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FK866 compromises mitochondrial metabolism and adaptive stress responses in cultured cardiomyocytes

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ABSTRACT

Aim: FK866 is an inhibitor of the NAD^+ synthesis rate-limiting enzyme nicotinamide phosphoribosyltransferase (NAMPT). Using FK866 to target NAD^+ synthesis has been proposed as a treatment for inflammatory diseases and cancer. However, use of FK866 may pose cardiovascular risks, as NAMPT expression is decreased in various cardiomyopathies, with low NAD^+ levels playing an important role in cardiovascular disease progression. In addition, low NAD^+ levels are associated with cardiovascular risk conditions such as aging, dyslipidemia, and type II diabetes mellitus. The aim of this work was to study the effects of FK866-induced NAD^+ depletion on mitochondrial metabolism and adaptive stress responses in cardiomyocytes.
Methods and results: FK866 was used to deplete NAD^+ levels in cultured rat cardiomyocytes. Cell viability, mitochondrial metabolism, and adaptive responses to insulin, norepinephrine, and H_2O_2 were assessed in cardiomyocytes. The drop in NAD^+ induced by FK866 decreased mitochondrial metabolism without changing cell viability. Insulin-stimulated Akt phosphorylation, glucose uptake, and H_2O_2-survival were compromised by FK866. Glycolytic gene transcription was increased, whereas cardiomyocyte hypertrophy induced by norepinephrine was prevented. Restoring NAD^+ levels *via* nicotinamide mononucleotide administration reestablished mitochondrial metabolism and adaptive stress responses.
Conclusion: This work shows that FK866 compromises mitochondrial metabolism and the adaptive response of cardiomyocytes to norepinephrine, H_2O_2, and insulin.

1. Introduction

There is mounting data associating increased nicotinamide adenine dinucleotide (NAD^+) levels with the pathogenesis of cancer and inflammatory diseases [1], making pharmacological inhibition of NAD^+ synthesis a target in treating both pathologies. NAD^+, an essential coenzyme involved in biochemical redox reactions, acts as a substrate for a wide range of enzymes, including NAD-dependent protein deacetylases, mono-ADP ribosylases, poly (ADP-ribose) polymerases (PARP), and NAD^+ glycohydrolases [2]. However, NAD^+ also regulates cell redox status, cell signaling, and gene transcription [3]. The classical pathways controlling NAD^+ production are *de novo* biosynthesis from tryptophan and the niacin salvage pathway [4]. In mammalian cells, NAD^+ consumption and production are mainly controlled by PARP-1 and nicotinamide phosphoribosyltransferase (NAMPT), which regulate NAD^+ consumption and biosynthesis, respectively [3]. NAMPT is the rate-limiting enzyme for NAD^+ synthesis and represents an attractive pharmacological target for achieving NAD^+ depletion [5–7].

The nicotinamide analog FK866 (also known as WK175 and APO866) acts as a competitive inhibitor of NAMPT [8]. FK866 was the first nanomolar-effective NAMPT inhibitor and is currently the most widely used. FK866 has shown positive results in treating

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inflammatory diseases [9,10] and cancer [11–13]. FK866-induced NAD^+ depletion is associated with lower levels of tumor necrosis factor alpha (TNFα) and interleukin 6 (IL-6) as well as neutrophil infiltration, providing a protective effect in autoimmune encephalitis, rheumatoid arthritis, and spinal cord injury. In addition, FK866 reduces tumor growth, volume, and metastatic processes in a wide variety of cancer cell lines and *in vivo* models [14]. FK866 is currently in phase II clinical trials [10]. A potential risk of using FK866 that has remained largely unexplored, however, is that tissues with elevated metabolic demand, such as cardiac tissue, could be particularly sensitive to an NAD^+ imbalance, creating a risk for detrimental long-term cardiac effects.

NAD^+ plays an important role in the cardiac physiology of ATP generation, which is achieved by fatty acid oxidation (~80%) and glycolysis (~20%) [15], followed by oxidation of NADH and FADH2 in the mitochondria. Several cardiovascular diseases, including cardiac hypertrophy and heart failure, have been associated with NAD^+ imbalances [16,17]. The potential effects of FK866 on cardiac function associated with NAD^+ homeostasis have not been previously reported. Given the above, we developed this study to investigate whether *in vitro* FK866 reduces mitochondrial metabolism and compromises the adaptive stress response in cultured cardiomyocytes.

2. Materials and methods

2.1. Materials

Antibodies against the total and phosphorylated forms of the insulin receptor β-subunit and Akt were from Cell Signaling (Danvers, MA). HRP-conjugated anti-rabbit and anti-mouse antibodies were from Calbiochem (La Jolla, CA). Tetramethylrhodamine-methyl-ester (TMRM), CM-H2DCFDA, MitoTracker Green-FM (MTG), rhodamine phalloidin, and Hoechst 33342 were from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS), fetal calf serum (FCS), and type II collagenase were from Gibco BRL (Carlsbad, CA). Anti-GAPDH antibody, propidium iodide (PI), carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP), insulin, norepinephrine (NE), FK866 hydrochloride hydrate, β-nicotinamide mononucleotide (NMN), Dulbecco's modified Eagle's medium (DMEM), M199 medium, and other reagents were from Sigma–Aldrich (St. Louis, MO). Protein assay reagents were from Bio-Rad (Hercules, CA).

2.2. Animals

Rats were obtained from the Animal Breeding Facility of the Faculty of Chemical and Pharmaceutical Sciences, University of Chile. All studies conform to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (8th Edition, 2011) and were approved by our Institutional Ethics Review Committee.

2.3. Cardiomyocyte culture

Cardiomyocytes were isolated from neonatal Sprague-Dawley rats as previously described [18]. In brief, rat ventricles were minced and cells dissociated in a solution of collagenase and pancreatin. After enzymatic digestion, the cells were plated in gelatin-coated plastic dishes and cultured in DMEM/M199 (4:1) containing 10% FBS, 5% FCS, and antibiotics (streptomycin and penicillin, 100 U/mL). To prevent the overgrowth of fibroblasts and smooth muscle cells, bromodeoxyuridine (100 μM) was used in the cell culture media. Cardiomyocytes were plated at a final density of $1–8 \times 10^3/mm^2$ on gelatin-coated Petri dishes. For microscopy experiments, cells were plated on gelatin-coated 18 mm glass coverslips in 12-well Petri dishes. Neonatal rat cardiomyocyte cultures were 95% pure through the identification with a β-myosin heavy chain antibody [18].

