Abnormal Ca\textsuperscript{2+} release and catecholamine-induced arrhythmias in mitochondrial cardiomyopathy

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Received January 3, 2005; Revised February 7, 2005; Accepted March 3, 2005

Mitochondrial dysfunction is implicated in numerous cardiac disorders. It has been assumed that the functional defects are directly related to a decreased rate of mitochondrial ATP production, but recent studies have challenged this idea. Here, we used mice with tissue-specific knockout of mitochondrial transcription factor A (\textit{Tfam}) that leads to progressive cardiomyopathy. The role of changes in the excitation–contraction (E–C) coupling in cardiomyocytes of these mice was studied by measuring the free cytosolic Ca\textsuperscript{2+} concentration and by analyzing the expression of genes encoding E–C coupling proteins. Action potential-mediated Ca\textsuperscript{2+} transients, measured with the fluorescent indicator fluo-3 in isolated cardiomyocytes, were smaller and faster in \textit{Tfam} knockout cardiomyocytes when compared with controls. The total sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} content was decreased in \textit{Tfam} knockout cells. The gene for the SR Ca\textsuperscript{2+} binding protein calsequestrin-2 (CASQ2), as well as other genes encoding proteins involved in SR Ca\textsuperscript{2+} handling, showed decreased expression in \textit{Tfam} knockout hearts. Decreased CASQ2 levels have been linked to severe arrhythmias triggered by \&-adrenergic stimulation. In line with this, application of the \&-adrenergic agonist isoproterenol resulted in frequent doublet Ca\textsuperscript{2+} transients in \textit{Tfam} knockout cardiomyocytes. In conclusion, our results show that mitochondrial dysfunction in the heart induces specific down-regulation of the expression of genes encoding proteins involved in E–C coupling. These changes predispose to cardiac arrhythmias and terminal heart failure and are thus important in the pathogenesis of mitochondrial cardiomyopathy.

INTRODUCTION

Mitochondrial dysfunction is implicated in many common diseases and also in the normally occurring aging process (1–3). Deficient mitochondrial function has been linked to dilated, hypertrophic, ischemic and alcoholic cardiomyopathy. In addition, decreased respiratory chain function in the heart has been reported in myocarditis, arrhythmias, sudden cardiac death and normal aging (4). A general assumption has been that the functional defects associated with mitochondrial dysfunction are directly related to a decreased mitochondrial ATP production rate (MAPR). However, there is little direct experimental support for this idea and recent studies on mouse models with genetically induced respiratory chain deficiencies suggest a more elaborate picture. We have recently generated mice with a knockout of the mitochondrial transcription factor A (\textit{Tfam}) in fast-twitch skeletal muscle fibers and observed a close to normal MAPR because of a major compensatory increase in the mitochondrial mass (5). The affected muscles in these mitochondrial myopathy mice produced significantly less force than control muscles but fatigue development was normal. Thus, these results suggest that other factors besides reduced MAPR are critical in the pathogenesis of mitochondrial myopathy (5).

We have generated three strains of tissue-specific \textit{Tfam} knockout mice with progressive cardiomyopathy leading to atrioventricular heart conduction blocks and terminal cardiac failure (6–8). We performed global gene expression analyses in the heart of one of these cardiomyopathy strains with a mean survival of 10–12 weeks (8). Unexpectedly, we found a switch in energy metabolism with decreased expression of genes encoding critical enzymes in fatty acid oxidation and increased expression of several genes encoding glycolytic enzymes at an early disease stage. It is questionable whether these metabolic changes are beneficial for the heart in sustaining energy production and they may instead facilitate...
development of cardiomyopathy (8). In the present study, we used the same mouse strain to study possible defects in intracellular Ca\(^{2+}\) handling in mitochondrial cardiomyopathy.

