Calcineurin transgenic mice have mitochondrial dysfunction and elevated superoxide production

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Calcineurin transgenic mice have mitochondrial dysfunction and elevated superoxide production. Am J Physiol Cell Physiol 284: C562–C570, 2003. First published October 23, 2002; 10.1152/ajpcell.00336.2002.—Introduction of the constitutively active calcineurin gene into neonatal rat cardiomyocytes by adenovirus resulted in decreased mitochondrial membrane potential (P < 0.05). Infection of H9c2 cells with calcineurin adenovirus resulted in increased superoxide production (P < 0.001). Transgenic mice with cardiac-specific expression of a constitutively active calcineurin cDNA (CaTG mice) exhibit a two- to threefold increase in heart size that progresses to heart failure. We prepared mitochondria enriched for the subsarcolemmal population from the hearts of CaTG mice and transgene negative littermates (control). Intact, well-coupled mitochondria prepared from one to two mouse hearts at a time yielded sufficient material for functional studies. Mitochondrial oxygen consumption was measured with a Clark-type oxygen electrode with substrates for mitochondrial complex II (succinate) and complex IV [tetramethylpentadecane (TMPD)/ascorbate]. CaTG mice exhibited a maximal rate of electron transfer in heart mitochondria that was reduced by ~50% (P < 0.002) without a loss of respiratory control. Mitochondrial respiration was unaffected in tropomodulin-overexpressing transgenic mice, another model of cardiomyopathy. Western blotting for mitochondrial electron transfer subunits from mitochondria of CaTG mice revealed a 20–30% reduction in subunit 3 of complex I (ND3) and subunits I and IV of cytochrome oxidase (CO-I, CO-IV) when normalized to total mitochondrial protein or to the adenine nucleotide transporter (ANT) and subunit 3 of cytochrome oxidase (CO-I, CO-IV) when normalized to total mitochondrial protein or to the adenine nucleotide transporter (ANT) and compared with littermate controls (P < 0.002). Impaired mitochondrial electron transport was associated with high levels of superoxide production in the CaTG mice. Taken together, these data indicate that calcineurin signaling affects mitochondrial energetics and superoxide production. The excessive production of superoxide may contribute to the development of cardiac failure.

mitochondria; bioenergetics; hypertrophy; genetic models of heart failure

EXPRESSION OF A CONSTITUTIVELY ACTIVE FORM of calcineurin in transgenic mice has been shown to result in cardiac hypertrophy and progression to failure by 8–12 wk (26). Calcineurin signaling is recognized to contribute to the progression of disease in a number of models, as its inhibition has been shown to prevent cardiac hypertrophy (36). Cardiac function depends upon several factors, including adequate cell mass, intact contractile machinery, and adequate production of ATP. Sarcomeric dysfunction, as occurs in tropomodulin-overexpressing transgenic (TOT) mice, leads to dilated cardiomyopathy within 3–4 wk after birth. Loss of heart muscle through apoptosis leads to heart failure, as seen in the Gαq transgenic mice and in the FKBP-caspase-8 transgenic mice (1, 42). Mitochondrial dysfunction also leads to heart failure, as has been described in a variety of mitochondrial DNA deletion syndromes and in the targeted deletion of the adenine nucleotide translocator 1 (ANT1) (11, 41). The relationship between calcineurin signaling and these aspects of cardiac function are unclear, although calcineurin does not seem to promote apoptosis; in fact, activated calcineurin protected cultured cardiomyocytes from apoptosis mediated by 2-deoxyglucose or staurosporine and was associated with reduced infarct size after ischemia-reperfusion (I/R) (9). We hypothesized that calcineurin might affect mitochondrial function. This was suggested by the observation that calcineurin dephosphorylated the family of transcriptional regulators known as nuclear factor of activated T cells (NFAT), thereby permitting their translocation from cytosol to nucleus (31). NFAT has been shown to interact with GATA4 in the heart (26) and with MEF2c (4, 22, 43). These transcription factors have been shown to play a role in the regulation of expression of mitochondrial proteins (27, 44).