2.4. Treatments

24 h after plating, cells were washed with PBS and deprived of fetal bovine serum (FBS) for the remaining experimental time. Cells were left for 2 h to allow their adaption to serum absence before any treatment. Cells were then incubated with 1 or 10 μM FK866 for 48 h. For hypertrophy experiments, cells were co-incubated with 10 μM NE for 48 h. For cell death induction, cardiomyocytes were treated with 100 μM H_2O_2 during the last 24 h of FK866 treatment. For assessment of insulin signaling, 100 nM insulin were added in the last 30 min of FK866 treatment. Finally, to restore NAD^+ levels, cells were treated with FK866 and NMN for 48 h.

2.5. NAD^+ assay

To assess cellular NAD^+ levels, the EnzyChromTM NAD/NADH Assay Kit (BioAssay Systems, Hayward, CA) was used following manufacturer recommendations.

2.6. Western blot analysis

Once treatments were completed, cells were washed 3 times with PBS solution and then lysed with the lysis buffer NP40 (Tris–HCl 100 mM pH 7.4; NaCl 300 mM; NP40 0.5% v/v) supplemented with commercial protease and phosphatase inhibitors (Roche Applied Science, Penzberg, Germany). The homogenate was centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant protein content was assessed using the Bradford method (BioRad protein assay, BioRad, Hercules, CA) and then denatured in SDS buffer. Equal amounts of protein were separated by SDS–PAGE (8% polyacrylamide gels) and electrotransferred to PVDF membranes. After blocking with 5% nonfat milk in Tris-buffered saline (pH 7.6, containing 0.1% (v/v) Tween 20 (TBST)), membranes were incubated with primary antibodies at 4 °C and re-blotted with horseradish peroxidase-linked secondary antibody (1:5,000 in 1% TBST). The bands were detected using the EZ-ECL kit (Biological Industries, Kibbutz Beit Haemek, Israel) and Diversity 4 System detector with GeneSys software and quantified by image densitometry using ImageJ software (NIH). Protein content was normalized by GAPDH level.

2.7. Fluorescence microscopy analysis

Cells were seeded in 12-well plates (0.25×10^6 cells/well) on 18-mm diameter coverslips coated with gelatin (2% w/v). Treated cells were fixed with paraformaldehyde 4% v/v, permeabilized with Triton X-100 0.1% v/v, and blocked with BSA 1% v/v. Cells were then incubated with rhodamine phalloidin (1:500) and Hoechst (1:1000). Once set on microscope slides using DAKO mounting medium (DAKO Corporation, Carpinteria, CA), samples were observed using an epifluorescence microscope Axioskop 20. Images were analyzed using ImageJ, tracing the edge of at least 100 cells per condition for evaluation of cell area. To assess sarcomerization, the fluorescence pattern was determined by tracing a line equivalent to 10 μm on actin fiber generating a histogram with peaks at regular intervals in cardiomyocytes with structured sarcomeres [19].

2.8. Flow cytometry

After treatments were completed, cells were loaded with one of the following: 1,2,3-dihydrorhodamine (DHR) to assess reactive

oxygen species (ROS) levels with H_2O_2 100 μM for 30 min as a positive control; propidium iodide to determine cell viability, using H_2O_2 500 μM for 24 h as a positive control; Mitotracker Green-FM to determine mitochondrial mass; or tetramethylrhodamine methyl ester to evaluate mitochondrial potential, using the uncoupling agent CCCP as a positive control of mitochondrial membrane depolarization. Once loaded with the respective probe, cells were released by trypsinization and analyzed in a FACS-SCAN flow cytometer.

2.9. ATP measurement

Intracellular ATP levels were determined with a luciferin/luciferase-based ATP detection kit CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI) according to manufacturer instructions. Sample luminescence was quantified in a TopCount NXT microplate luminescence counter (PerkinElmer, Waltham, MA). Data was normalized as-fold over control.

2.10. Oxygen consumption

Oxygen consumption was measured by Clark oxygraphy. Cells were trypsinized, pelleted, and resuspended in PBS. The suspension was placed in a temperature-controlled chamber coupled to a Clark electrode 5331 (Yellow Springs Instruments, Yellow Springs, OH) where oxygen uptake was measured polarographically. Recording and data analysis were performed using Strathkelvin respirometry/monitoring software (North Lanarkshire, ML1 5RX).

2.11. Glucose uptake

Cardiomyocytes were rinsed twice with HEPES-buffered saline. Glucose uptake was measured using 10 mM [^{3}H]2-deoxyglucose as previously described [20].

2.12. Real time qPCR

Real-time PCR was performed with SYBR green (Applied Biosystems, Carlsbad, CA) as previously described [20]. The relative quantification in gene expression was determined using the $2^{-\Delta\Delta Ct}$ method [21]. Using this method, we obtained the fold changes in gene expression normalized to an internal control gene (18S rRNA). Primers used were as follows: *Hk2*rat forward 5′-CGAATCAAAGAGAACAAGGG-3′; *Hk2*rat reverse 5′-CAAAATGGGGATGTTTCTTG-3′; *Pfkfb2*rat forward 5′-CTGGAGGTAAAAGTGTCAAG-3′; *Pfkfb2*rat-reverse 5′-ACGAGAGGTCCTTATCATAG-3′; *Slc2a1*rat forward 5′-CAATATGTGGAGCAACTGTG-3′; *Slc2a1*rat reverse 5′-AGTAGGTGAAGATGAAGAAGAG-3′; *Slc2a4*rat forward 5′-AAGTGATTGAACAGAGCTAC-3′; *Slc2a4*rat reverse 5′- CTTTTCCTTCCCAACCATTG-3′. The mean values of the control samples Cp's for the genes were: 18S rRNA (15.5 ± 0.4), HK2 (33.2 ± 0.4), Pfkfb2 (35.4 ± 0.8), Slc2a1 (32.7 ± 0.6), Slc2a4 (37.1 ± 0.5). Data are shown as fold of change of each sample respect to the internal control sample (18S rRNA).

2.13. ROS intracellular levels assay

Cardiomyocytes were seeded in opaque 96-well plates and exposed to 1–10 μM FK866 for 48 h and treated for 30 min with chloromethyl-dichlorofluorescein (CM-H2DCFDA, 10 μM). Cells were then washed three times with PBS and fluorescence was determined in a microplate reader (excitation: 490, emission: 525 nm). H_2O_2 (100 μM, 15 min) was used as positive control.

