Original Paper

AMP-Activated Protein Kinase $\alpha 1$ Regulates Cardiac Gap Junction Protein Connexin 43 and Electrical Remodeling Following Pressure Overload

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Key Words

Electrical remodeling • Conduction • Ampk $\alpha 1$ • Cx43 • Ubiquitination

Abstract

Background/Aims: Adenosine 5'-monophosphate (AMP)-activated protein kinase (Ampk) modulates a wide array of cellular functions and regulates various ion channels and transporters. In failing human hearts an increased Ampk $\alpha 1$ activity was observed. The present study aimed to uncover the impact of Ampk $\alpha 1$ on cardiac electrical remodeling. **Methods:** Gene-targeted mice lacking functional Ampk $\alpha 1$ (Ampk $\alpha 1^{-/-}$) and corresponding wild-type mice were exposed to pressure overload by “transverse aortic constriction” (TAC). *In vivo* electrophysiology was performed with a single catheter technique, myocardial conduction velocities and conduction characteristics investigated in isolated hearts, transcript levels quantified by RT-PCR and protein abundance determined by Western blotting. Moreover, connexin 43 (Cx43) was expressed in *Xenopus* oocytes with or without coexpression of wild-type or mutant AMPK and Cx43 protein abundance quantified utilizing confocal microscopy. **Results:** TAC treatment increased Ampk $\alpha 1$ protein expression in cardiac tissue from wild-type mice. TAC further increased left ventricular conduction inhomogeneity and triggered conduction blocks, effects blunted in the Ampk $\alpha 1^{-/-}$ mice. TAC treatment decreased Cx43 protein abundance in cardiac tissue, an effect significantly blunted in the Ampk $\alpha 1^{-/-}$ mice. TAC treatment did not modify Cx43 mRNA levels but increased ubiquitination of Cx43 protein, an effect mitigated by Ampk $\alpha 1$ deficiency. As shown in *Xenopus* oocytes, Cx43 cell membrane protein abundance

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was significantly downregulated by wild-type AMPK^{WT} and constitutively active AMPK^{Y70Q}, but not by catalytically inactive AMPK^{K45R}. **Conclusion:** Ampk α 1 stimulates ubiquitination of the gap junction protein Cx43, thereby contributing to gap junction remodeling following pressure overload.

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Introduction

Electrical coupling by gap junctions is essential for action potential propagation throughout cardiac muscle [1]. Connexin 43 (Cx43) is the major gap junction protein in ventricular myocytes [1]. Cx43-dependent gap junction coupling underlies dynamic alterations [2]. In failing hearts, “electrical remodeling” modifies activity of ion channels, excitation-contraction coupling and intercellular gap junctions [3]. Electrical remodeling is a hallmark of cardiac failure [3] which is associated with high morbidity and mortality at least partially due to fatal arrhythmias [4]. Cardiac electrical remodeling involves altered function of the gap junction protein Cx43 [1].

A crucial regulator involved in the myocardial stress response is adenosine 5'-monophosphate (AMP)-activated protein kinase (Ampk) [5]. Ampk is activated by increase of cytosolic Ca²⁺ concentration as well as AMP/ATP concentration ratio and thus senses cellular Ca²⁺ overload and energy depletion [6]. Ampk is a heterotrimer consisting of a catalytic alpha (α) subunit and regulatory beta (β) and gamma (γ) subunits [5]. Two isoforms of the catalytic α subunit exist with distinct subcellular localization: Ampk α 1 (encoded by the *Prkaa1* gene) found in the non-nuclear fraction and Ampk α 2 (encoded by the *Prkaa2* gene) localized in both nucleus and non-nuclear fraction [7].

In cardiac tissue, Ampk α 2 is the dominant isoform and plays an important role in cardiac ischemia, hypertrophy and failure [5]. Ampk activity is increased in left ventricular (LV) hypertrophy, which reduces expression of the Ampk α 2 isoform but increases the expression of the Ampk α 1 isoform [8]. The contribution of Ampk α 1 to Ampk activity is more pronounced in human than in mouse hearts [9]. In the failing human heart, the activity of the catalytic Ampk α isoforms is shifted towards Ampk α 1 [9].

