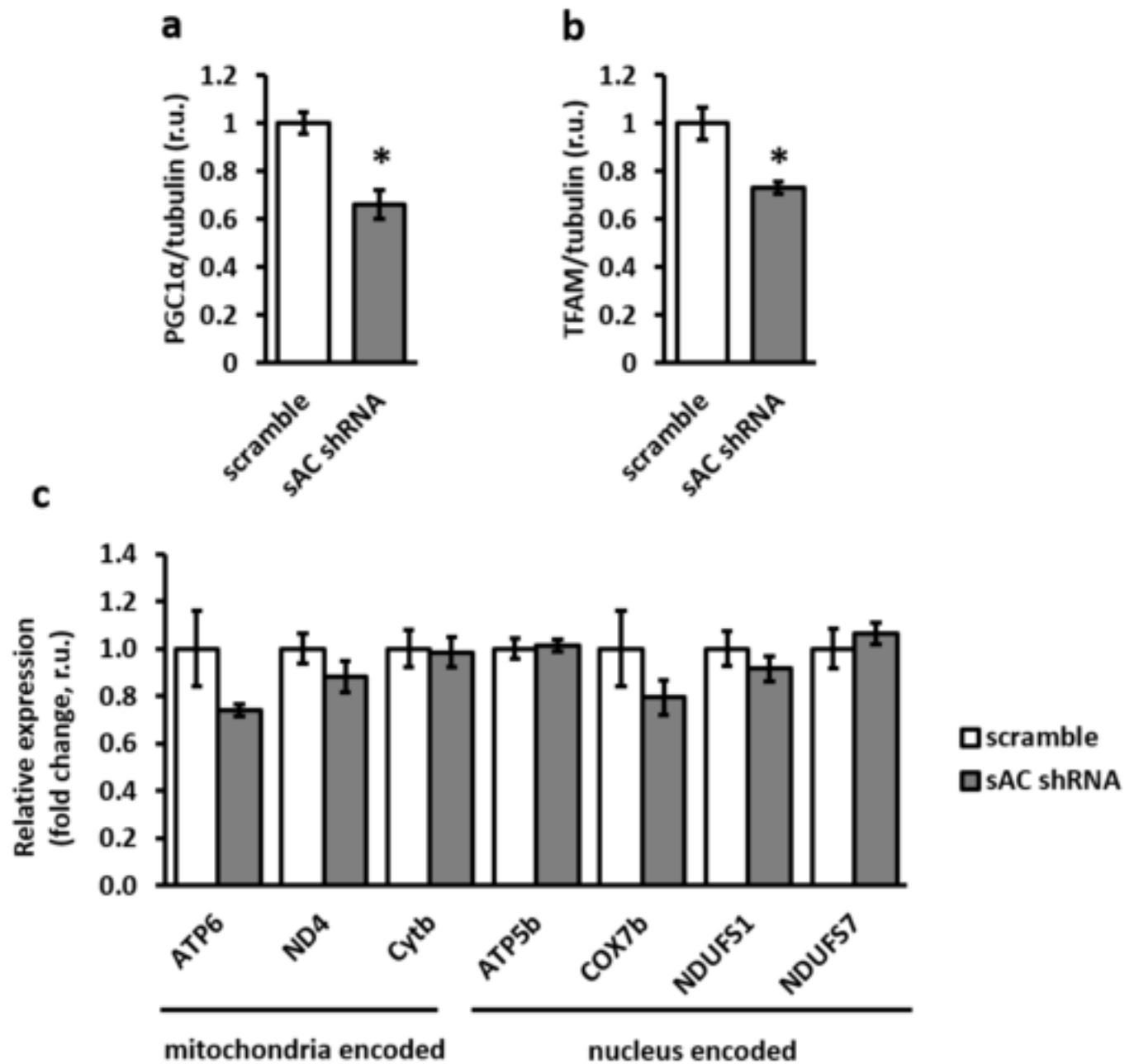


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