2.14. Statistical analysis

Results are shown as representative images or as mean ± SEM of at least three independent experiments. Data was analyzed by one-way ANOVA with either Bonferroni, Tukey's or Dunnett's post-test. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Inhibition of NAMPT by FK866 depletes NAD$^+$ levels

Several studies have shown that inhibition of NAMPT by the competitive inhibitor FK866 depletes NAD$^+$ levels [8,9,22]. However, the effects of FK866 on cardiac cells remain unknown. To test this, cultured cardiomyocytes were treated with FK866 for

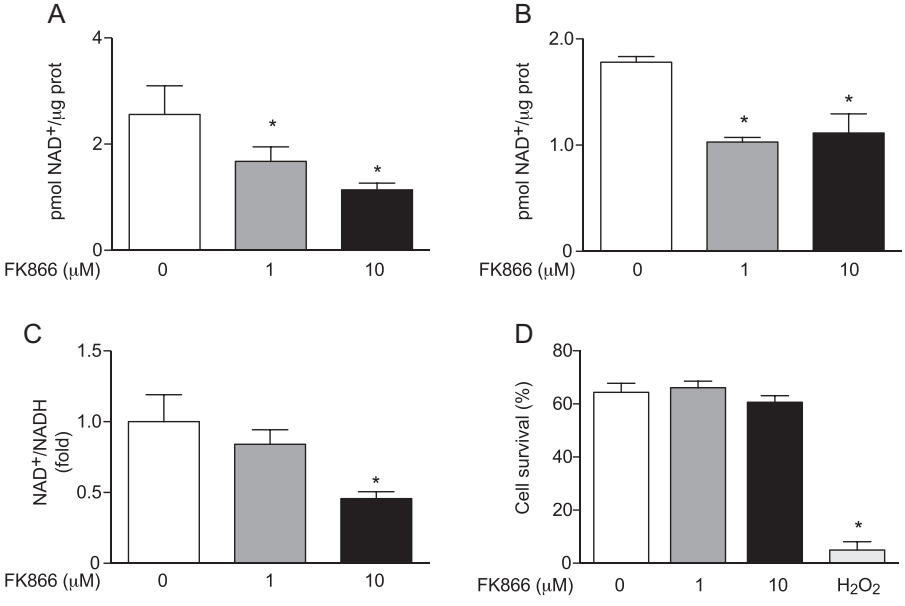

Fig. 1. FK866 depletes NAD$^+$ and NADH levels without affecting cell survival.
Cardiomyocytes were treated with FK866 1 or 10 μM for 48 h. NAD$^+$ (A) and NADH (B) contents (pmol/ug protein) were assessed with a commercial kit ($n = 3$). (C) NAD$^+$/NADH ratio was calculated for each treatment condition. (D) Cell viability was determined by PI incorporation by non-permeabilized cells. Treatment with H_2O_2 500 μM for 24 h was used as a positive cell death control $^*P < 0.05$ vs. control. Data were analyzed by one-way ANOVA followed by Dunnett's test. Bar graphs represent mean ± SEM ($n = 7$).

48 h. As shown in Fig. 1A, FK866 (1 and 10 μM) decreased NAD^+ levels from 3.0 ± 0.7 to 1.8 ± 0.4 and 1.1 ± 0.2 pmol/μg prot, respectively. FK866 (1 and 10 μM) also decreased NADH levels from 1.8 ± 0.1 to 1.0 ± 0.1 and 1.1 ± 0.3 pmol/μg prot, respectively (Fig. 1B). The NAD^+/NADH ratio is an important indicator of the redox state of a cell and controls the activity of several key enzymes, including glyceraldehyde 3-phosphate dehydrogenase and pyruvate dehydrogenase [23,24]. Our results show that FK866 reduces the NAD^+/NADH ratio in cultured cardiomyocytes treated with FK866 (Fig. 1C) but without any effect on cell viability (Fig. 1D).

3.2. FK866 compromises mitochondrial function

Mitochondrial metabolism is essential to maintaining cardiomyocyte function, and altered metabolism is associated with the development of cardiovascular diseases [25,26]. Therefore, we evaluated various parameters reflecting mitochondrial state and function in cardiomyocytes treated with FK866. Fig. 2A shows that mitochondrial membrane potential ($\Delta\Psi$mt) did not change after treatment with FK866; however, intracellular ROS levels were decreased (21.3 ± 0.2% of reduction) (Fig. 2B). We then assessed mitochondrial oxidative phosphorylation function (OXPHOS) by measuring oxygen consumption rate, as described in the Methods section. Significant decreases in oxygen consumption were observed in cells treated with 1 and 10 μM FK866 (29.8 ± 0.2% and 38.5 ± 0.2% of reduction, respectively) (Fig. 2C). These results were consistent with decreased intracellular ATP levels (Fig. 2D). Taken together, these results suggest that the reduced NAD^+ levels induced by FK866 lead to mitochondrial dysfunction.

It is well known that increased glycolytic enzyme expression serves as an important homeostatic mechanism for energy balance recovery in cells with mitochondrial dysfunction. In order to investigate this aspect of mitochondrial function, we measured mRNA levels for key components of glycolysis in cardiomyocytes treated with FK866. As shown in Fig. 2E, mRNA levels for hexokinase 2 (*HK2*) and 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2 (*Pfkfb2*) increased in cells treated with FK866. Increased mRNA levels for glucose transporter 1 (*Slc2a1*) and glucose transporter 4 (*Slc2a4*) were also observed in these cells.