Ampk is a powerful regulator of channels, carriers and Na⁺/K⁺ ATPase [10–19]. The kinase is involved in the regulation of cardiac sodium and potassium channels [11, 17]. Ampk α 1 further downregulates gap junction protein beta 2 [12]. Mutations in the Ampk γ 2 subunit leading to increased Ampk activity are associated with cardiac electrophysiologic abnormalities, hypertrophy and glycogen storage disease [20].

The present study investigated the role of Ampk α 1 on cardiac electrophysiology and gap junction remodeling during pressure overload. To this end, cardiac electrical remodeling and Cx43 expression were measured in gene-targeted mice lacking functional Ampk α 1 (Ampk α 1^{-/-}) and corresponding wild-type mice exposed to pressure overload by “transverse aortic constriction” (TAC).

Materials and Methods

Murine aortic banding

All animal experiments were conducted according to the German law for the welfare of animals and were approved by local authorities. Ampk α 1 knockout mice were described previously [21, 22]. Male mice of 9–12 weeks of age were used for the experiments. Cardiac pressure overload was induced by transverse aortic constriction (TAC) [23]. Briefly, mice were anaesthetized and placed on a heating pad. After orotracheal intubation and ventilation (Harvard minivent, Harvard Apparatus, Holliston, USA), the intercostal space was opened by a small incision in the muscular thoracic wall. The transverse aorta was exposed and constricted between the brachiocephalic artery and the left carotid artery by the width of a 27-G canula using a 7-0 filament. Sham treatment was performed similarly but without constriction of the filament. Animals were treated with buprenorphine (0.05mg/kg BW) after the procedure.

Electrophysiological investigation

The *in vivo* electrophysiological investigations (EPI) were carried out with a single catheter technique [24]. Under inhalative anaesthesia (isoflurane; induction period 2.5 vol.%, maintenance 1.2 vol.% in 70% N₂O/ 30% O₂) the jugular vein was dissected and a 2-French octapolar mouse electrophysiological catheter (Ciber Mouse, NuMed Inc., NY, USA) was positioned into the right cardiac cavities on atrial and ventricular level.

Functional electrophysiological parameters were examined using a universal isolated stimulator (Hugo Sachs Elektronik, Type 263, Germany) and custom written software (Stimulus V3.2, H. Begerau, Bonn, Germany). Refractory periods were determined according to established procedure protocols [25]. The susceptibility to ventricular tachyarrhythmias (VTs) was determined by ventricular extrastimulus pacing (S1S1: 120 ms, 100 ms, and 80 ms followed by up to 3 extra beats) and ventricular burst stimulation (1s at S1S1: 50–10 ms, 10 ms stepwise reduction; stimulus voltages 1.5 and 3.0 V). VTs were defined as ≥4 consecutive ventricular ectopic beats.

Langendorff-perfusion and epicardial mapping

Myocardial conduction velocities and conduction characteristics were investigated after excorporation of the hearts and Langendorff-perfusion with Krebs-Henseleit buffer at constant pressure (80 mmHg; Langendorff apparatus by Radnoti Technologies, Monrovia, CA, USA; perfusate composition: NaCl 110 mM, KCl 4.6 mM, MgSO₄ 1.2 mM, CaCl₂ 2 mM, NaH₂PO₄ 2 mM, NaHCO₃ 25 mM, glucose 8.3 mM, Na-pyruvate 2 mM, gassed with carbogen (O₂ 95%, CO₂ 5%), pH 7.35–7.45, temperature 37°C) as previously described [26]. Using a 72-electrode array (FlexMEA72, Multi Channel Systems, Reutlingen, Germany, interelectrode distance: longitudinal 750 μm, transversal 625 μm) 76 activation maps in spontaneous sinus rhythm were analyzed from left ventricular epicardium (Cardio 2D Software, V2.0.3, Multi Channel Systems, Reutlingen, Germany). Considering the direction of the electrode array in relationship to the myocardial fiber orientation, longitudinal and transversal conduction velocities could be calculated. The inhomogeneity index as an index of local conduction slowing for each electrode [27] was calculated using custom-programmed software (Latency version 3.0; H. Begerau, Bonn, Germany). Significant conduction blocks were defined as conduction delay of >4 ms between adjacent electrodes.