To examine mitochondrial function by polarography, we adapted previously described techniques for isolating mitochondria from rat and rabbit hearts and optimized them for use with much smaller and more fragile mouse hearts (14, 17, 24, 29, 39). As we describe in this report, isolated mitochondria from transgenic mice expressing activated calcineurin exhibit impaired oxidative phosphorylation and increased superoxide production that may contribute to heart failure.
Neonatal cardiomyocyte culture and analysis of mitochondrial membrane potential. Neonatal rat cardiomyocytes were prepared by collagenase digestion as previously described (16), with the following changes: 100 μM bromodeoxyuridine (BrdU) was added to inhibit proliferation of nonmyocytic cells. Cells were plated on fibronectin-coated chamber slides at 24,000 cells/cm². After 24 h, they were infected with adenovirus for activated calcineurin (8), Adβgal, or empty vector AdSR and cultured for 48 h. Cells were loaded with rhodamine 123 (R123) (Molecular Probes, Eugene, OR) at 1 μg/ml for 30 min and costained with Hoechst 33342 at 30 μg/ml for 5 min for nuclear detection. Successive fields were imaged with a Nikon TE300 fluorescence microscope (Nikon, Tokyo, Japan) and digitally collected with a Spot2 digital camera (Universal Imaging, Westchester, PA) using identical parameters. Field selection was performed in bright-field illumination and was restricted to areas in which cell density was <85 cells per field. This was necessary to ensure that cells in close proximity could be scored as individual cells. Mitochondrial membrane potential was assessed as the ratio of relative fluorescence intensity of R123 to the number of nuclei in each field using MetaMorph imaging software. To rule out loss of mitochondrial mass, parallel wells of cells were stained with MitoTracker Green (Molecular Probes), which stains mitochondria but is independent of mitochondrial membrane potential. Cells (500–1,000) were scored from each condition. Five fields from each slide were analyzed, and three to four slides were averaged from each experiment. Experiments were repeated in two independent myocyte preparations with essentially identical results.

Mice. Mice were generated and characterized as previously described (26, 37). All animal procedures were approved by the Animal Care and Use Committee of The Scripps Research Institute.

Isolation of heart mitochondria. This protocol was based on several previously published methods (17, 24, 29, 39). Hearts were removed while still beating from mice anesthetized with ketamine/xylazine. Two mouse hearts were pooled and rapidly minced in ice-cold MSE buffer [in mmol/l: 220 mannitol, 70 sucrose, 2 EGTA, 5 MOPS (pH 7.4), and 2 taurine supplemented with 0.2% fatty acid-free bovine serum albumin (BSA)]. Heart tissue was homogenized in MSE buffer with a Polytron-type tissue grinder at 11,000 RPM for 2.5 s, followed by 2 quick strokes at 500 RPM with a loose fit Potter-Elvehjem tissue grinder. The homogenate was centrifuged at 500 g twice for 5 min, and the supernatant was saved. Mitochondria were pelleted from the supernatant by centrifugation at 3,000 g twice, and the pellet was rinsed with MSE buffer. The supernatant was saved as crude cytosol. The final pellet was rinsed and resuspended in 50 μl of incubation medium [in mmol/l: 220 mannitol, 70 sucrose, 1 EGTA, 5 MOPS (pH 7.4), 2 taurine, 10 MgCl₂, and 5 KH₂PO₄, supplemented with 0.2% fatty acid-free BSA] (33). Mitochondria were incubated for 15 min on wet ice, and protein concentration was determined with BSA as a standard by a Bradford assay. All work was performed on wet ice at 0°C.