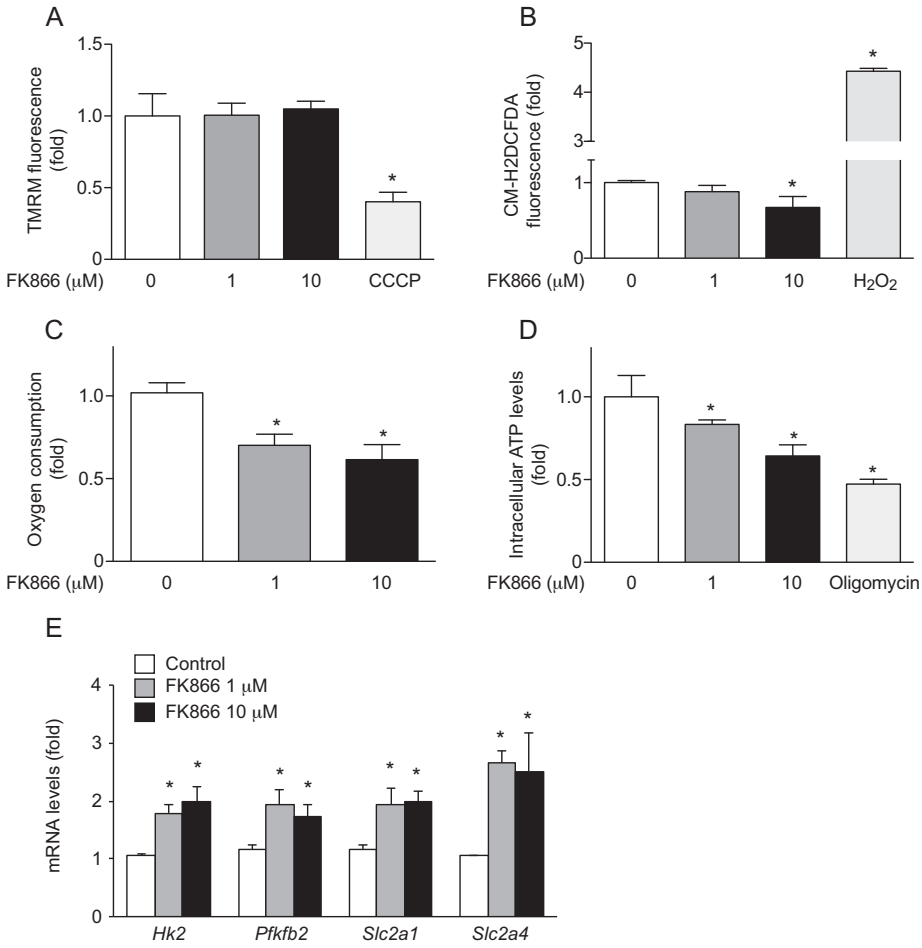

Fig. 2. FK866 triggers mitochondrial dysfunction.
Cardiomyocytes were treated with FK866 1 or 10 μM for 48 h. (A) Mitochondrial membrane potential was determined by TMRM staining (200 nM, 30 min) and flow cytometry. The uncoupling agent CCCP (10 μM, 30 min) was used as a positive control for mitochondrial depolarization (relative value of control, 1 = 2620 ± 1120 arbitrary fluorescence units) (n = 5). (B) Total intracelullar ROS levels were measured using a fluorescence plate reader and the dye CM-H_2DCFDA. H_2O_2 (100 μM, 30 min) was used as a ROS positive control (relative value of control, 1 = 1859 ± 349 arbitrary fluorescence units) (n = 3). (C) Basal oxygen consumption rates were measured using a Clark's electrode (relative value of control, 1 = 260 ± 45 nmol O_2/min/10^6 cells) (n = 5). (D) Intracellular ATP levels were measured with a commercial kit. Oligomycin (10 μM, 1 h) is shown as a positive control for ATP drop (n = 3). (E) mRNA levels of Glut4 (Slc2a4), Glut1 (Slc2a1) hexokinase 1 (Hk1) and hexokinase 2 (Hk2) were measured by RT-qPCR and analyzed using the ΔΔCt method. Data are shown as fold of change of each sample respect to the internal control gene (18RNA) (n = 4). $^*P < 0.05$ vs. control. Data were analyzed by one-way ANOVA followed by Dunnett's test. Bar graphs represent mean ± SEM (n = 4).

3.3. NMN restores NAD$^+$ levels and prevents mitochondrial dysfunction induced by FK866

NMN is the product of NAMPT activity and is used by nicotinamide mononucleotide adenylyltransferase (NMNAT), the second enzyme of the NAD$^+$ salvage pathway, to catalyze the final step of NAD$^+$ biosynthesis [23]. To confirm that FK866 depletes NAD$^+$ levels through inhibition of NAMPT activity, cultured cardiomyocytes were supplemented with NMN 1 mM and simultaneously treated with FK866 (1 or 10 μM) for 48 h. Fig. 3A shows that adding NMN prevents the decrease in NAD$^+$ levels in cardiomyocytes treated with both concentrations of FK866 and increases baseline levels in untreated cells. We also observed a recovery of NADH levels (Fig. 3B) and NAD$^+$/NADH ratio (Fig. 3C).

Because FK866 decreased cardiomyocyte mitochondrial function (Fig. 2), we investigated the effect of NMN supplementation on mitochondrial metabolism in cardiomyocytes treated with FK866. As shown in Fig. 4, NMN prevented the decreases in both oxygen consumption (Fig. 4A) and intracellular ATP levels (Fig. 4B) in cells treated with 1 or 10 μM of FK866. Moreover, treatment with NMN prevented increases in *Hk2*,*Pfkfb2*, *Slc2a1*, and *Slc2a4*mRNA levels (Fig. 4C).

3.4. FK866 impairs insulin response

Insulin contributes to the supply of substrates for ATP production in the heart, regulating energy balance through its actions in glucose transport, glycolysis, pyruvate, fatty acids, and glycogen metabolism [27]. Abnormal insulin signaling plays an important role in various pathological conditions [28], and increased glucose availability improves cardiomyocyte function [29]. To study the effects of NAD$^+$ depletion on the insulin signaling pathway, cells were treated with FK866 for 48 h and then stimulated with insulin for 30 min. Fig. 5A shows a representative Western blot of the phosphorylated and total forms of the insulin receptor and Akt. As expected, phospho-Akt levels increased after exposure to insulin for 30 min in comparison to untreated cardiomyocytes. No changes were observed in the phospho-IR levels of FK866-treated cells. However, insulin-dependent Akt phosphorylation was significantly lower in cardiomyocytes treated with FK866. This result correlated with a significant reduction in glucose uptake (Fig. 5B). Additionally, increased mRNA levels for *Hk2*, *Pfkfb2, Slc2a1*, and *Slc2a4*were observed in cardiomyocytes stimulated with insulin, similar to those observed in cells treated with FK866. Surprisingly, this increase was inhibited in cells exposed to both stimuli (Fig. 5C). Finally, to determine the effect of NAD$^+$ recovery on insulin activity, cells were treated with NMN (1 mM) and FK866 (10 μM) for 48 h and stimulated with insulin (100 nM) for 30 min. The results show that NMN was able to restore insulin-mediated Akt activation in cardiomyocytes treated with FK866 (Fig. 6), consistent with the key role of NAD$^+$ levels in the activation of the insulin/Akt pathway and expression of diverse glycolytic components in cardiac cells.

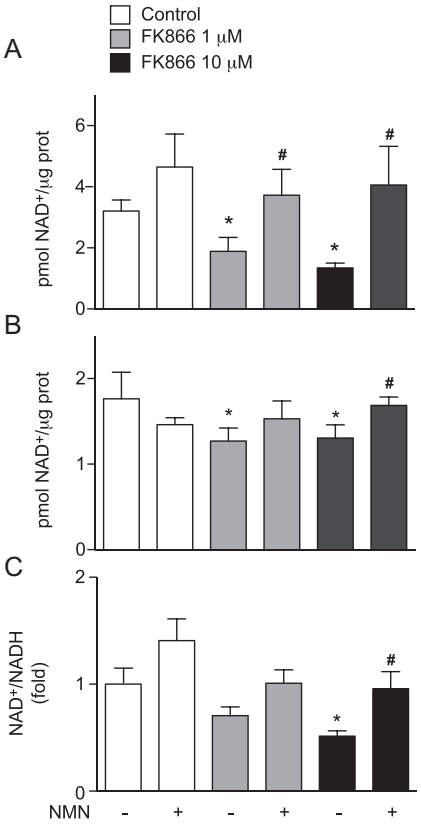

Fig. 3. NMN administration restores NAD$^+$ levels in cells treated with FK866. Cardiomyocytes were treated with FK866 1 or 10 μM for 48 h with or without co-incubation with NMN 1 mM. NAD$^+$ (A) and NADH (B) levels were assessed with a commercial kit ($n = 3$). (C) NAD$^+$/NADH ratio was calculated for each treatment condition. $^*P < 0.05$ vs. control; $^\#P < 0.05$ vs. FK866. Data were analyzed by one-way ANOVA followed by Bonferroni correction. Bar graphs represent mean ± SEM ($n = 3$).