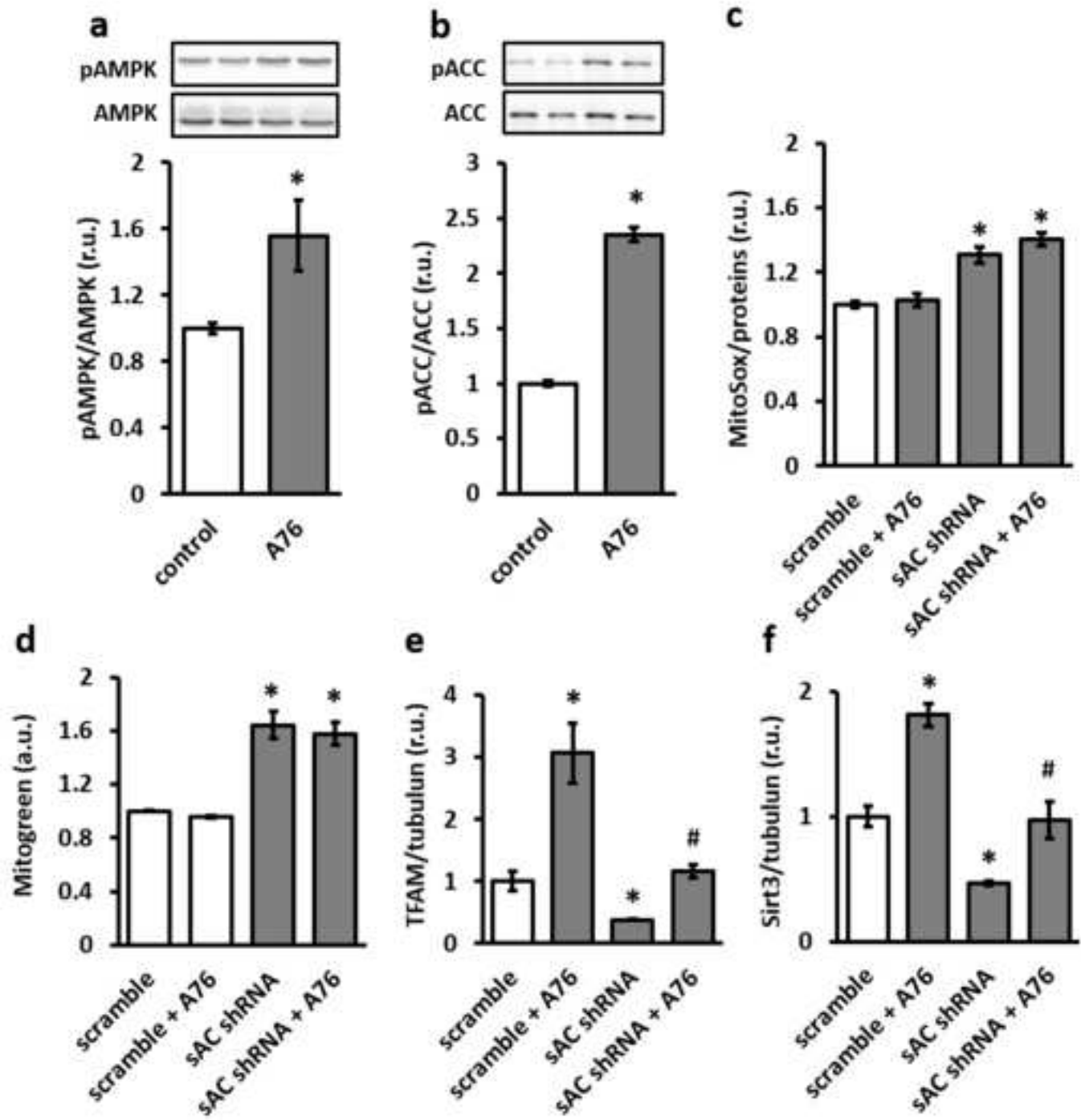


Figure 7

