Mitochondrial uncoupling downregulates calsequestrin expression and reduces SR Ca\(^{2+}\) stores in cardiomyocytes

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Aims
Mitochondrial cardiomyopathy is associated with deleterious remodelling of cardiomyocyte Ca\(^{2+}\) signalling that is partly due to the suppressed expression of the sarcoplasmic reticulum (SR) Ca\(^{2+}\) buffer calsequestrin (CASQ2). This study was aimed at determining whether CASQ2 downregulation is directly caused by impaired mitochondrial function.

Methods and results
Mitochondrial stress was induced in cultured neonatal rat cardiomyocytes by means of the mitochondrial uncoupler carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP). Ca\(^{2+}\) transients and reactive oxygen species (ROS) were measured by confocal microscopy using the indicators fluo-4 and MitoSOX red, respectively. Mitochondrial stress led to concentration-dependent downregulation of calsequestrin (CASQ2) and changes in the Ca\(^{2+}\) signals of the cardiomyocytes that were accompanied by reduction in SR Ca\(^{2+}\) content and amplitude and duration of Ca\(^{2+}\) sparks. Caspase 3, p38, and p53 inhibitors had no effect on FCCP-induced CASQ2 downregulation; however, it was attenuated by the ROS scavenger N-acetylcysteine (NAC). Importantly, NAC not only decreased FCCP-induced ROS production, but it also restored the Ca\(^{2+}\) signals, SR Ca\(^{2+}\) content, and Ca\(^{2+}\) spark properties to control levels.

Conclusion
Mitochondrial uncoupling results in fast transcriptional changes in CASQ2 expression that manifest as compromised Ca\(^{2+}\) signalling, and these changes can be prevented by ROS scavengers. As impaired mitochondrial function has been implicated in several cardiac pathologies as well as in normal ageing, the mechanisms described here might be involved in a wide spectrum of cardiac conditions.

Keywords
E-C-coupling • Calcium release • ROS • Antioxidant therapy • Mitochondrial cardiomyopathy

1. Introduction
Mitochondrial dysfunction poses severe challenges to cells that are heavily energy-dependent, such as cardiomyocytes, and it has been shown to underlie pathological conditions including hypertrophic cardiomyopathy, arrhythmias, doxorubicin-induced cardiomyopathy, and sudden cardiac death (for reviews, see references 3,4). It is known that impaired oxidative phosphorylation (OXPHOS) contributes to disease progression via energy depletion, oxidative stress, and regulation of apoptosis. While the mechanisms linking mitochondrial dysfunction and cardiac performance are subjects of ongoing investigation, animal models have shed some light on disturbances in cardiomyocyte function. Significant changes in cardiomyocyte Ca\(^{2+}\) handling were found in a mouse model of mitochondrial cardiomyopathy with cardiac-specific knockout of mitochondrial transcription factor A; Ca\(^{2+}\) handling was impaired possibly due to changes in sarcoclemmal Ca\(^{2+}\) buffering capacity, with marked downregulation of the sarcoplasmic reticulum (SR) Ca\(^{2+}\) buffer calsequestrin 2 (CASQ2).6

CASQ2 is normally expressed at constant levels throughout life and not only acts as an SR Ca\(^{2+}\) sensor, but also regulates SR Ca\(^{2+}\) release via interactions with triadin, junctin, and the ryanodine receptor.8–10 Dysregulation of CASQ2 expression leads to altered Ca\(^{2+}\) release and contractile dysfunction, is involved in the pathogenesis of doxorubicin-induced cardiomyopathy, and contributes to the development of arrhythmias and sudden cardiac death in patients with catecholaminergic polymorphic ventricular tachycardia (CPVT).13

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At the cellular level, impaired Ca\(^{2+}\) handling due to mitochondrial dys-function would have serious consequences for cardiac cells, as Ca\(^{2+}\) signals regulate cell contractions as well as a myriad of cell signalling cascades involved in metabolism and gene expression.\(^{14,15}\)