3.5. FK866 decreases cardiomyocyte hypertrophic response triggered by norepinephrine

Cardiomyocyte hypertrophy is an adaptive cellular mechanism triggered in response to ischemia, hemodynamic overload, and adrenergic system activation [30,31]. At the cellular level, hypertrophic cardiomyocytes display several changes, such as increased cell area and sarcomere assembly [32]. We investigated the effects of NAD$^+$ depletion on the NE-induced hypertrophic response. Cultured cardiomyocytes were treated with NE (10 μM for 48 h) and stained with rhodamine phalloidin to detect structured sarcomeres. Our results showed that after treatment with NE, cells displayed the characteristic morphology of the hypertrophic phenotype, with increased sarcomere structure and abundance. These NE-induced morphological changes were prevented by FK866 and restored by NMN (Fig. 7A). To evaluate sarcomere organization we determined the fluorescence pattern associated with a linear path (approx. 10 μm) traced on one actin fiber of cells stained with rhodamine phalloidin. Hypertrophic cardiomyocytes exhibit greater sarcomeric structure. Therefore the line passes through fluorescence ordered sequences, generating a histogram with peaks at regular intervals. In agreement with this, relative linear fluorescence profile was observed as a structured pattern in cells treated with NE. A decrease in this structured pattern was observed in cells treated with NE and FK866 but restored in cells cultured with NE, FK866, and NMN (Fig. 7B). Similar results were observed when we used cell area as a hypertrophy parameter (Fig. 7C).

3.6. FK866 promotes susceptibility to H_2O_2-induced cell death

NAD$^+$ is required for key cellular processes such as DNA repair, transcriptional regulation, redox reactions, and other mechanisms associated with the regulation of cell survival and adaptation to stress [24,33,34]. Cardiomyocytes are terminally differentiated cells, unable to proliferate, and progressive loss of these cells is one of the most important components in the development of heart failure [35]. To evaluate the effect of NAD$^+$ depletion on cardiomyocyte survival, cells were treated with FK866 for 48 h and exposed to H_2O_2 (100 μM for 24 h). As shown in Fig. 8A, cultured cardiomyocytes exposed to H_2O_2 in the presence or absence of FK866 showed decreased cell viability (29.8 ± 9.5% and

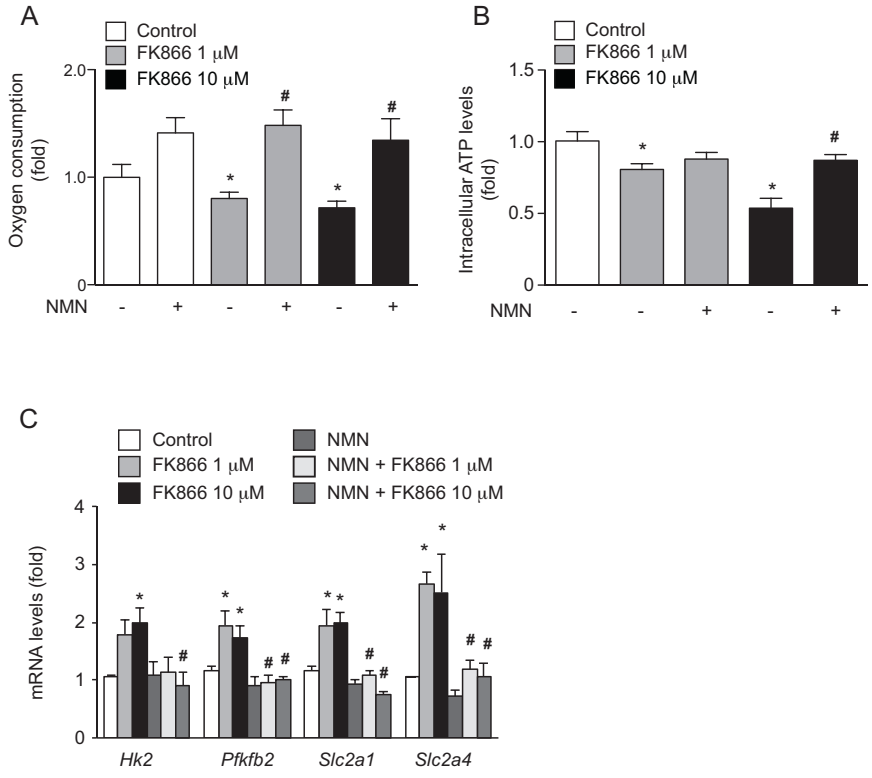

Fig. 4. NMN administration prevents mitochondrial dysfunction induced by FK866.
Cardiomyocytes were treated with FK866 1 or 10 μM for 48 h with or without co-incubation with NMN 1 mM. (A) Baseline oxygen consumption rates were measured using a Clark electrode (relative value of control, 1 = 257 ± 37 nmol O_2/min/10^6 cells) (n = 3). (B) Intracellular ATP levels were measured with a commercial kit (n = 3). (C) mRNA levels of Glut4 (Slc2a4), Glut1 (Slc2a1) hexokinase 1 (Hk1), and hexokinase 2 (Hk2) were measured by RT-qPCR and analyzed using the ΔΔCt method. Data are shown as fold of

54.8 ± 4.8% living cells, respectively) compared to control (78.9 ± 3.9% living cells). To corroborate these results, the same experiment was done in cardiomyocytes co-incubated with NMN to recover NAD^+ levels. NMN treatment by itself did not prevent H_2O_2-induced death; however in cardiomyocytes treated with H_2O_2 plus FK866 and NMN, cell survival restored the levels observed in cardiomyocytes treated with H_2O_2 alone (Fig. 8B).

4. Discussion

This study shows that FK866-induced NAD^+ depletion in cardiomyocytes stimulates mitochondrial dysfunction and compromises the adaptive stress response, and that these effects can be prevented by restoring NAD^+ levels with NMN. At baseline, cardiomyocytes seem to be able to adapt to FK866-induced NAD^+ depletion, but not when exposed to a stress condition. Collectively, our data show that NAD^+ plays a critical role in the cardiomyocyte adaptive stress response and that FK866 may alter cardiomyocyte function.