Measurement of respiration in mitochondria from mouse or rat hearts. Oxygen consumption was measured at 30°C with a Clark-type oxygen electrode (Instech) in 600 μl of KCl respiration buffer [in mmol/l: 140 KCl, 1 EGTA, 10 MOPS (pH 7.4), 10 MgCl₂, and 5 KH₂PO₄, supplemented with 0.2% fatty acid-free BSA] (6, 23, 33). Complex II activity was measured using 200 μg of mitochondria with 5 mM succinate as a substrate. Complex IV activity was measured using 150 μg of mitochondria with 0.4 mM TMPD and 1 mM ascorbate as a substrate. For each complex, the ADP-stimulated respiration rate (state 3) was measured after the addition of 2 mM ADP; the ADP-independent respiration rate, oligomycin-inhibited...
sensitive (state 4), was measured after the addition of 2 μM oligomycin, and the maximal respiration rate was measured after uncoupling the mitochondria with 2 μM FCCP. Rates were calculated as nA O₂·min⁻¹·mg⁻¹ protein after subtracting the rate that was insensitive to the inhibitors, 1 μM antimycin A for complex III and 1 mM KCN for complex IV. As a measure of mitochondrial integrity, the respiratory control ratio (RCR) state 3 divided by state 4 was calculated. The data reported herein represent five independent mitochondrial preparations comprising two hearts each from a total of ten control and ten transgenic mice. Comparison between calcineurin transgenic (CalTG) and wild-type (WT) mice was analyzed using Student’s t-test.

**Immunoblotting for mitochondrial proteins.** Mitochondria (50 μg) and crude cytosol (60 μg) were resolved by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) nylon membranes. Membranes were probed for ND3 and Rieske iron-sulfur protein with antibodies kindly provided by Dr. Akemi Matsuno-Yagi of the Scripps Research Institute for cytochrome oxidase subunits I and IV (Molecular Probes), ANT (Oncogene Research Products, Boston, MA), and calcineurin (Santa Cruz Biotechnology, Santa Cruz, CA). Detection was performed with enhanced chemiluminescence (ECL; Amersham, Piscataway, NJ). Nonsaturated autoradiographs were quantitated with Scion/NIH Image. Comparison between CalTG and WT mice was analyzed using Student’s t-test.

**Langendorff heart perfusions.** All procedures were approved by the Animal Care and Use Committee at the Scripps Research Institute. In brief, mice were anesthetized with 150 μg/kg pentothal, and hearts were removed and quickly cannulated on a chilled template, and arranged in separate thick sections on a chilled template, and arranged in separate wells of a 24-well plate in a solution consisting of 2 μM DHE (Molecular Probes) in PBS. Plates were incubated in the dark at 37°C for 20 min. Sections were then placed on glass slides and imaged using an ultraviolet transilluminator (Fisher Scientific). All images were identically captured using a Kodak DC120 digital camera (Kodak) using Kodak Digital Science 1D software (Kodak). Images were treated identically and saved as TIFF files and analyzed using Adobe Photoshop 5.5. The percentage of superoxide production was quantified as the ratio of fluorescent (white) pixels to the total heart area. Statistical analysis was performed using ANOVA with Tukey-Kramer post test (InStat statistical software version 4.10; GraphPad, San Diego, CA).

**Measurement of superoxide production in cultured cells.** H9c2 cells were plated in 12-well tissue culture plates at 5,000 cells per cm² in DMEM supplemented with 10% FBS. At 24 and 48 h after being plated, cells were infected with replication deficient adenovirus expressing activated calcineurin or β-galactosidase at a multiplicity of infection (MOI) of 10–20 plaque-forming units (PFU) per cell. At 24 h after the second infection, fresh medium containing 10 μM DHE was added to wells (40). DHE fluorescence (excitation 518 nm/emission 605 nm) was measured at the indicated time with a Gemini fluorescence microplate reader (Molecular Devices). Images of cells were also captured to confirm intracellular fluorescence.