The importance of mitochondria in maintaining cardiac cell function is well known, but what is less understood is how mitochondrial dysfunction contributes to the pathological changes of cardiomyocytes, which later lead to heart disease. Mitochondrial cardiomyopathy is associated with progressive changes in the morphology of mitochondria, an adaptive, transcriptional switch from oxidative to anaerobic metabolism\(^3\) and suppressed expression of SR proteins, such as calsequestrin 2. However, it is not known to what extent these changes are directly caused by impaired mitochondrial function. In this study, we exposed cultured mouse ventricular cardiomyocytes to the mitochondrial uncoupler carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), and interestingly, changes in Ca\(^{2+}\) handling and CASQ2 expression were found similar to those reported in animal models of cardiomyopathy. Further, co-exposure of cells to FCCP and the reactive oxygen/nitrogen scavenger N-acetylcysteine (NAC) not only improved Ca\(^{2+}\) handling, but also attenuated the downregulation of CASQ2. Together, these data show a link between impaired mitochondrial function and cardiomyocyte Ca\(^{2+}\) handling and highlight the importance of reactive oxygen species (ROS) at the levels of function and gene expression.

2. Methods

2.1. Cell isolation and culturing

All protocols conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) and were approved by the Animal Use and Care Committee of the University of Oulu. Rat neonatal ventricular cardiomyocytes were isolated 1–2 days after birth as described previously.\(^7\) Briefly, ventricles were excised, cut into small pieces, and incubated for 1 h in a solution containing (in mM): 100 NaCl, 10 KCl, 1.2 K\(_2\)HPO\(_4\), 4.0 MgSO\(_4\), 50 taurine, 20 glucose, 10 HEPES, 2 mg/mL collagenase type II (Worthington, Lakewood, NJ, USA), 2 mg/mL pancreatin (P-3292, Sigma, St Louis, MO, USA), and 1% penicillin–streptomycin. After incubation, the detached cells were collected in 15 mL Falcon tubes and centrifuged for 5 min at 160 g. The supernatant and the top layer of the pellet containing damaged cells were discarded. For mRNA and protein measurements, the isolated cardiomyocytes were plated on 35 mm fibronectin-coated plastic dishes, and for confocal imaging, on laminin-coated, glass-bottom Petri dishes as described previously.\(^8\) The cells were cultured to reach confluence in Dulbecco’s modified Eagle’s medium (DMEM) of function and gene expression.

2.2. Ca\(^{2+}\) and ROS imaging

Ca\(^{2+}\) signals were measured with a confocal microscope as described previously.\(^7\) Briefly, cells were loaded in DMEM + 1 mM probenecid solution for 1 h at 37°C with fluo-4-AM-ester (10 \(\mu\)M, dissolved in pluronic DMSO, Molecular Probes, Eugene, OR, USA). The cells were incubated at room temperature (20–22°C) at least 30 min for the dye to de-esterify. To measure Ca\(^{2+}\) signals, the culture dishes were placed in a custom-made perfusion system built into an Olympus Fluoview 1000 confocal inverted microscope. Cells were maintained at a 32–35°C by continuous superfusion with pre-heated DMEM + Glutamax I (Gibco, Carlsbad, CA, USA) culturing medium (pH 7.4, bubbled with 95% O\(_2\)/5% CO\(_2\)). For Ca\(^{2+}\) imaging, fluo-loaded myocytes were excited at 488 nm, and the emitted light was collected with a spectral detector from 520 to 620 nm through a \(\times 20\) or \(\times 60\) objective lens. When electrically excited, the cells were paced with 1 ms voltage pulses at 50% over the excitation threshold through two platinum wires located one on each side of the Petri dish. Cells were line-scanned at 400–600 Hz, depending on the length of the scanning line, with a fixed pixel time of 10 ms with the \(\times 20\) objective and 2 ms with the \(\times 60\) objective. To estimate SR Ca stores, 10 mM of caffeine was applied rapidly (<2 s) on fluo-loaded myocytes. In order to equalize the SR storing conditions, the cells were paced at 0.5 Hz, and pacing was stopped 3 s before the caffeine was applied. Fluo-fluorescence intensity is expressed as an F/F\(_0\) ratio, where F is the background-subtracted fluorescence intensity and F\(_0\) is the background-subtracted minimum fluorescence value measured from each cell at rest. Ca\(^{2+}\) sparks were analysed by SparkMaster\(^9\) as described previously.\(^10\)