The intrinsically high metabolic rate of cardiomyocytes makes this cell type especially susceptible to drugs like FK866 that target mitochondrial energy production. This specific chemical inhibitor of NAMPT has been mainly tested in tumoral cell lines [36] and only in a few non-tumoral cells, including hepatocytes [12] and kidney cells [13]. Our work is the first report showing the effects of FK866 in normal cardiomyocytes. FK866 was highly efficient in depleting intracellular NAD^+ levels (a nearly 70% decrease) in cardiomyocytes. Hsu et al. reported a 33% reduction of NAD^+ levels in cultured rat cardiomyocytes transfected with siRNA against NAMPT [37]. Both studies suggest an essential role for NAD^+ in cardiomyocyte survival. In our hands, FK866 also stimulated a mild and dose-independent decrease in NADH levels. This differential effect of FK866 on NAD^+ and NADH levels in cardiomyocytes could be explained by the pyruvate–lactate conversion mediated by lactate dehydrogenase [38,39] and by the activity of ubiquinone oxidoreductase and other dehydrogenases [40]. However, this hypothesis should be tested in future studies.

Our results also show that FK866 induces mitochondrial dysfunction, as this inhibitor decreased ATP, ROS levels, and oxygen consumption, consistent with the key role of NAD^+ in mitochondrial metabolism (reviewed in [41]). Furthermore, glycolytic markers increased as a compensatory mechanism to cope with metabolic stress. Under our experimental conditions, mitochondrial membrane potential did not change, suggesting that substrate deficiency is responsible for the drop in mitochondrial metabolism [42] rather than mitochondrial damage, as shown in cancer cells treated with FK866 [43]. This result is also consistent with maintenance of cardiomyocyte viability in contrast with the activation of the apoptotic intrinsic pathway observed in cancer cells [44]. Cardiomyocytes are highly plastic cells, capable of adapting to numerous types of stress [45]. In this work, we measured three forms of adaptive response: metabolic response to insulin, hypertrophic response to NE, and survival response to H_2O_2.

Insulin regulates numerous aspects of cardiomyocyte physiology, including glucose transport and utilization, glycolytic rate, glycogen synthesis, mitochondrial dynamics [18], contractility [46], cardiac hypertrophy [47], autophagy [48], and survival [49]. Insulin coordinates nutrient availability with efficiency and economy of energetic processes, as well as activation of catabolic reactions [27]. Akt regulates those cellular functions and others, such as autocrine and paracrine factor production, through the action of its multiple substrates [50]. Our results show that FK866-

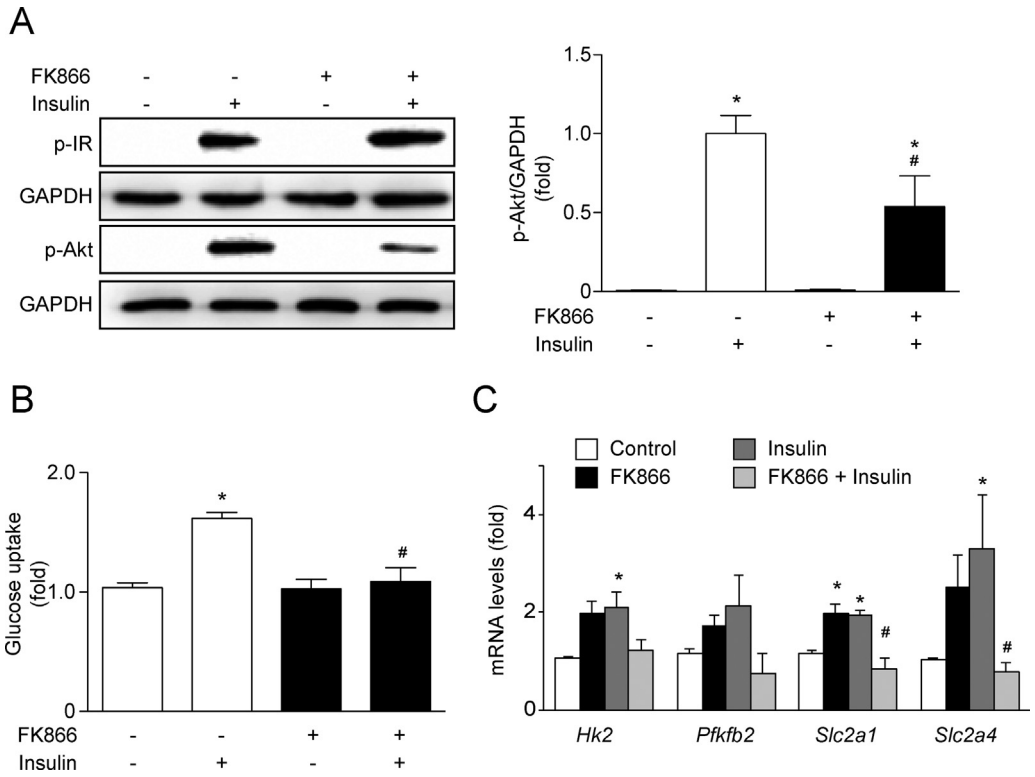

Fig. 5. FK866 compromises insulin action in cultured cardiomyocytes.
Cells were incubated with FK866 for 48 h and then treated with or without insulin (100 nM, 30 min). (A) Representative Western blot for phosphorylated insulin receptor (p-IR; $Tyr^{1150/1151}$) (left panel) and phosphorylated Akt (p-Akt; Ser^{473}) (right panel) Densitometric quantification of p-Akt levels vs. GAPDH (relative value of insulin, $1 = 0.89 \pm 0.17$ arbitrary densitometry units) ($n = 3$). (B) Glucose uptake was measured by incorporation of [^{3}H] 2-deoxyglucose (relative value of control, $1 = 9.6 \pm 3.2$ cpm/μg protein) ($n = 3$). (C) mRNA levels of Glut4 (Slc2a4), Glut1 (Slc2a1) hexokinase 1 (Hk1) and hexokinase 2 (Hk2) were measured by RT-qPCR ($n = 4$). $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. Insulin. Data were analyzed by one-way ANOVA followed by Bonferroni correction. Bar graphs represent mean ± SEM ($n = 4$).