Quantitative RT-PCR

Total RNA was isolated from snap frozen murine heart samples using Trifast Reagent (Pqlab, Erlangen, Germany) according to the manufacturer's instructions. Reverse transcription of 2 μg RNA was performed using oligo(dT)_{12–18} primers (Invitrogen, Karlsruhe, Germany) and SuperScript III Reverse Transcriptase (Invitrogen, Karlsruhe, Germany). cDNA samples were treated with RNase H (Invitrogen, Karlsruhe, Germany). Quantitative real-time PCR was performed with the iCycler iQ™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) and iQ Sybr Green Supermix (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. The following primers were used (5'→3' orientation):

Cx43 fw: ACAAGGTCCAAGCCTACTCCA;

Cx43 rev: CCGGGTTGTTGAGTGTTACAG;

Gapdh fw: AGGTCGGTGTGAACGGATTTG;

Gapdh rev: TGTAGACCATGTAGTTGAGGTCA;

Prkaa1 fw: GACCGGACATAAAGTGGCTGT;

Prkaa1 rev: CCACGTCAAGGCTCCGAAT;

Prkaa2 fw: ATGGTTGTCCATAGGGACCTG;

Prkaa2 rev: GCGATCCACAGCTAGTTCGTAG.

The specificity of the PCR products was confirmed by analysis of the melting curves and in addition by agarose gel electrophoresis. All PCRs were performed in duplicate and relative mRNA fold changes were calculated by the 2^{−ΔΔCt} method using *Gapdh* as internal reference.

Immunostaining and confocal microscopy

For immunohistochemistry [28], cardiac tissues were immediately frozen in mounting medium (Tissue-Tek, Sakura Finetek) and sectioned at a thickness of 8 μm on coated slides. Heart sections were dried for 30 min at RT and fixed in methanol for 10 min at RT. To reduce nonspecific background staining, slides were incubated with 5% normal goat serum in PBS/ 0.1% Triton X-100 for 1 hour at RT. Sections were

incubated overnight at 4°C with rabbit polyclonal anti-Cx43 antibody (diluted 1:50, Cell Signaling). Binding of primary antibody was visualized using goat anti-rabbit Alexa Fluor488-conjugated antibody (diluted 1:1,000, Invitrogen) incubated for 1 hour at RT. Nuclei were stained using DRAQ-5 dye (diluted 1:1,000, Biostatus) and actin using Rhodamine Phalloidin (diluted 1:100, Invitrogen). The slides were mounted with Prolong Gold antifade reagent (Invitrogen). Images were collected with a confocal laser-scanning microscope (LSM 510, Carl Zeiss MicroImaging GmbH) using a water immersion A-Plan $\times 40/1.2$ W DICIII. Confocal images are representative for 4 mice/ group. Negative controls were carried out simultaneously with all experiments by omitting incubation with primary antibody.

Analysis of Cx43 ubiquitination

Murine heart tissues were lysed with ice-cold IP lysis buffer (Thermo Fisher Scientific, Rockford, IL, USA) supplemented with complete protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Rockford, IL, USA). After centrifugation at 10000 rpm for 5 min, protein concentration was determined by Bradford assay (Biorad Laboratories, Hercules, CA). Immunoprecipitation of Cx43 from heart tissue samples was performed by using Pierce Direct IP kit (Thermo Fisher Scientific, Rockford, IL, USA) according to the manufacturer's instructions. To pre-clear the lysate, fixed amounts of protein (600 μ g) were incubated with control agarose resin (Thermo Fisher Scientific, Rockford, IL, USA) for 1 hour at 4 °C on a rotator. Coupling of 4 μ l rabbit polyclonal anti-Cx43 antibody (Cell Signaling) to the AminoLink Plus coupling resin (Thermo Fisher Scientific, Rockford, IL, USA) was performed for 2 hours at RT. The pre-cleared lysate was incubated with the immobilized antibody to form the immune complex overnight at 4 °C on a rotator. Then, immune complexes were washed to remove non-bound proteins and low pH elution buffer was used to dissociate the bound antigen from the antibody. The eluted proteins were boiled in Roti-Load1 Buffer (Carl Roth GmbH, Karlsruhe, Germany) by heating for 5 min at 95°C. Proteins were separated on 10% SDS-polyacrylamide gels and transferred to PVDF membranes. The membranes were incubated overnight at 4°C with rabbit anti-ubiquitin antibody (diluted 1:1000, Cell Signaling, Danvers, MA, USA) and then with goat anti-rabbit HRP-conjugated antibody (diluted 1:1000, Cell Signaling) for 1 hour at RT. To remove antibodies, membranes were incubated in stripping buffer (Thermo Fisher Scientific, Rockford, IL, USA) for 10 min at RT and re-probed with rabbit polyclonal anti-Cx43 antibody (diluted 1:1000, Cell Signaling). Antibody binding was detected with the ECL detection reagent (Thermo Fisher Scientific, Rockford, IL, USA) and bands were quantified using Quantity One Software (Bio-Rad, München, Germany). Results are shown as the ratio of ubiquitin to Cx43 protein normalized to the sham treated wild-type mice.