Superoxide production was measured using the indicator MitoSOX Red (Molecular Probes) according to the manufacturer’s instructions. Briefly, cells were loaded with 5 \(\mu\)M MitoSOX reagent working solution and incubated for 10 min at 37°C. During measurements, culture dishes were perfused with warm, buffered DMEM with or without FCCP and NAC. Confocal images were taken at 1024 \(\times\) 1024 pixels with a \(\times 40\) oil objective, with a scanning rate of 10 \(\mu\)s per pixel. MitoSOX was excited at 515 nm, and emitted light was measured from 520–620 nm. Images were analysed using ImageJ.

2.3. RNA isolation and quantitative real-time PCR

Total RNA from cell cultures was isolated using the GenElute Mammalian Total RNA Miniprep Kit (Sigma). cDNA was synthesized using the First Strand cDNA Synthesis Kit (MBI Fermentas), and quantitative PCR (qPCR) reactions were performed with the ABI 7700 Sequence Detection System (Applied Biosystems, USA) using TaqMan chemistry. The mouse calsequestrin 2 (CASQ2, BC072547) primers were 5′-AAAGGAGCA TCAAAGACCCACC-3′ and 5′-TCGTCTTCCATGTTTCAACAA-3′, and the fluorogenic probe was 5′-Fam-CTGCCTTGGCCGCCCAAG AGG-Tamra-3′. The results were normalized to 18S rRNA quantified from the same samples using the forward and reverse primers 5′-TGGTTGCAAAAGCTAACTTAAAG-30 and 5′-AGTCAAATTAAG CGCGAAGGC-3′ and the fluorogenic probe 5′-Vic-CCTGTGTTGTC CCTTCCGTCCA-Tamra-3′.

2.4. Western blotting

Cardiomyocytes were prepared from 2- to 4-day-old rats. Cells were plated on fibronectin-coated six-well plates at a density of 1 \(\times\) 10\(^5\) cells/cm\(^2\). Cells were cultured in DMEM (Invitrogen) containing 10% FBS and 1% penicillin–streptomycin for 48 h. On the third and fourth day, 1 \(\mu\)M FCCP with or without 1 mM NAC was added to the culture medium. After 48 h of culture with FCCP + NAC, myocytes were lysed by buffer containing (in mM): 20 Tris–HCl (pH 7.5), 150 NaCl, 1 ethylenediaminetetraacetic acid, 1 ethylene glycol-bis(\(\beta\)-amino ethylether) tetra-acetic acid, 2.5 sodium pyrophosphate, 1 \(\beta\)-glycerophosphate, 1 Na\(_2\)VO\(_4\), 2 benzamide, 1 phenylmethylsulfonylfouride, 50 NaF, 2 dithiothreitol, 1% Triton, 10 \(\mu\)g/mL leupeptin, 10 \(\mu\)g/mL pepstatin, and 10 \(\mu\)g/mL aprotinin. Samples were sonicated and centrifuged. The crude protein fraction was collected, and total protein concentration was measured with the Bio-Rad Protein Assay Kit II (Bio-Rad).

For western blot analysis, 20 \(\mu\)g of protein was used. Samples were electrophoresed on an 8% SDS–polyacrylamide gel and transferred to a nitrocellulose membrane (0.2 \(\mu\)m, Bio-Rad Laboratories). The membrane was blocked in 5% non-fat milk and incubated with calsequestrin 2 (PA1–913, Thermo Scientific) or GAPDH (Cell Signaling 2118) primary antibody. After incubation with horseradish peroxidase-linked secondary antibody, blots were visualized with the ECL Plus Detection Kit.
analysed using one-way ANOVA and Bonferroni’s test. Results showed that mitochondrial inhibition alters 
Ca$^{2+}$ handling of cardiomyocytes.