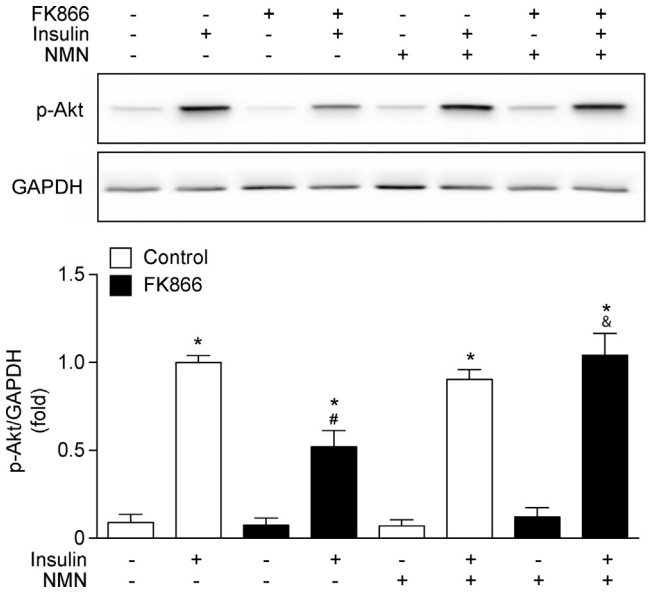

Fig. 6. NMN counteracts the effects of FK866 on insulin action.
Cells were treated with FK866 10 μM for 48 h with or without co-incubation with NMN 1 mM. Cells were then treated with insulin (100 nM for 30 min), and phospho-Akt levels were determined by Western blot as described in Methods. Upper panel, Representative Western blot for phosphorylated Akt (p-Akt; Ser^{473}); Lower panel, Densitometric quantification of p-Akt levels vs. GAPDH (relative value of insulin, $1 = 1.43 \pm 0.09$ arbitrary densitometry units) ($n = 3$). $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. Insulin, $^{\&}P < 0.05$ vs. FK866 + Insulin. Data were analyzed by one-way ANOVA followed by Tukey's test. Bar graphs represent mean ± SEM ($n = 3$).

induced NAD^+ depletion reduces Akt phosphorylation and lower glucose uptake in response to insulin, suggesting a loss of response to insulin by cardiomyocytes. Complete Akt activation involves phosphorylation in Thr^{308} and Ser^{473} residues [51]. We assessed Ser^{473} phosphorylation, which relies on Akt binding to phosphatidylinositol 3,4,5-triphosphate (PIP_3) and promotes its translocation to the plasma membrane and further phosphorylation by phosphoinositol dependent kinase 1 (PDK1). Both Akt and PDK1 are deactivated by acetylation in their pleckstrin homology (PH) domains, and both are also targets of NAD^+ dependent deacetylase SIRT1 [52]. Along the same lines, Li et al. conducted an experiment with SIRT1 overexpression, and found a drop in Akt acetylation and an increase in Akt activity [53]. Therefore, FK866-induced NAD^+ depletion might impair the insulin response of cardiomyocytes by decreasing the activity of SIRT1 on Akt and PDK1; however, the mechanisms remain to be investigated. The reduction of glucose utilization and insulin-induced Akt phosphorylation in the myocardium are associated with cardiovascular risk factors such as diabetes, hypertension, and coronary artery disease [50]. In addition, cardiac insulin resistance has been associated with higher acetylation and lower phosphorylation of Akt in HFD-fed mice [54]. Given the above findings, decreased insulin response due to NAD^+ depletion could pose an increase in cardiovascular risk.

We also assessed the effect of FK866 on NE-induced pathological hypertrophy. Cardiac hypertrophy is an initially adaptive response to work overload that results in increased cardiac output [55], although chronic work overload leads to myocardium dysfunction, cardiomyocyte loss, fibrosis, and heart failure [56]. Our results show that FK866 prevents cardiomyocyte hypertrophy triggered by NE [19]. This effect could be explained by the inhibition of the ADP-ribosyl