The contraction of cardiac muscle is mediated by Ca\(^{2+}\) influx through voltage-activated L-type Ca\(^{2+}\) channels, which activates sarcoplasmic reticulum (SR) Ca\(^{2+}\) release channels, ryanodine receptor-2 (RyR2), via a process known as Ca\(^{2+}\)-induced Ca\(^{2+}\) release (9,10). Relaxation occurs when Ca\(^{2+}\) release is stopped and Ca\(^{2+}\) removed from the cytoplasm mainly by active reuptake into the SR by Ca\(^{2+}\) ATPase 2A (SERCA2A) and by Ca\(^{2+}\) extrusion out of the cell via Na\(^+\)/Ca\(^{2+}\) exchange (10). Cardiac failure is generally associated with defect excitation–contraction (E–C) coupling in cardiomyocytes manifested as slow Ca\(^{2+}\) transients with decreased amplitudes (11). Our aim was to elucidate whether mitochondrial cardiomyopathy involves changes in E–C coupling similar to those generally seen in cardiac failure. Unexpectedly, we found an E–C coupling phenotype that was different from the phenotype commonly seen in cardiomyocytes with decreased amplitudes (11). Our aim was to elucidate whether mitochondrial cardiomyopathy involves changes in E–C coupling similar to those generally seen in cardiac failure. Unexpectedly, we found an E–C coupling phenotype that was different from the phenotype commonly seen in cardiac failure; the Ca\(^{2+}\) transients were faster and \(\beta\)-adrenergic stimulation triggered frequent doublet Ca\(^{2+}\) transients in respiratory chain deficient cardiomyocytes. These changes closely resemble those observed in cardiomyocytes with decreased SR Ca\(^{2+}\) stores caused by mutated calsequestrin-2 (CASQ2), an SR high-capacity Ca\(^{2+}\)-binding protein (12). In line with this, we observed a marked down-regulation in the expression of the CASQ2 gene in the Tfam knockout hearts. Our results thus show specific effects on E–C coupling in mitochondrial cardiomyopathy that may predispose to arrhythmias and sudden death.

RESULTS

**Tfam knockout cardiomyocytes display smaller and faster Ca\(^{2+}\) transients**

Ca\(^{2+}\) signals and cell shortening were measured in ventricular cardiomyocytes isolated from 8-week-old control and Tfam knockout mice. Action potential-induced Ca\(^{2+}\) transients (0.5 Hz stimulation) were significantly \((P < 0.001; n = 12)\) smaller and faster in Tfam knockout cardiomyocytes when compared with controls (Fig. 1A–C). The associated shortening of the cells was also smaller in Tfam knockout cardiomyocytes (10.1 \(\pm\) 0.8% of the resting cell length versus 16.7 \(\pm\) 0.7% in controls; \(P < 0.001\)) and the relaxation was faster (time to 90% relaxation 67.4 \(\pm\) 6.0 versus 140.3 \(\pm\) 8.0 ms; \(P < 0.001\)). On the other hand, the shortening velocity was not changed in Tfam knockout cardiomyocytes (195 \(\pm\) 17 versus 201 \(\pm\) 11% of the resting cell length per second; \(P > 0.05\)), indicating that there was no change in myosin isoform composition in Tfam knockout hearts.

Next, we studied the effect of different stimulation frequencies on SR Ca\(^{2+}\) handling. An increased stimulation frequency normally accelerates SR Ca\(^{2+}\) uptake in cardiomyocytes via activation of calmodulin kinase II, which phosphorylates SERCA2A and its associated inhibitory protein phospholamban (13,14). The faster SR Ca\(^{2+}\) uptake leads to an increased SR Ca\(^{2+}\) load and hence maintained or increased amplitude of the Ca\(^{2+}\) transients when the stimulation frequency is increased in normal cardiomyocytes (11,12). We obtained the expected result when we increased the stimulation frequency from 0.5 to 3 Hz in control cells and observed a maintained Ca\(^{2+}\) transient amplitude and a markedly faster rate of Ca\(^{2+}\) decline (Fig. 1D–F). In Tfam knockout cells, on the other hand, the Ca\(^{2+}\) transient amplitude was reduced by \(\sim\)20% when the stimulation frequency was increased and the rate of Ca\(^{2+}\) decline showed little change (Fig. 1D–F).