3.1. FCCP-induced CASQ2 downregulation is concentration-dependent

CASQ2 is the main SR Ca$^{2+}$ buffer in neonatal and adult cardiomyocytes, and it is expressed throughout life at nearly constant levels. Dysregulation of CASQ2 expression leads to altered Ca$^{2+}$ release and contractile dysfunction, as in the case of models of mitochondrial cardiomyopathy and chronic doxorubicin cardiomyopathy. Therefore, we first studied whether CASQ2 expression is sensitive to mitochondrial uncoupling by FCCP. Treatment of neonatal cardiomyocytes for 48 h with FCCP induced a concentration-dependent inhibition of CASQ2 expression. We found that CASQ2 mRNA decreased with increasing concentrations of FCCP, with 5 μM FCCP causing a 95% reduction in CASQ2 (Figure 1).

3.2. Mitochondrial inhibition alters Ca$^{2+}$ handling of cardiomyocytes

Reduced SR Ca$^{2+}$ buffering due to decreased CASQ2 expression should have an impact on the cardiomyocyte Ca$^{2+}$ signals. Therefore, Ca$^{2+}$ transients were measured by confocal Ca$^{2+}$ imaging; cultured cardiomyocytes were exposed to 1 μM FCCP for 48 h, then Ca$^{2+}$ signals were measured from fluo-4-loaded cells at 1 Hz pacing. Cells were perfused without FCCP in order to study only the chronic changes in Ca$^{2+}$ transients. After 48 h treatment with FCCP, cardiomyocytes maintained confluency in culture and continued to spontaneously contract at a rate not significantly different from control cells (0.13 ± 0.16 vs. 0.15 ± 0.13 s in controls, n = 11 and 28 culture dishes, respectively). However, there were significant changes in Ca$^{2+}$ transients following the inhibition of mitochondrial function (Figure 2). The time constant of cytosolic Ca$^{2+}$ removal, represented by the decay of the Ca$^{2+}$ transient, was significantly decreased by ~40% (186.5 ± 15.7 vs. 320.4 ± 25.1 ms in controls, P < 0.001, n = 61). The upstroke of the Ca$^{2+}$ transient, or the time to peak, was ~34% faster (106.5 ± 5.6 vs. 162.2 ± 6.8 ms in controls, P < 0.001, n = 61) (Figure 2B–D).

3.3. CASQ2 downregulation by FCCP is unaffected by caspase 3, p38, and p53 inhibitors

Under conditions of cell stress, numerous signalling cascades can be initiated that lead to the remodelling of cardiomyocyte gene expression and metabolism, and possibly even apoptosis. Responses to mitochondrial stress, in particular, are known to involve, e.g. caspases, protein kinases, and transcription factor p53. The effect of these pathways on cardiac-specific gene transcription in response to FCCP-induced mitochondrial stress was studied using inhibitors of caspase 3 (100 μM DEVd-CHO), p38 (10 μM sb203580), and p53 (2 μM pifithrin-α). Inhibitors were used at concentrations shown to be effective but not toxic in cultured cells in previous studies. Cells were cultured in the presence of 1 μM FCCP with and without inhibitor for 48 h, and CASQ2 mRNA was quantified by qPCR. The FCCP-induced repression of CASQ2 gene expression was unaffected by these inhibitors (Figure 3), suggesting that CASQ2 downregulation is not a direct consequence of the activation of these cascades.

3.4. NAC counters the effect of FCCP on Ca$^{2+}$ handling and gene expression

Mitochondrial uncouplers are known to affect mitochondrial ROS production, and elevated ROS levels are involved in various signalling cascades that alter gene expression. Therefore, the effect of FCCP on ROS production in cultured neonatal cardiomyocytes as well as the potential effect of ROS on the observed functional and gene expression changes in FCCP-treated cardiomyocytes was studied. ROS production in cultured cardiomyocytes was monitored by confocal microscopy using the superoxide indicator MitoSOX red (Molecular Probes) following exposure to FCCP and the reactive oxygen/nitrogen species scavenger NAC. There was an ~41.5% increase in superoxide levels during a 40 min perfusion with 1 μM FCCP, followed by a rapid decrease in fluorescence following the addition of 1 mM NAC (Figure 4A and B). Superoxide levels fell 25% after 8 min co-perfusion of FCCP and NAC (Figure 4B), and returned to control levels 12 min after the addition of NAC. Next we studied whether FCCP-induced downregulation of CASQ2 could be similarly reversed by NAC. Co-exposure of 1 μM FCCP and 1 mM NAC for 48 h led to a partial rescue of CASQ2 mRNA expression (Figure 4C). NAC attenuated the decrease in CASQ2 expression caused by FCCP alone by ~50% (FCCP: 0.30 ± 0.049 vs. FCCP + NAC: 0.59 ± 0.027, fold changes from control, n = 6).