**Fig. 3.** Representative oxygen electrode tracings from calcineurin and control mice. Scale indicates nA O consumed per unit time. Each tracing represents mitochondria pooled from 2 mouse hearts. Infecions represent the following additions: a, mitochondria; b, substrate [succinate for complex II, tetramethylpentadecane (TMPD)/ascorbate for complex IV]; c, ADP; d, oligomycin; e, FCCP; f, inhibitor (antimycin A for complex II/III, KCN for complex IV).
Fig. 4. Respiration in 3- to 4-wk-old and from 9- to 10-wk-old calcineurin transgenic mice (CalTG) is impaired relative to control mice. Rates are normalized to mitochondrial protein. Filled bars are control, transgene negative animals, whereas open bars are CalTG mice. State 3 is defined as the rate obtained after the addition of 2 μM ADP, whereas state 4 is the rate after addition of 2 μM oligomycin. Maximal stimulated rate is obtained with the addition of 2 μM FCCP, whereas the respiratory control ratio (RCR) is defined as the ratio of state 3 to state 4. Error bars represent SD (n = 6 independent analyses from 12 animals per genotype for all conditions, except for FCCP, where n = 4 analyses, 8 animals per genotype). P values between wild-type (WT) and CalTG are shown below each condition.
RESULTS

Adenoviral-mediated gene transfer of the activated calcineurin has been shown to induce neonatal cardiomyocyte hypertrophy and to upregulate expression of atrial natriuretic peptide 48 h after infection (9). Accordingly, we infected neonatal rat cardiomyocytes with the activated calcineurin adenovirus and examined mitochondrial membrane potential using rhodamine 123 (Fig. 1). We found that calcineurin-overexpressing myocytes consistently exhibited less rhodamine 123 fluorescence per cell than control or adenovirus-β-galactosidase-infected myocytes. Moreover, the diminished mitochondrial membrane potential was not merely a consequence of hypertrophy, because cells stimulated with phenylephrine possessed normal mitochondrial membrane potential. These results suggest that calcineurin regulates mitochondrial function.

These results led us to examine the effects of calcineurin on mitochondrial function in the CalTG mice. To examine the possibility that calcineurin might directly regulate mitochondrial function through dephosphorylation, we isolated mitochondria from WT and CalTG hearts and performed subcellular fractionation. However, we did not detect the activated calcineurin transgene in the mitochondrial fraction (Fig. 2). This finding suggests that the mechanism is indirect, possibly through transcriptional pathways.

Mitochondria from the hearts of CalTG and transgene negative littermates were prepared and mitochondrial electron transfer capacity was analyzed. Representative tracings are shown in Fig. 3. Respiration in CalTG mitochondria was impaired when substrates for complex II (succinate) and complex IV (TMPD/ascorbate) were used. ADP stimulated respiration fourfold in CalTG and control mice when succinate was used as a substrate. The respiratory control ratio is used as an indication of mitochondrial integrity and demonstrates that the mitochondria isolated from the CalTG hearts did not sustain greater damage. We examined the maximal stimulated rate using FCCP to un-couple the mitochondria. We examined mice at 3–4 wk of age. We found that respiratory control ratios were similar to control mitochondria but that state 3, state 4, and FCCP-uncoupled respiration was reduced by 29–37% in mitochondria prepared from CalTG hearts (Fig. 4).

Fig. 5. A: Western blotting demonstrates a decrease in the abundance of cytochrome oxidase (COX) subunits I and IV and a decrease in ND3 of complex I, with no change in the abundance of the Rieske iron-sulfur protein or the adenine nucleotide translocator (ANT). The first 4 lanes are from WT mice heart mitochondria, whereas the last 5 lanes are from the activated CalTG mice. Each lane represents material from 2 hearts. Equal amounts of mitochondrial protein were loaded in each lane. B: quantitation of mitochondrial proteins in control (n = 4 pairs of hearts) and CalTG (n = 5 pairs of hearts). After densitometry, samples were normalized to the nuclear-encoded Rieske iron-sulfur protein. Error bars represent SD. P values are shown below each comparison.
These studies were repeated on 9- to 10-wk-old mice when heart failure had set in (9). Overall, CalTG mitochondrial chondria demonstrated a statistically significant reduction by 50% in oxygen consumption per milligram of mitochondrial protein for state 3, state 4, and FCCP-uncoupled using succinate or TMPD/ascorbate as substrates. To further address the possibility that these changes in mitochondrial respiration were secondary to heart