Western blot analysis

Murine heart tissues were lysed with ice-cold IP lysis buffer (Thermo Fisher Scientific) supplemented with complete protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific). After centrifugation at 10000 rpm for 5 min, protein concentration was estimated by Bradford assay (Biorad Laboratories, Hercules, CA) and proteins were boiled in Roti-Load1 Buffer (Carl Roth GmbH) at 100°C for 10 min. Proteins were separated on SDS-polyacrylamide gels and transferred to PVDF membranes. The membranes were incubated overnight at 4°C with the following primary antibodies: rabbit monoclonal anti-Ampk α 1 antibody (diluted 1:1000, Novus Biologicals), rabbit monoclonal anti-phospho-Ampk α (Thr¹⁷²) antibody, rabbit monoclonal anti-Ampk α antibody, rabbit polyclonal anti-phospho-ACC (Ser⁷⁹) antibody, rabbit polyclonal anti-ACC antibody, rabbit polyclonal anti-Cx43 antibody and rabbit anti-GAPDH antibody (diluted 1:1000, Cell Signaling) and then with secondary goat anti-rabbit HRP-conjugated antibody (diluted 1:1000, Cell Signaling) for 1 hour at RT. For loading controls, the membranes were stripped with stripping buffer (Thermo Fisher Scientific, Rockford, IL, USA) for 10 min at RT. Antibody binding was detected with the ECL detection reagent (Thermo Fisher Scientific). Bands were quantified with Quantity One Software (Bio-Rad Laboratories) and results are shown as the ratio of phosphorylated to total protein to Gapdh and as the ratio of total protein to Gapdh normalized to the sham treated wild-type mice.

Constructs

For generation of cRNA, constructs were used encoding AMPK α 1-HA, AMPK β 1-Flag, AMPK γ 1-HA [29], constitutively active mutant ^{R70Q}AMPK γ 1-HA [30] and kinase-dead mutant ^{K45R}AMPK α 1-HA [31] in POL1, *Xenopus* oocyte expression vector and mouse tagged Cx43-eGFP inserted in the plasmid pSGEM, *Xenopus* oocyte expression vector. The construct for mouse Cx43-eGFP was obtained by subcloning the insert into

pSGEM vector from pMJ-Cx43-eGFP (kind gift from Prof. Dr. Klaus Willecke) using EcoRI – NotI restriction sites. The eGFP-tagged Cx43 construct was sequenced to confirm the insert and tag in frame. cRNA was synthesized as described previously [32, 33].

Confocal microscopy in *Xenopus* oocytes

Xenopus oocytes were prepared as previously described [34, 35]. cRNA (4.6 ng) encoding either AMPK α 1-HA + AMPK β 1-Flag + AMPK γ 1-HA (AMPK^{WT}), or AMPK α 1-HA + AMPK β 1-Flag + ^{R70Q}AMPK γ 1-HA (AMPK^{R70Q}) or ^{K45R}AMPK α 1KD-HA + AMPK β 1-Flag + AMPK γ 1-HA (AMPK^{K45R}) were injected on the first day and 23 ng of cRNA encoding Cx43-eGFP on the third day after preparation of the *Xenopus* oocytes [19, 36]. All experiments were performed 3 days after the first injection. Oocytes were maintained at 17°C in ND96-A solution containing 88.5 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, 0.11 mM tetracycline, 4 μ M ciprofloxacin, 0.22 mM gentamycin (Refobacin), 0.5 mM of the oocyte maturation inhibitor theophylline (Euphyllong) [37], and 5 mM sodium pyruvate. The pH was adjusted to 7.4.