Ca$^{2+}$ transients of cultured cells were monitored following exposure to 1 μM FCCP ± 1 mM NAC for 48 h; during measurements, cells were perfused without FCCP or NAC. As before, there were significant decreases in the time to peak and decay of Ca$^{2+}$ transients of cells exposed to FCCP compared with controls (P < 0.001, n = 50). Cells exposed to both FCCP and NAC,
The differences between the time to peak and decay of FCCP vs. controls was a marked reduction in total SR Ca$^{2+}$ buffering capacity. Therefore, the SR Ca$^{2+}$ content was measured from control and FCCP-treated cells by the application of caffeine, which causes sudden and complete extrusion of Ca$^{2+}$ from the SR. Indeed, although FCCP-treated cells displayed Ca$^{2+}$ transients similar in amplitude to control cells, there was a marked reduction in total SR Ca$^{2+}$ content by 0.33-fold ($P < 0.05$; $n = 11$; Figure 5A and B). The fractional release of Ca$^{2+}$ from the SR, calculated as the ratio of Ca$^{2+}$ extruded during each Ca$^{2+}$ transient compared with the total SR Ca$^{2+}$ content, was also significantly increased in FCCP-treated cells [0.8 ± 0.02 (n = 11) vs. 0.66 ± 0.02 (n = 14) in controls, $P < 0.05$; Figure 5B]. In parallel, CASQ2 protein levels were also greater in cells co-exposed to FCCP and NAC (FCCP: 0.70 ± 0.05 vs. FCCP + NAC: 0.90 ± 0.12, fold changes from control, $n = 6$; Figure 5C).

### 3.6. NAC prevents the FCCP-induced changes in Ca$^{2+}$ sparks

Whereas CASQ2 protein levels define the functional size of the SR Ca$^{2+}$ stores, CASQ2 also directly affects the ryanodine receptor, modulating its open probability. As an example, expression of mutated CASQ2 with deficient Ca-binding decreases the amplitude and duration of individual Ca$^{2+}$ sparks, which are transient, rapid Ca$^{2+}$ releases from clusters of ryanodine receptors in the SR membrane (Figure 6A). Supporting this, analysis of Ca$^{2+}$ sparks revealed significant decreases in the amplitude and duration (FDHM, full-duration-at-half-maximum) of sparks from FCCP-treated cells. Co-exposure of cells to FCCP and NAC restored spark characteristics to near control levels, indicating that NAC fully restores the functional SR in FCCP-treated cardiomyocytes (Figure 6A and B).

### 4. Discussion

Mitochondrial cardiomyopathy is a progressive syndrome induced by defective OXPHOS involving deleterious metabolic, morphological, and functional changes of the heart. At the level of cardiomyocyte function, these changes include selective transcriptional downregulation of genes involved in Ca$^{2+}$ signalling. Among these is calsequestrin 2, the major Ca$^{2+}$ buffer in the cardiomyocyte SR lumen. In this study, we demonstrate that a relatively short period of mitochondrial uncoupling induces changes commonly associated with chronic mitochondrial cardiomyopathy. We show that chemical uncoupling of mitochondria by FCCP decreased CASQ2 expression in 48 h, enough to alter Ca$^{2+}$ signals in cultured neonatal cardiomyocytes. The reduction in the CASQ2 protein level was reflected in the size of the SR Ca$^{2+}$ stores and the properties of spontaneous Ca$^{2+}$ sparks, which were significantly smaller and shorter than in controls. Although the Ca$^{2+}$ transient amplitude of FCCP-treated cells was similar to controls, caffeine pulse measurements demonstrated that this was due to greater fractional release of Ca$^{2+}$ from the SR in FCCP-treated cells. Overall, these changes in CASQ2 expression and Ca$^{2+}$ signalling are in accordance with those previously found in myocytes from a mouse model of mitochondrial cardiomyopathy. Moreover, we demonstrate that FCCP increases the production of ROS and that the effects of FCCP on CASQ2 expression,
Figure 4  NAC affects ROS production, CASQ2 expression, and Ca\textsuperscript{2+} transients of FCCP-treated cardiomyocytes. (A–B) NAC decreases superoxide levels in cells exposed to FCCP. Cells were acutely exposed to 1 \mu M FCCP for 40 min, and then the perfusion was switched to 1 \mu M FCCP + 1 mM NAC (arrow in B). Control cells were perfused with unmodified DMEM (n = 10). (C) Addition of NAC attenuates the FCCP-induced reduction in CASQ2 mRNA expression (n = 6). (D–F) NAC restores Ca\textsuperscript{2+} transient parameters to control values. Cells were cultured 48 h with 1 \mu M FCCP + 1 mM NAC. During fluo-4 measurements, cells were perfused with DMEM and paced at 1 Hz. TTP denotes time to peak (n = 50). **P < 0.01 from control; §§§P < 0.001 from FCCP.