Fig. 6. Superoxide production in hearts from WT and CalTG mice was measured by quantifying dihydroethidium (DHE) conversion. A shows representative stained heart slices from WT and CalTG mice at baseline and after ischemia-reperfusion (I/R). B shows mean ± SE for 4 hearts for each condition. P values were determined by ANOVA.
failure, we examined a model of dilated cardiomyopathy due to overexpression of the actin-capping molecule tropomodulin (37). Structural alterations are apparent as early as 1 wk of age (37). We examined respiration in heart mitochondria in 9- to 10-wk-old TOT mice and found that it did not differ from age-matched controls (data not shown). These results indicate that the mitochondrial alterations are directly associated with the activated calcineurin transgene rather than with hypertrophy or heart failure itself.

To assess the basis for impaired mitochondrial respiration, we hypothesized that components of the electron transfer complexes might be altered. We used antibodies to both nuclear-encoded and mitochondrial-encoded components of the respiratory chain. Westerns were loaded with equal amounts of mitochondrial protein, and loading was further normalized to the mitochondrial proteins ANT and Rieske iron-sulfur protein. Blots were probed for the proteins shown in Fig. 5A. We found that CalTG mitochondria exhibited similar amounts of ANT and Rieske iron-sulfur protein compared with control mitochondria but consistently showed less protein for complex I subunit 3 (ND3) and cytochrome oxidase subunits I and IV, summarized in Fig. 5B. Loss of these subunits is consistent with the impaired respiration we observed. In addition, we probed three samples of control and CalTG mitochondria for nuclear-encoded complex I subunits (75, 51, 49, 42, 39, 24, 18, and 9 kDa) and complex V subunits (α, β, b, and OCSP) and found no change in the abundance of these proteins relative to nontransgenic controls (data not shown).

Many studies have shown that a limitation to electron flow can result in increased superoxide production via the ubiquinone. To determine whether the observed mitochondrial abnormalities resulted in increased superoxide production, we stained heart slices with DHE, which is converted to the fluorescent ethidium by superoxide anion (3). We found that superoxide production was 4.5-fold greater in the CalTG hearts than WT ($P < 0.001$)(Fig. 6).

Ischemia and reperfusion are also known to result in increased superoxide production. We assessed superoxide production in CalTG and WT hearts after 30 min ischemia and 15 min reperfusion (I/R). In WT hearts, superoxide production increased 3.4-fold over control levels after I/R ($P < 0.01$). In contrast, CalTG hearts increased superoxide production <1.2-fold over their already high baseline level ($P = NS$) (Fig. 6).

To determine whether the increased superoxide production was causally related to calcineurin activity, we infected H9c2 cells for 48 h with AdCal or Adβgal and assessed superoxide production by DHE conversion. Activated calcineurin caused an increased rate of superoxide production (Fig. 7). No increase in apoptosis was observed over this time period. We conclude that calcineurin directs cellular alterations that include mitochondrial alterations and elevated superoxide production.

**DISCUSSION**

We have developed methodology to isolate mitochondria from the hearts of transgenic mice for polarography studies. This technique will allow investigators to better characterize mitochondrial alterations in a variety of genetically modified mice. To maximize the quality of the mitochondria obtained, we chose to use only the mitochondria released by brief homogenization of the minced tissue. This results in an enrichment of subsarcolemmal mitochondria, with relatively poor recovery of interfibrillar mitochondria. Thus our conclusions are based on the properties of the subsarcolemmal population of mitochondria, and it should be noted that interfibrillar mitochondria might exhibit different properties, as reported by Hoppel (19).