To visualize Cx43-eGFP cell surface expression, *Xenopus* oocytes were fixed in 4% paraformaldehyde/PBS for 2 hours at RT. After washing with PBS, *Xenopus* oocytes were analyzed by a fluorescence laser scanning microscope (LSM510, CarlZeiss MicroImaging GmbH, Germany) with A-Plan 10x/0.25, focused on the midline of the oocytes. Brightness and contrast settings were kept constant during imaging of all oocytes in each injection series. Confocal images are representative for three independent experiments. The fluorescence intensity reflecting Cx43-eGFP membrane abundance was quantified by ZEN2009 software (Zeiss, Göttingen, Germany) [38].

Statistics

Data are shown as arithmetic means $\pm$ SEM unless indicated otherwise. Normality was tested with Shapiro-Wilk test. Non-normal data were transformed (log or reciprocal) prior to statistical testing to provide normality according to Shapiro-Wilk test. Statistical testing was performed by one-way Anova followed by Tukey-test for homoscedastic data or Games-Howell test for heteroscedastic data. Non-normal data was tested by the Steel-Dwass method. Two groups were compared by unpaired two-tailed t-test. Left ventricular conduction blocks are shown as individual measurements and box-plots (generalized linear model, exponential distribution). The probability of VT is shown descriptive. A $p < 0.05$ was considered statistically significant.

Results

A first series of experiments explored whether cardiac Ampk α 1 expression is modified following pressure overload induced by transverse aortic constriction (TAC). As a result, in wild-type mice (Ampk α 1^{+/+}), 2 weeks of TAC treatment significantly increased cardiac Ampk α 1 protein abundance compared to sham treated mice (Fig. 1A). TAC treatment did not significantly modify *Prkaa1* and *Prkaa2* mRNA levels (Fig. 1B). The Ampk α protein abundance was increased following TAC treatment without modifying ACC (Acetyl-CoA carboxylase) phosphorylation, a target phosphorylated by Ampk [39] (Fig. 1C).

To investigate whether Ampk α 1 is involved in electrical remodeling of the heart, electrophysiological investigations and cardiac mapping were performed on hearts from Ampk α 1^{+/+} mice and Ampk α 1 knockout mice (Ampk α 1^{-/-}) following TAC or sham procedure (Fig. 2). TAC treatment led to an increase of the LV inhomogeneity index in Ampk α 1^{+/+} hearts as compared to sham treated hearts, effects significantly blunted in the Ampk α 1^{-/-} hearts (Fig. 2B). Furthermore, following TAC treatment, Ampk α 1^{+/+} hearts displayed more conduction blocks >4ms than Ampk α 1^{-/-} hearts (Fig. 2C). Surprisingly, both longitudinal and transversal conduction velocity tended to be lower in Ampk α 1^{-/-} hearts as compared to Ampk α 1^{+/+} hearts, irrespective of TAC treatment (Fig. 3 A,B). The conduction velocity was not further reduced by TAC treatment in Ampk α 1^{-/-} hearts. Longitudinal conduction velocity was significantly reduced (Fig. 3A) and ventricular refractory period was significantly increased (Fig. 3C) following TAC treatment in Ampk α 1^{+/+} hearts, but not in Ampk α 1^{-/-} hearts. The probability to induce ventricular tachycardias following TAC treatment was low irrespective of the genotype (Fig. 3D). Taken together, Ampk α 1 is involved in cardiac electrical remodeling following pressure overload.