Figure 5  NAC restores the SR Ca\textsuperscript{2+} stores of FCCP-treated cells. (A) Representative caffeine-induced (10 mM) Ca\textsuperscript{2+} releases (fluo-4 fluorescence) measured from neonatal cardiomyocytes treated with FCCP or FCCP and NAC (48 h). (B) Effects of FCCP (n = 11) and FCCP and NAC (n = 10) on the amplitude of the caffeine-induced Ca\textsuperscript{2+} release and fractional release (amplitude of the action potential-induced Ca\textsuperscript{2+} transient/amplitude of the caffeine-induced Ca\textsuperscript{2+} release) compared with control cells (n = 14). Before measurements, cells were incubated in solution without FCCP or NAC for at least 30 min. (C) Calsequestrin protein levels measured from neonatal cardiomyocytes treated with FCCP or FCCP and NAC for 48 h compared with control myocytes (n = 6 in each group). Representative western blots are shown below the bar graphs. *P < 0.05.
cardiomyocyte Ca$^{2+}$ signals, and SR Ca stores can be reversed by the co-application of the ROS scavenger NAC.

Among the transcriptional targets commonly associated with mitochondrial cardiomyopathy, changes in CASQ2 expression have a drastic impact on the function of the heart due to the fundamental role of CASQ2 in establishing cardiomyocyte Ca$^{2+}$ signalling. The amount of CASQ2 defines the size of the releasable SR Ca pool and thereby limits the Ca$^{2+}$ signals and contractile force. CASQ2 also acts as a Ca$^{2+}$ sensor by binding and inhibiting triadin/junctin-induced RyR activation at low luminal Ca$^{2+}$ concentrations, although at higher [Ca$^{2+}]_{SR}$, its inhibitory influence is relieved.

Hence, in FCCP-treated cells, decreased CASQ2 expression might result in an overall reduced inhibition of RyRs, increasing their open probability at lower [Ca$^{2+}$] levels. This would induce faster activation of RyRs during increasing [Ca$^{2+}$], subsequently leading to greater fractional release. In accordance with this, FCCP-treated cells had faster time-to-peak of the Ca$^{2+}$ transients and increased fractional SR Ca$^{2+}$ release. As such, these changes should increase the SR Ca$^{2+}$ release, but because the SR Ca$^{2+}$ stores were reduced, the amplitude of the whole-cell Ca$^{2+}$ transients remained unchanged.

The decreases in amplitude and duration (FDHM) of the individual Ca$^{2+}$ sparks of the FCCP-treated cells also reflect the decrease in the available SR Ca$^{2+}$ and are in line with results from myocytes expressing mutated CASQ2 with reduced Ca-binding. Changes in spark characteristics have been linked to arrhythmias; therefore, these changes are significant, as they imply that mitochondrial uncoupling directly affects the elementary mechanisms of Ca$^{2+}$ release. It is known that insufficient levels of CASQ2 have a serious impact on Ca$^{2+}$ release and can lead to contractile dysfunction and arrhythmias, as in the case of induced cardiomyopathy and CPVT. Even a modest 25% reduction in CASQ2 has been shown to increase SR Ca$^{2+}$ leak and increase the susceptibility of heterozygous CASQ2+/- mutant cardiomyocytes to stress-induced ventricular tachycardia.