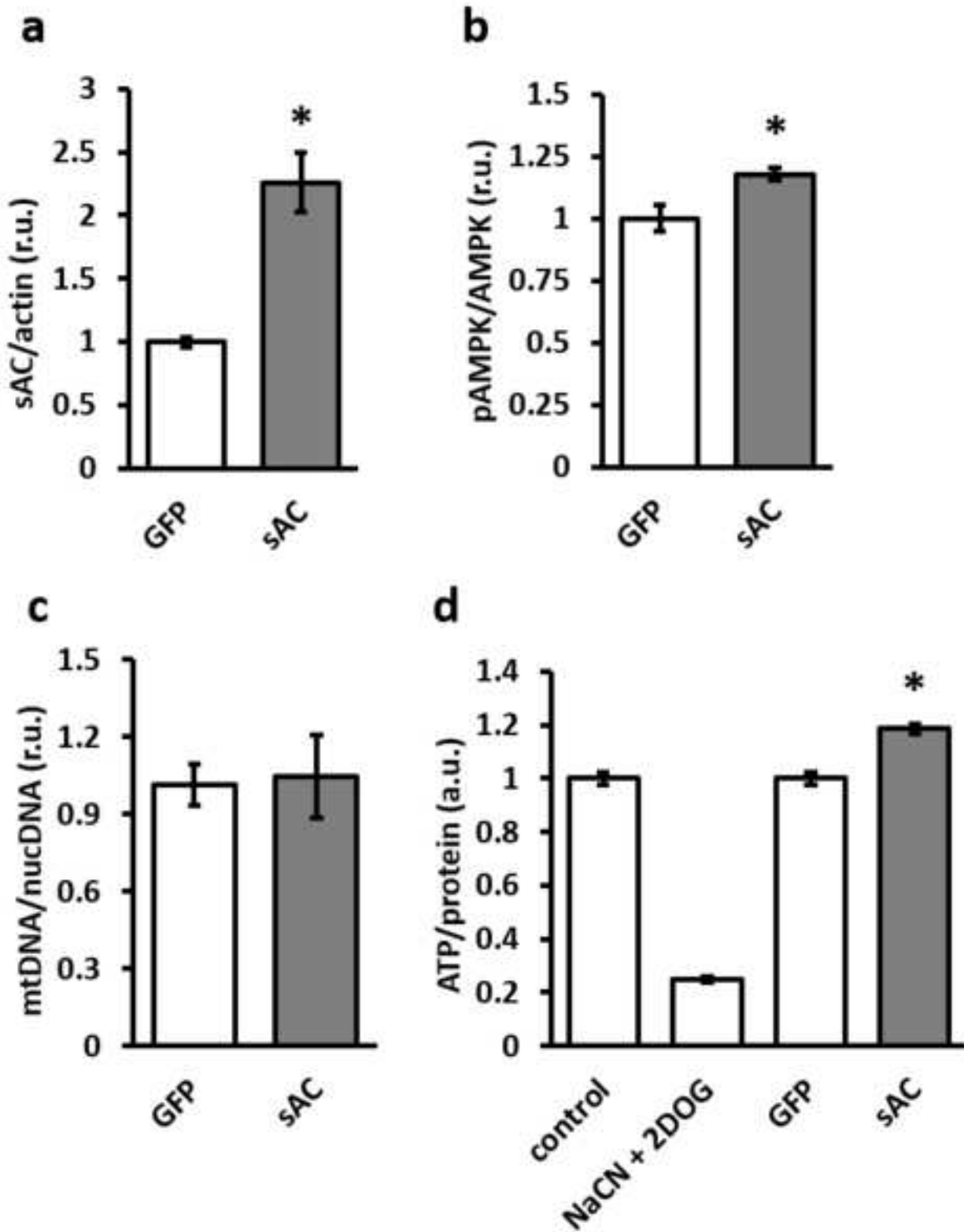
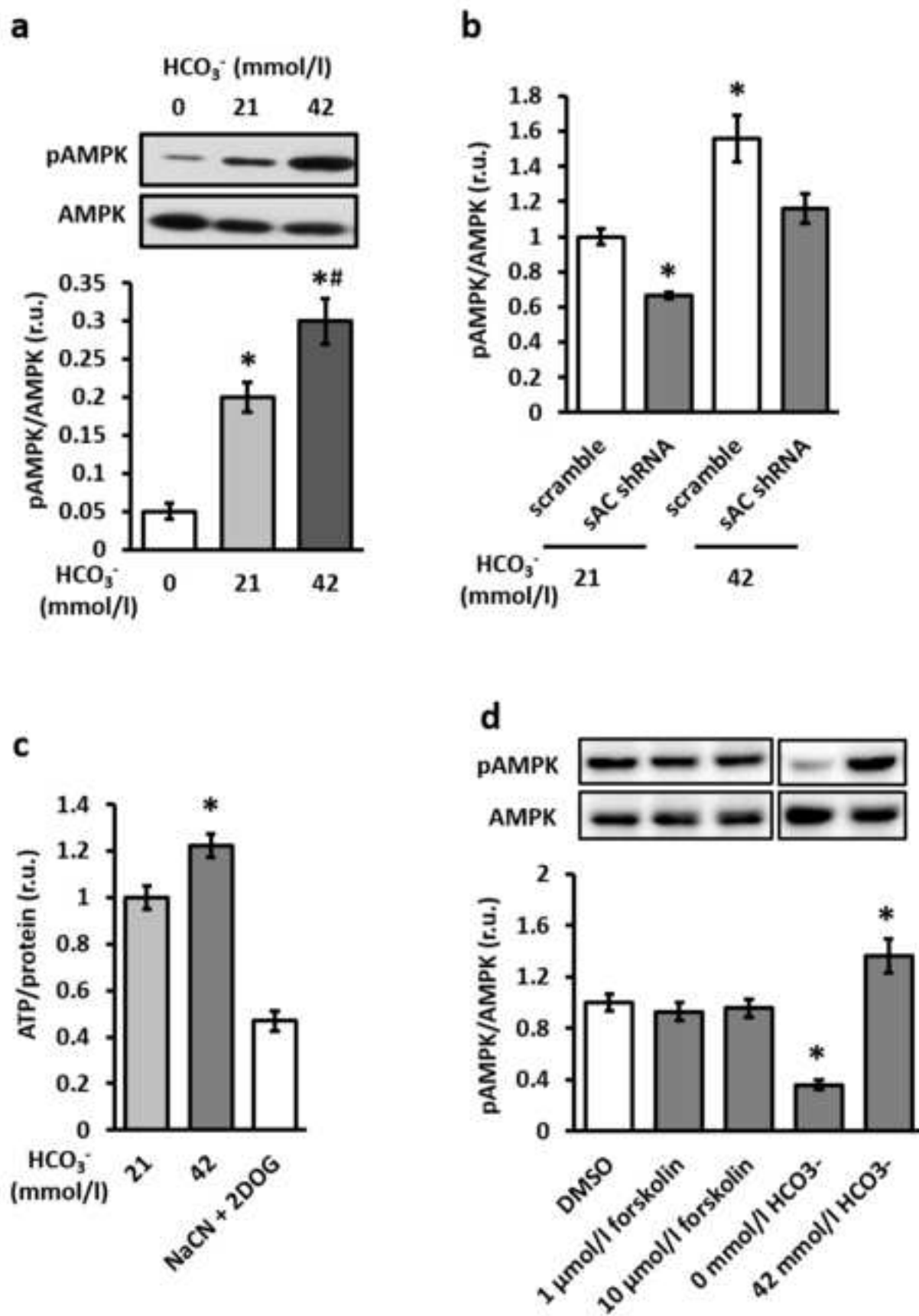


Figure 8





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