Fig. 1. Ampkα isoform expression in cardiac tissue from Ampkα1-deficient mice following TAC. **A.** Representative original Western blots showing Ampkα1 and Gapdh protein expression in cardiac tissue from Ampkα1 knockout mice (Aα1^{-/-}) and respective wild-type mice (Aα1^{+/+}) following sham procedure (sham) or transverse aortic constriction (TAC). Arithmetic means ± SEM (n=10 mice/group; arbitrary units, a.u.) of normalized Ampkα1/Gapdh protein ratio in cardiac tissue from wild-type mice following sham procedure or TAC. **B.** Arithmetic means ± SEM of *Prkaa1* and *Prkaa2* relative mRNA expression (n=10 mice/group; a.u.) in cardiac tissue from Ampkα1 knockout mice (Ampkα1^{-/-}) and respective wild-type mice (Ampkα1^{+/+}) following sham procedure or TAC. **C.** Representative original Western blots showing phospho-Ampkα (Thr¹⁷²), Ampkα, phospho-ACC (Ser⁷⁹), ACC and Gapdh protein expression in cardiac tissue from Ampkα1^{-/-} mice (Aα1^{-/-}) and Ampkα1^{+/+} mice (Aα1^{+/+}) following sham procedure or TAC. Arithmetic means ± SEM of normalized Ampkα/Gapdh protein ratio and normalized phospho-ACC/ACC/Gapdh protein ratio (n=12 mice/group, a.u.) in cardiac tissue from Ampkα1^{-/-} mice and Ampkα1^{+/+} mice following sham procedure or TAC. *(p<0.05), ***(p<0.01) statistically significant vs. respective sham mice.

In search for a structural substrate accounting for the increased conduction inhomogeneity in TAC treated Ampkα1^{+/+} mice, protein abundance and localization of the gap junction protein connexin 43 (Cx43) were investigated. As shown by confocal imaging and Western blotting, TAC treatment decreased the expression and deranged the organization of Cx43 protein in cardiac tissue from Ampkα1^{+/+} mice, effects mitigated in the Ampkα1^{-/-} mice (Fig. 4A,B). Furthermore, TAC treatment increased ubiquitination of Cx43 protein in Ampkα1^{+/+} mice, an effect again significantly blunted in Ampkα1^{-/-} mice (Fig. 4C). Cx43 mRNA levels were not significantly modified by TAC treatment and were not different between Ampkα1^{-/-} and Ampkα1^{+/+} mice (Fig. 4D). Thus, Ampkα1 down-regulates Cx43 protein abundance in cardiac tissue by increasing the ubiquitination-mediated degradation.

To further elucidate the Ampkα1-sensitive regulation of the gap junction protein Cx43, *Xenopus* oocytes were injected with cRNA encoding Cx43-eGFP alone or together with wild-type AMPK^{WT} (AMPKα1 + AMPKβ1 + AMPKγ1), with catalytically inactive mutant AMPK^{αK45R} (AMPKα1 + AMPKβ1 + AMPKγ1) or with constitutively active mutant AMPK^{γR70Q} (AMPKα1-HA + AMPKβ1 + AMPKγ1) and Cx43 protein abundance in the cell membrane was determined by confocal microscopy in Cx43-eGFP-expressing *Xenopus* oocytes. As shown in Fig. 5A,B, the expression of Cx43-eGFP protein in the *Xenopus* oocytes cell membrane was significantly decreased following coexpression of wild-type AMPK^{WT} and of constitutively active AMPK^{γR70Q}. In contrast, coexpression of the catalytically inactive AMPK^{αK45R} did not significantly modify Cx43-eGFP protein cell surface expression. Thus, kinase activity is required for the effect of AMPK on Cx43 protein cell membrane abundance.

Fig. 2. Ampk1 deficiency ameliorates left ventricular conduction inhomogeneity following TAC. A. Representative left ventricular epicardial activation maps of Ampk1 knockout mice (Ampk1^{-/-}) and respective wild-type mice (Ampk1^{+/+}) following sham procedure (sham) or transverse aortic constriction (TAC). Activation starts with red color and propagates towards blue. Black lines represent isochrones; each 1ms apart. Inlets demonstrate electrode orientation in relationship to the heart. B. Arithmetic means $\pm$ SEM of LV inhomogeneity index (n = 6-11 mice/group; (P5-P95)/P50) measured in Langendorff heart preparations from Ampk1^{-/-} mice and Ampk1^{+/+} mice following sham procedure or TAC. ** (p < 0.01) statistically significant vs. respective sham mice; † (p < 0.05) statistically significant vs. respective Ampk1^{+/+} mice. C. Individual measurements and box-plots of LV conduction blocks >4ms (points represent individual mice) in hearts from TAC treated Ampk1^{-/-} mice and Ampk1^{+/+} mice (p < 0.01 generalized linear model, exponential distribution).