**Decreased expression of genes encoding for SR Ca\(^{2+}\) handling proteins**

Global gene expression analyses with microarrays showed up-regulation of commonly used marker genes of pathological cardiac remodeling, such as atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP) and skeletal \(\alpha\)-actin in Tfam knockout hearts (Fig. 3A). On the other hand, genes encoding proteins involved in E–C coupling showed a pattern of alterations in Tfam knockouts, which is distinctly different from that normally associated with heart failure. Tfam knockout hearts showed markedly reduced CASQ2, RyR2, SERCA2A and the L-type Ca\(^{2+}\) channel subunit \(\alpha\) (L-type \(\alpha\)) transcript levels, whereas the expression of the Na\(^+\)/Ca\(^{2+}\) exchanger was moderately reduced (Fig. 3B). Quantitative real-time PCR (RT-PCR) was used to further quantify changes in expression of transcripts encoding proteins involved in SR Ca\(^{2+}\) release in hearts of 8-week-old Tfam knockout and control mice. The results showed that the mRNA expression of CASQ2, RyR2 and L-type \(\alpha\) was decreased to \(<30\%\) of normal levels in Tfam knockout hearts (Fig. 3C). The marked decrease in CASQ2 expression is of particular interest because the expression of this protein is rigidly controlled (16) and remains unaltered in dilated cardiomyopathy and end-stage cardiac failure in humans (17,18).
-Adrenergic stimulation triggers doublet Ca\(^{2+}\) transients in Tfam knockout cardiomyocytes. Decreased expression of CASQ2 or inactivating mutations in this protein predispose ventricular cardiomyocytes to a distinct type of arrhythmia, which is characterized by specific patterns of irregular Ca\(^{2+}\) release, especially in response to β-adrenergic stimulation (12). We exposed isolated control cardiomyocytes to the β-adrenergic agonist isoproterenol (100 nM) and found an increase in the action potential-induced Ca\(^{2+}\) amplitude and faster Ca\(^{2+}\) decay but no alteration of rhythmic activity (Fig. 4A). In contrast, β-adrenergic stimulation of Tfam knockout cells resulted in additional Ca\(^{2+}\) transients and spontaneous Ca\(^{2+}\) waves in all eight cells tested (Fig. 4B). The occurrence of a second Ca\(^{2+}\) transient prior to the complete decay of the initial externally triggered Ca\(^{2+}\) transient constitutes a pattern that highly resembles that of cardiomyocytes with CASQ2 mutation (12).

**DISCUSSION**

Heart-specific Tfam knockout mice display a progressive cardiomyopathy that leads to terminal cardiac failure,
atrioventricular heart conduction blocks and death (6–8). These mitochondrial cardiomyopathy mouse strains provide a powerful tool to investigate mechanisms underlying the cardiac dysfunction caused by respiratory chain deficiency. In the present study, we used cardiomyocytes of one heart-specific Tfam knockout mouse strain that displays severe respiratory chain deficiency and also secondary changes such as decreased expression of genes encoding critical enzymes in fatty acid oxidation and increased expression of several genes encoding glycolytic enzymes (8). We now studied E–C coupling in these Tfam knockout cardiomyocytes and found that the Ca\(^{2+}\) transients were smaller and faster. We also observed frequent doublet Ca\(^{2+}\) transients upon β-adrenergic stimulation. We found reduced transcript levels for genes encoding SR Ca\(^{2+}\) handling proteins consistent with a specific effect on E–C coupling in mitochondrial cardiomyopathy.

Mitochondrial diseases are often associated with increased heart size due to hypertrophy and/or dilation (19). We observed smaller and faster Ca\(^{2+}\) transients in Tfam knockout cardiomyocytes and these changes were accompanied by decreased cell shortening and faster relaxation. The Tfam knockout mouse strain used in the present study displays an increased heart weight to body weight ratio (8), which probably reflects a compensatory hypertrophic response to the impaired contractile function of cardiomyocytes.