Apart from being one of the genes altered in mitochondrial cardiomyopathy and doxorubicin-induced cardiotoxicity, very little is known about the transcriptional regulation of CASQ2. It has been reported that cardiac expression of CASQ2 requires the common cardiac transcription factors MEF-2 and SRF, and overexpression of Egr-1 leads to CASQ2 repression, but the specific mechanisms regulating CASQ2 expression are not well understood. Although we showed here that mitochondrial uncoupling downregulates CASQ2 expression, we also showed that the inhibition of common apoptotic pathways (caspase 3, p38, and p53) had no effect on CASQ2 downregulation. However, ROS scavenger NAC was found to reverse the effects of FCCP on CASQ2 expression and Ca$^{2+}$ signalling. Although the entire cascade between FCCP-induced ROS production and transcriptional downregulation of the nuclear CASQ2 gene was not determined in this study, an increasing number of studies have shown that ROS not only have direct effects on proteins, but are also key players in cell signalling cascades leading to transcriptional regulation (for reviews, see references 38–40). Our findings suggest that part of the effect of a promising antioxidid therapy, which protects heart muscle cells from the acute direct effects of ROS, might be based on the reduction of the mitochondria-induced transcriptional changes that negatively affect

**Figure 6** NAC prevents FCCP-induced changes on spontaneous Ca$^{2+}$ sparks in neonatal cardiomyocytes. (A) Representative 3D pseudocolor plots showing Ca$^{2+}$ sparks (flu-4 fluorescence) measured from neonatal cardiomyocytes treated 48 h with FCCP or FCCP and NAC. (B) Properties of the sparks, amplitude (left), full-duration-at-half-maximum (FDHM, middle), and full-width-at-half-maximum (FWHM, right). Before measurements, cells were incubated in solution without FCCP or NAC for at least 30 min (n = 42–65 spontaneously occurring sparks/group measured from at least five cells/group). *P < 0.05.
cardiomyocyte Ca\(^{2+}\) signalling. Indeed, NAC has been shown in vivo to improve cardiac function in hypertrophic hearts \(^{42}\) and decrease the incidence of arrhythmias following infarction, \(^{43}\) ischaemia, \(^{44}\) and heart surgery. \(^{45}\) In addition to reducing markers of oxidative stress in hearts of animal models of cardiomyopathy, \(^{46,47}\) NAC has also been implicated in restoring cardiac mitochondrial function in ageing rats. \(^{48}\)

Impaired function of the respiratory chain has been reported in the ageing heart as well as in cardiac pathologies such as myocarditis, arrhythmias, and sudden cardiac death. Our findings suggest that all of these conditions may involve mitochondria-initiated changes in nuclear gene expression as CASQ2 downregulation and subsequent changes in Ca\(^{2+}\) signalling can be induced by a relatively short period of mitochondrial uncoupling. To what extent these mechanisms contribute to symptoms in mitochondrial diseases is not known, but the results suggest that defective Ca\(^{2+}\) signalling does not require chronic mitochondrial impairment, but instead can be induced within days. This relates our findings to causes of acute episodes of arrhythmias in cardiac pathologies as well as sudden cardiac death.

In summary, we show that, in cardiomyocytes, mitochondrial uncoupling induces changes in Ca\(^{2+}\) handling which correlated with the decreased expression of the SR Ca\(^{2+}\) buffer calsequestrin. Uncoupling increases the production of reactive oxygen/nitrogen species, and a reduction in reactive oxygen/nitrogen species by NAC restored the CASQ2 expression, SR Ca\(^{2+}\) stores, and Ca\(^{2+}\) signalling of the cells. Collectively, our data suggest that mitochondrial uncoupling results in fast transcriptional changes manifested as compromised Ca\(^{2+}\) signalling and that these changes can be prevented by ROS scavengers. As impaired mitochondrial function has been implicated in several cardiac pathologies as well as in normal ageing, the mechanisms described here might be involved in a wide spectrum of cardiac conditions.

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