Optimal function of mitochondria in the heart is essential for ATP production and calcium homeostasis.

![Fig. 7. Superoxide production in H9c2 cells infected with adenovirus for activated calcineurin or β-galactosidase was measured by DHE conversion. Error bars denote SE for 3 experiments done in triplicate. The rate of superoxide production in the calcineurin expressing cells was significantly greater ($P < 0.005$).](http://ajpcell.physiology.org/)
Mitochondrial dysfunction may contribute to heart failure, as is well recognized in the mitochondrial deletion syndromes. Calcineurin is upregulated/activated in conditions that lead to cardiac hypertrophy, such as pressure overload and inhibition of calcineurin suppresses hypertrophy (7, 21). Although calcineurin is upregulated in the TOT mice, the level of activity is still considerably lower than that obtained in the activated calcineurin transgenic mice, which might explain why we do not observe mitochondrial dysfunction in the TOT mice. Overexpression of activated calcineurin leads to cardiac hypertrophy and eventual failure through a process that does not seem to involve apoptosis but likely involves new gene transcription, because the process can be recapitated by NFAT3, a key target of calcineurin regulation in the heart (26).

We have described mitochondrial alterations that arise as a result of overexpression of activated calcineurin in cell culture and the transgenic mouse model. Alterations in energy metabolism due to calcineurin have not been heretofore described. The impairment of oxidative phosphorylation is associated with a decrease in the amount of protein corresponding to components of the electron transfer complexes in the transgenic mouse. Both nuclear-encoded (cytochrome oxidase subunit IV) and mitochondrial-encoded (ND3, cytochrome oxidase subunit I) proteins appear to be downregulated. This may be mediated by increased degradation, a change in mitochondrial and/or nuclear transcription or translation. Proper assembly and stabilization of electron transfer components requires the coordinated synthesis of both nuclear and mitochondrial-encoded proteins (2, 28). Therefore, dysregulation of nuclear or mitochondrial transcription or translation could result in the destabilization of electron transfer complexes. Moreover, protein import into the mitochondria depends upon membrane potential. Diminished mitochondrial membrane potential, as we observed in the neonatal cardiomyocytes infected with adenovirus for activated calcineurin, could lead to impaired import of nuclear-encoded proteins. Further work will be required to establish the mechanism for the loss of electron transfer capacity mediated by calcineurin.

Calcineurin is known to regulate the activity of transcription factors GATA4, NFAT3, and MEF2C (10, 20, 22). These transcription factors have been shown to regulate the expression of some nuclear genes of mitochondrial energy metabolism (18, 27, 44). Furthermore, it has recently been reported that calcineurin activates transcription of PGC-1, a transcriptional corepressor that regulates mitochondrial biogenesis (32). PGC-1 and the other transcription factors would be expected to increase mitochondrial biogenesis, a common feature of cardiac hypertrophy (30, 34, 45, 46). One would predict that PGC-1 overexpression would also lead to hypertrophy and elevated levels of superoxide production.

The excessive superoxide production observed in the CaITG mice may explain several features of their phenotype. Superoxide at low levels has been shown to stimulate hypertrophy (35), but chronic or high levels of superoxide will result in cumulative cellular damage and dilated cardiomyopathy (5, 15). Although dysregulated mitochondrial biogenesis may be responsible for excessive superoxide production and progression to heart failure, it is also possible that calcineurin activates superoxide production through other mechanisms, including upregulation of other enzyme systems that can generate superoxide, such as cytochrome P450 monooxygenases (12, 13, 38). Excessive superoxide production would lead to secondary mitochondrial damage and eventual progression to heart failure.

This work was supported by National Heart, Lung, and Blood Institute Grant HL-61518 (to R. A. Gottlieb) and National Institute of Diabetes and Digestive and Kidney Diseases Training Grant DK-07022 (to A. B. Gustafsson).

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