The expression of genes encoding the L-type Ca\(^{2+}\) channel and RyR2 was down-regulated in Tfam knockout hearts (Fig. 3). Although both of these proteins have an essential role in SR Ca\(^{2+}\) release, a decreased concentration of L-type Ca\(^{2+}\) channels or RyR2 cannot explain the reduced SR Ca\(^{2+}\) content in Tfam knockout hearts (Fig. 2) (20). The mRNA expression of SERCA2A was decreased in Tfam knockout hearts, which may lead to reduced SR Ca\(^{2+}\) filling and hence smaller Ca\(^{2+}\) transients. Many types of heart failure, including ischemic and non-ischemic human heart failure, show a decrease in SERCA expression that is accompanied by an increase in Na\(^{+}/Ca\(^{2+}\) exchanger expression. These expression changes will limit the slowing of the Ca\(^{2+}\) transient decay phase and enhance the SR Ca\(^{2+}\) loss because Ca\(^{2+}\) extrusion from the cell will be favored over SR uptake (10). However, Tfam knockout hearts showed little change in the Na\(^{+}/Ca\(^{2+}\) exchanger mRNA levels and there was no significant change in the rate of decay of caffeine-induced Ca\(^{2+}\) transients, which indicates normal activity of the Na\(^{+}/Ca\(^{2+}\) exchanger (Fig. 2D).

Figure 2. Tfam knockout cardiomyocytes have a reduced SR Ca\(^{2+}\) content. (A) Representative caffeine-induced (10 mM) Ca\(^{2+}\) transients of Tfam knockout and control cardiomyocytes. Tfam knockout cardiomyocytes show reduced amplitude of the Ca\(^{2+}\) transient (B) and increased fractional release (ratio of the action potential-induced to the caffeine-induced Ca\(^{2+}\) transient amplitude) (C), whereas there was no change in the time constant of the exponential decay of the caffeine-induced Ca\(^{2+}\) transient (D). Values are expressed as mean ± SEM (n = 6). Filled and open bars represent controls and Tfam knockouts, respectively. Asterisks indicate statistical significance: **P < 0.01; ***P < 0.001; ns, not significant.
CASQ2 has an important role as an SR Ca\textsuperscript{2+} buffer and regulates SR Ca\textsuperscript{2+} release by forming a functional complex with RyR2 and other SR proteins, such as triadin and junctin (10,21,22). Recent studies employing genetically modified rat cardiomyocytes with markedly decreased functional CASQ2 showed a decreased SR Ca\textsuperscript{2+} content as well as smaller and faster Ca\textsuperscript{2+} transients (12,23). Furthermore, \beta\text-supraadrenergic stimulation of rat cardiomyocytes with decreased CASQ2 results in spontaneous, premature Ca\textsuperscript{2+} transients occurring during the initial Ca\textsuperscript{2+} transient decay. Thus, the respiratory chain deficient and CASQ2 deficient cardiomyocytes display strikingly similar defects in intracellular Ca\textsuperscript{2+} handling, which are likely to predispose to ventricular arrhythmias. In line with this, mild exogenous stress frequently caused sudden death of mice with \textit{Tfam} deficient hearts (7). Furthermore, humans with a mutation in the \textit{CASQ2} gene have a syndrome with syncope, seizures and even sudden death in response to physical or emotional stress, called catecholaminergic polymorphic ventricular tachycardia (24,25).

A down-regulation of SR Ca\textsuperscript{2+} handling genes, including CASQ2, is also associated with the cardiomyopathy induced by the anti-neoplastic agent doxorubicin that interferes with mitochondrial function (26,27). Doxorubicin-induced cardiomyopathy is characterized by specific defects in SR Ca\textsuperscript{2+} release and decreased cardiac function together with the occurrence of various types of tachycardias (28). Interestingly, the cardiotoxicity of doxorubicin is believed to be mediated by increased mitochondrial production of highly reactive free radical species of molecular oxygen (27) and an increased oxidative stress has also been suggested as a major causative factor in mitochondrial cardiomyopathies (19). We speculate that oxidative stress may be involved in generating a decrease in the expression of SR Ca\textsuperscript{2+} handling genes after doxorubicin exposure as well as in the \textit{Tfam} knockout hearts.