Fig. 3. Cardiac conduction velocity and arrhythmia induction in Ampk1 deficient mice following TAC. Arithmetic means $\pm$ SEM of longitudinal (A) and transversal (B) LV conduction velocity (n = 8-12 mice/group; cm/s) and of ventricular refractory period (C, n = 8-12 mice/group; ms) measured in hearts from Ampk1^{-/-} mice and Ampk1^{+/+} mice following sham procedure or TAC. * (p < 0.05) statistically significant vs. respective sham mice. D. Probability of induction of ventricular tachycardias (n = 8-12 mice/group; %) in hearts from Ampk1^{-/-} mice and Ampk1^{+/+} mice following sham procedure or TAC.

Fig. 4. Connexin 43 remodeling following TAC is mitigated by Ampk α 1 deficiency. **A.** Confocal microscopy images showing connexin 43 (Cx43) protein expression in cardiac tissue from Ampk α 1 knockout mice (Ampk α 1^{-/-}) and respective wild-type mice (Ampk α 1^{+/+}) following sham procedure (sham) or transverse aortic constriction (TAC). Cx43 expression is represented by green labeling, in merged images nuclei are labeled in blue, actin staining is labeled in red. Images are representative for four mice/group. Scale bar indicates 20 μ m. **B.** Representative original Western blots and arithmetic means $\pm$ SEM of normalized Cx43/Gapdh protein ratio (n = 10 mice/group; arbitrary units, a.u.) in cardiac tissue from Ampk α 1^{-/-} mice (A α 1^{-/-}) and Ampk α 1^{+/+} mice (A α 1^{+/+}) following sham procedure or TAC. **C.** Representative original Western blots and arithmetic means $\pm$ SEM of normalized Cx43 ubiquitination (n = 7 mice/group; a.u.) measured by ubiquitin/Cx43 protein ratio in Cx43-immunoprecipitated cardiac samples from Ampk α 1^{-/-} mice and Ampk α 1^{+/+} mice following sham procedure or TAC. **D.** Arithmetic means $\pm$ SEM of Cx43 relative mRNA expression (n = 8 mice/group; a.u.) in cardiac tissue from Ampk α 1^{-/-} mice and Ampk α 1^{+/+} mice following sham procedure or TAC. * (p < 0.05), ** (p < 0.01), *** (p < 0.001) statistically significant vs. respective sham mice; † (p < 0.05) statistically significant vs. respective Ampk α 1^{+/+} mice.

Discussion

The present study reveals a novel function of the AMP-activated protein kinase α 1 (Ampk α 1). The kinase regulates the protein abundance of cardiac gap junction protein connexin 43 (Cx43) and affects electrical remodeling of the heart. Cx43 dysregulation is pivotal for the electrical cardiac remodeling and may underlie enhanced susceptibility to arrhythmia in the failing heart [40]. Downregulation of Ampk α 2 and upregulation of Ampk α 1 pointing to an isoform shift is observed in the failing human heart [9]. Accordingly, Ampk α 1 protein abundance was increased in cardiac tissue from wild-type mice following pressure overload induced by transverse aortic constriction (TAC).

According to the present observations, Ampk α 1 regulates ubiquitination of the gap junction protein Cx43 in cardiac tissue. In contrast, Ampk α 1 does not appreciably alter Cx43

Fig. 5. Coexpression of AMPK down-regulates Cx43 cell membrane protein abundance in *Xenopus* oocytes. Representative confocal microscopy images showing Cx43-eGFP cell surface expression (A) and arithmetic means $\pm$ SEM of normalized Cx43-eGFP fluorescence intensity (B, $n = 14$ -17 oocytes/group from three independent experiments; a.u.) in *Xenopus* oocytes injected with water (H₂O), expressing Cx43-eGFP alone (Cx43-eGFP) or together with wild-type AMPK^{WT} (Cx43-eGFP+AMPK^{WT}), with catalytically inactive mutant AMPK ^{α 45R} (Cx43-eGFP+AMPK ^{α 45R}) or with constitutively active mutant AMPK ^{γ R70Q} (Cx43-eGFP+AMPK ^{γ R70Q}). Cx43-eGFP expression is represented by green fluorescence. Images are representative for 3 independent experiments. Scale bar indicates 100 μ m. ***($p < 0.001$) statistically significant vs. *Xenopus* oocytes expressing Cx43 alone. †††($p < 0.001$) statistically significant vs. *Xenopus* oocytes coexpressing Cx43 and AMPK ^{α 45R}.