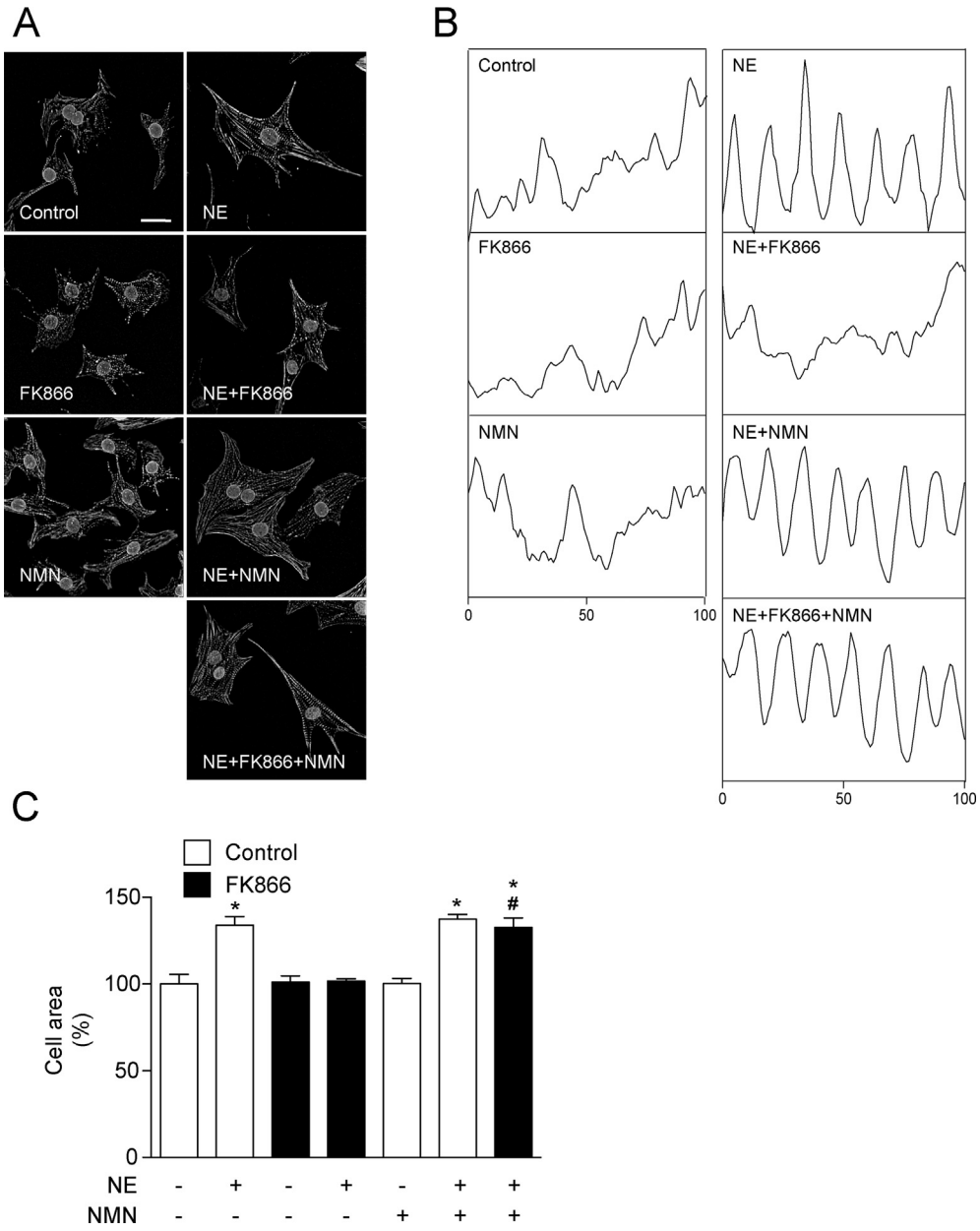

Fig. 7. FK866 compromises cardiomyocyte hypertrophy, and this effect is prevented by NMN.
Cells were incubated with FK866 for 48 h with or without NMN (1 mM). Cells were then treated additionally with NE (10 μM for 48 h). (A) Representative fluorescence microscopy images of rhodamine–phalloidin-stained cells ($n = 4$). Scale bar = 10 μm. (B) Assessment of structured sarcomeres by a relative fluorescence profile equivalent to a 10 μm-long line traced on actin fiber. (C) Quantification of relative mean cell area of at least 100 cells per condition (relative value of control, 1 = 58,619 ± 44,223 pixels^2) ($n = 4$). $^*P < 0.05$ vs. control; $^\#P < 0.05$ vs. NE + FK866. Data were analyzed by one-way ANOVA followed by Bonferroni correction. Bar graphs represent mean ± SEM.

cyclase CD38. CD38 uses NAD^+ to produce cyclic ADP-ribose (cADPR), a second messenger that mobilizes intracellular Ca^{2+}, which is part of the NE signaling pathway for hypertrophic development *via* calcineurin activation [19]. cADPR has been reported to participate in the hypertrophic response to angiotensin II; however, the molecular mechanisms underlying cADPR signaling remain elusive [57]. It must be emphasized that while preventing hypertrophic response may seem to be a favorable effect, hypertrophy is initially beneficial since it improves cardiac function in conditions of higher demand [58].

FK866 also increased susceptibility to H_2O_2-dependent cardiomyocyte death. There are multiple possible explanations for this result. First, this finding may be attributable to mechanisms related to regulation of gene transcription and protein synthesis that depend on ATP availability. Second, NAD^+ depletion is associated with inhibition of autophagy flux [59], required for nutrient supply and elimination of damaged molecules [60]. Moreover, Akt exerts a cardioprotective effect, *via* inhibition of gene transcription [61] and activation of pro-apoptotic factors [50]. Thus, diminished Akt activity might impede cytoprotection.

Some limitations of our study are: (a) the use of cultured neonatal cardiomyocytes does not allow to extrapolate the effect of FK866 to adult heart. Additionally, these cells do not contract spontaneously due to serum deprivation and have lower energy needs that may moderate the impact of NAD^+ reduction. (b) Chronic treatment and the accumulation of FK886 in cardiomyocytes were not assessed also due to the experimental model used. This point is relevant because FK866 is being evaluated as an anticancer drug. Despite these limitations, neonatal rat cardiomyocyte cultures are a well-established model to study cell survival,

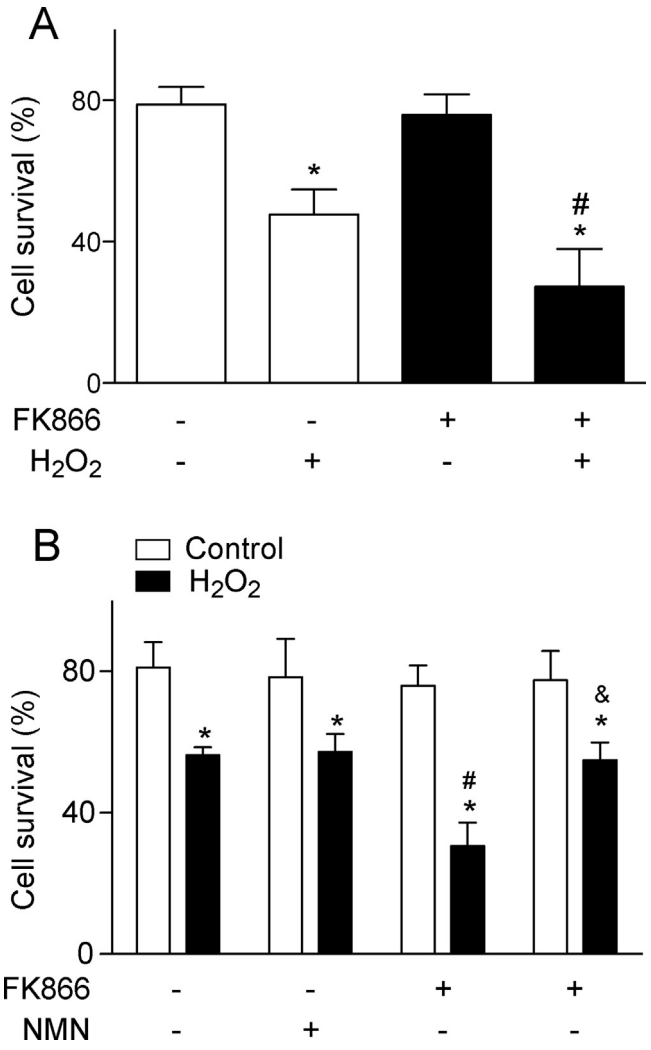

Fig. 8. FK866 increases susceptibility to H_2O_2-induced death, which is prevented by restoring NAD^+ levels with NMN.
Cell viability was determined by PI incorporation by non-permeabilized cells. (A) Cells were treated with FK866 for 48 h with or without 100 μM H_2O_2 during the last 24 h. Cell death was assayed as described in Methods ($n = 3$). Bar graphs represent mean ± SEM. Data were analyzed by one-way ANOVA followed by Bonferroni correction. $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. H_2O_2. (B) Cells were co-incubated additionally with NMN 1 mM for 48 h when indicated. Bar graphs represent mean ± SEM ($n = 3$). $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. H_2O_2; $^{\&}P < 0.05$ vs. FK866 + H_2O_2. Data were analyzed by one-way ANOVA followed by Bonferroni correction.

hypertrophy and response to insulin. Our results clearly show that FK866 has deleterious effects on cardiomyocyte metabolism and adaptive response.

In conclusion, our study reinforces the importance of the NAD^+ salvage pathways in cardiomyocytes, especially the NAMPT pathway, for maintaining mitochondrial function and sounds an alarm regarding the safety of FK866 as a therapy drug, especially in cardiovascular patients.

Conflict of interest

None declared.

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