**Figure 3.** \textit{Tfam} knockout mice show specific down-regulation in the expression of genes encoding Ca\textsuperscript{2+} handling proteins. Gene expression changes measured with microarrays are indicated as fold change (ratio between \textit{Tfam} knockouts and controls) at given time points. (A) Up-regulation of the expression of previously described cardiac failure marker genes: ANP (4 weeks and 5–9 weeks \(P = 0.03\)), BNP (4 weeks \(P = 0.007\), 5–9 weeks \(P = 0.0001\)) and skeletal \(\alpha\)-actin (2 weeks \(P = 0.02\), 4 weeks \(P = 0.07\) and 5–9 weeks \(P = 0.03\)). (B) Down-regulation of the expression of genes involved in cardiomyocyte Ca\textsuperscript{2+} handling: CASQ2 (5–9 weeks \(P = 0.015\)), SERCA2A (5–9 weeks \(P = 0.002\)), RyR2 (4 weeks \(P < 0.05\), 5–9 weeks \(P = 0.001\)), L-type \(\alpha\) (4 weeks \(P < 0.01\), 5–9 weeks, \(P < 0.0001\)) and Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (5–9 weeks \(P = 0.05\)). (C) Quantitative RT–PCR analysis of the mRNA expression of genes encoding Ca\textsuperscript{2+} handling proteins from 8-week-old \textit{Tfam} knockout and control mice. Mean expression in control mice is indicated by 100%. Data are shown as mean ± SEM (\(n = 3\)). Asterisks indicate statistical significance: *\(P < 0.05\), **\(P < 0.01\).
Cardiac arrhythmias and conduction defects are common in pathological conditions with deficient mitochondrial function and likely contribute to the high mortality (29). Our results show that mitochondrial defects can re-program the nuclear gene expression to produce specific alterations in the expression of mRNAs encoding cardiac E–C coupling proteins, which, in turn, will predispose to arrhythmias and sudden cardiac death. Different types of arrhythmias are common in aging humans (30) and recent experimental studies show that elevated levels of somatic mtDNA mutations in the mouse heart lead to age-associated pathology (31). The specific alterations of Ca\(^{2+}\) handling in respiratory chain deficient cardiomyocytes, documented in the present study, thus suggest that bioenergetic decline of the aging heart may predispose to the commonly observed age-associated cardiac arrhythmias.

Tfam knockout cardiomyocytes have smaller and faster Ca\(^{2+}\) transients and display frequent doublet transients in response to \(\beta\)-adrenergic stimulation. These changes can be explained by reduced SR Ca\(^{2+}\) content because of decreased CASQ2 expression. Thus, heart-specific Tfam knockout results in re-programing of nuclear gene expression that causes severe cardiac dysfunction not directly related to the impaired mitochondrial ATP production.

**MATERIALS AND METHODS**

**Animals**

\(Tfam^{loxP/loxP}\), \(Ckmm-Cre\) knockout mice were generated as described elsewhere (8) and compared to \(Tfam^{loxP/loxP}\) littermate controls. Mice were euthanized by cervical dislocation and the heart was excised. All animal experiments were approved by the local animal ethics committee.