transcript levels. Thus, Ampk α 1 downregulates Cx43 protein abundance in cardiac tissue presumably by accelerating Cx43 protein degradation. The Cx43 protein is regulated by ubiquitination [41-43]. Ampk has previously been shown to regulate ubiquitin ligases, and thus protein degradation through the “ubiquitin proteasome system” [44-47]. Our findings suggest that Ampk α 1 regulates the ubiquitination with subsequent internalization and degradation of the Cx43 protein. Along those lines the decline of Cx43 abundance in cardiac hypertrophy involves posttranscriptional mechanisms [48].

The reduction of Cx43 protein in the cell membrane presumably contributes to the observed increased LV inhomogeneity index in wild-type mice following TAC treatment. Despite the changes in the LV inhomogeneity index and Cx43 protein, the probability to induce ventricular tachyarrhythmias was low in both genotypes following TAC procedure. Any differences in tachyarrhythmia induction between the genotypes might be masked by the low susceptibility to tachyarrhythmia induction in this model. The conduction velocity tended to be lower in Ampk α 1^{-/-} mice than in Ampk α 1^{+/+} mice, but decreased significantly following TAC treatment only in Ampk α 1^{+/+} mice. The scatter of the data precludes safe conclusions but other mechanisms than Ampk α 1-sensitive Cx43 regulation may contribute to modifications of conduction velocity. Ampk sensitivity of cardiac conduction velocity may be influenced by regulation of further ion channels, such as the voltage-gated Na⁺ channels, which are activated by Ampk [11]. Furthermore, Cx43 may not be the major determinant of cardiac conduction velocity [49, 50]. Cx43 also exerts effects independent of gap junction function [51]. Nonetheless, reduction and lateralization of Cx43 protein causes conduction defects in the failing heart [52, 53]. Cx43 is reduced following pressure overload induced by TAC and congestive heart failure, but Cx43 is not decisive for cardiac contractile function [54-56].

The downregulation of connexins by Ampk presumably serves to protect neighbours of energy depleted cells from Na⁺ and K⁺ fluxes through gap junctions due to impaired Na⁺/K⁺-ATPase activity in energy depleted cells [57]. Cx43 internalization is an important mechanism during cardiac ischemia [58]. Cardiac ischemia activates Ampk α 1 [59], which exerts protective effects in the ischemic heart [60, 61]. Similarly, inhibition of Cx43 mitigates cardiac ischemia reperfusion injury [62]. Inhibition of Cx43 by Ampk could serve to protect the heart from ischemic episodes. Chronic pressure overload causes activation and increased activity of Ampk [8]. During chronic activation, such as in heart failure, the inhibitory actions of Ampk on Cx43 might lead to detrimental loss of gap junctions and impaired cardiac conductance.

Mutations in the gamma subunit of Ampk are associated with increased Ampk activity, ventricular preexcitation and conduction defects [63, 64]. The effects of the mutated Ampk are not solely due to increased glycogen storage [36]. Ampk activity is increased following pressure overload, increasing the expression of the Ampk α 1 isoform, but rather reducing the Ampk α 2 isoform [8]. Ampk α 2 activation is protective in the failing heart [5]. Ampk α 2-deficient mice, but not Ampk α 1-deficient mice show dysfunctional expression of energy metabolism-related genes and exacerbated remodeling following TAC [65, 66]. Therefore, the effects of Ampk α 1 could be of significant importance especially in the failing heart.

In conclusion, Ampk α 1 negatively regulates Cx43 protein expression by increasing the ubiquitination-mediated degradation and thus impacts on gap junction coupling. These observations suggest that the previously observed isoform shift towards Ampk α 1 in human heart failure and an increased Ampk α 1 activity [9] may cause detrimental effects on gap junction remodeling.

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Disclosures Statement

None.

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