**Isolation and confocal Ca\(^{2+}\) imaging of cardiac myocytes**

Cardiomyocytes were isolated according to the protocols developed by the Alliance for Cellular Signalling (AfCS Procedure Protocol ID PP00000125) (32). Isolated cardiomyocytes were incubated in medium containing the fluorescent Ca\(^{2+}\) indicator fluo-3-AM (20 \(\mu\)M) for 60 min at room temperature followed by 10 min in medium without fluo-3. After being loaded with the dye, cardiomyocytes were placed in a perfusion chamber on glass coverslips pre-coated with laminin (Sigma, 1 \(\mu\)g/1 \(\mu\)l in PBS for 2 h and washed with PBS). They were superfused with standard Tyrode’s solution (mM): NaCl, 121; KCl, 5.0; CaCl\(_2\), 1.8; MgCl\(_2\), 0.5; Na\(_2\)HPO\(_4\), 0.4; NaHCO\(_3\), 24.0; EDTA, 0.1; glucose, 5.5. To study the effect of \(\beta\)-adrenergic stimulation, isoproterenol (100 nM) was added to the solution. The solution was bubbled with 5% CO\(_2\)/95% O\(_2\), which gives a bath pH of 7.4. A three-way solenoid valve system allowed rapid exchange of solutions. Experiments were performed at room temperature (~24°C). Cells were stimulated by 1–2 ms current pulses delivered via two platinum electrodes, one on each side of the perfusion chamber.

To measure the free cytosolic Ca\(^{2+}\) concentration, we used a BioRad MRC 1024 confocal unit equipped with a krypton/argon laser run at 15 mW (BioRad Microscopy Division,
Hertfordshire, UK). The confocal unit was attached to a Nikon Diaphot 200 inverted microscope and a Nikon Plan Apo 40x oil immersion objective (N.A. 1.3) was used. Fluo-3 was excited at 488 nm and the emitted light was collected through a 522 nm narrow band filter. The laser power used (3–6% of the maximum) did not have any noticeable deleterious effect on the fluorescent signal or the cell function over the time-course of an experiment. Fluo-3 intensity was measured by line scanning at 6 ms intervals along with the long axis and with focus in the middle of the cell. Stored images were analyzed with Image J (NIH, USA; http://rsb.info.nih.gov/ij/).

Changes in the fluo-3 fluorescent signal at each time point ($F$) are expressed relative to that measured in the rested state ($F_0 =$ diastolic $F$ at 0.5 Hz stimulation). This procedure allowed comparison of the fluo-3 signal in different cardiomyocytes.

**Microarray analysis**

Gene expression was assayed using Affymetrix Gene Chip Mul1K arrays as was previously described (8). The 2-week-old knockouts were compared with 2-week-old controls. The 4-week- and 5- to 9-week-old knockouts were compared with a 4- to 9-week old common control group.

**Quantitative RT–PCR**

We performed quantitative RT–PCR analysis on selected genes. Total RNA was isolated with the Trizol reagent and cDNA was synthesized using the Superscript First-Strand System for RT-PCR (Invitrogen). Gene expression was assayed with the ABI PRISM 7700 sequence detection system (Applied Biosystems) using Platinum CYBR Green qPCR Supermix UDG (Invitrogen). Primers were designed using the Primer Express v1.5 software (Applied Biosystems): CASQ2, forward 5’-TCTATACCAAGAGCCTGTCTCTCA-3’, reverse 5’-AAATCCACGACAAAGCTATGT-3’; RyR2, forward 5’-TGGATGTGAATCAGCAGAAT-3’, reverse 5’-GATAAGTTCAACCATCAGTCTGT-3’; L-type $\alpha$, forward 5’-GAGGAAAGGCGAGTACTGCAGTGA-3’, reverse 5’-TCTATTACCACGAACCTGTCTCA-3’; $\beta$-actin internal control.

**Statistical analysis**

Analyses of the microarray results were performed as described previously (8). Gene expression levels assayed by quantitative PCR analysis were compared using a two-sample $t$-test assuming unequal variances. In experiments on isolated cardiomyocytes, unpaired $t$-tests were used when comparing two groups. For comparison between multiple groups, we used one-way ANOVA followed by the Bonferroni test. $P$-values <0.05 were considered statistically significant. Values are presented as mean ± SEM.

**ACKNOWLEDGEMENTS**

The study was supported by the Swedish Research Council, the Göran Gustafsson Foundation for Research in Natural Sciences and Medicine, the Torsten and Ragnar Söderbergs Foundation, Sigrid Juselius Foundation, the Swedish Heart and Lung Foundation, the Swedish Foundation for Strategic Research (Functional Genomics and INGVAR), the Muscular Dystrophy Association and Funds of Karolinska Institutet.  

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