Uncoupling Protein-2 Modulates Myocardial Excitation–Contraction Coupling

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Rationale: Uncoupling protein (UCP2) is a mitochondrial inner membrane protein that is expressed in mammalian myocardium under normal conditions and upregulated in pathological states such as heart failure. UCP2 is thought to protect cardiomyocytes against oxidative stress by dissipating the mitochondrial proton gradient and mitochondrial membrane potential (ΔΨm), thereby reducing mitochondrial reactive oxygen species generation. However, in apparent conflict with its uncoupling role, UCP2 has also been proposed to be essential for mitochondrial Ca2+ uptake, which could have a protective action by stimulating mitochondrial ATP production.

Objective: The goal of this study was to better understand the role of myocardial UCP2 by examining the effects of UCP2 on bioenergetics, Ca2+ homeostasis, and excitation–contraction coupling in neonatal cardiomyocytes.

Methods and Results: Adenoviral-mediated expression of UCP2 caused a mild depression of ΔΨm and increased the basal rate of oxygen consumption but did not affect total cellular ATP levels. Mitochondrial Ca2+ uptake was examined in permeabilized cells loaded with the mitochondria-selective Ca2+ probe, rhod-2. UCP2 overexpression markedly inhibited mitochondrial Ca2+ uptake. Pretreatment with the UCP2-specific inhibitor genipin largely reversed the effects UCP2 expression on mitochondrial Ca2+ handling, bioenergetics, and oxygen utilization. Electrically evoked cytosolic Ca2+ transients and spontaneous cytosolic Ca2+ sparks were examined using fluo-based probes and confocal microscopy in line scan mode. UCP2 overexpression significantly prolonged the decay phase of [Ca2+]i transients in electrically paced cells, increased [Ca2+]i spark activity and increased the probability that Ca2+ sparks propagated into Ca2+ waves. This dysregulation results from a loss of the ability of mitochondria to suppress local Ca2+-induced Ca2+ release activity of the sarcoplasmic reticulum.

Conclusion: Increases in UCP2 expression that lower ΔΨm and contribute to protection against oxidative stress, also have deleterious effects on beat-to-beat [Ca2+]i handling and excitation-contraction coupling, which may contribute to the progression of heart disease. (Circ Res. 2010;106:730-738.)

Key Words: uncoupling protein-2 ▪ mitochondria ▪ calcium ▪ calcium sparks ▪ excitation–contraction coupling
mitochondrial permeability transition pore in apoptosis. Therefore, regulation of mitochondrial Ca\(^{2+}\) transport in the cardiomyocyte is essential for maintaining proper metabolic function, excitation–contraction (EC) coupling, and cell viability.

Mitochondrial uncoupling proteins (UCPs) emerged almost 30 years ago with the discovery of the thermogenic properties of UCP1 in brown adipose tissue. UCP1 was described as a regulated proton channel capable of dissipating the mitochondrial proton gradient, releasing the stored electrochemical energy in the form of heat. To date, 5 UCP isoforms have been identified in mammalian tissue and named UCP1–5 in the order of their discovery. In contrast to UCP1, which is expressed exclusively in brown adipose tissue, UCP2 is expressed in a variety of tissues, including the heart. The role of UCP2 in the heart is not yet clear, but it has been shown to be upregulated under pathophysiological conditions, such as heart failure. It is essential for maintaining proper metabolic function, excitation–contraction (EC) coupling, and cell viability.

Here, we use adenovirus-mediated delivery of UCP2 to study the impact of elevated UCP2 on bioenergetics, mitochondrial function, and mitochondrial and cytosolic Ca\(^{2+}\) handling in neonatal cardiomyocytes (NCMs). Our data demonstrate that elevated expression of UCP2 has a negative impact on mitochondrial Ca\(^{2+}\) uptake in excitable cells and indicate that upregulation of this protein can disrupt normal E-C coupling and lead to increased arrhythmogenic potential.

Methods
An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Cardiomyocyte Isolation and Culture
Primary cultures of NCMs were prepared from 1-day-old Wistar rats (Harlan) as previously described. The protocol was approved by the UMDNJ Institutional Animal Care and Use Committee in accordance with the NIH Guide for the Care and Use of Laboratory Animals. This isolation protocol produces an enriched culture containing more than 95% myocytes. See the Online Data Supplement for details.

Adenovirus Synthesis
Adenovirus containing human UCP2 (Ad-UCP2) was generated from human UCP2 cDNA using the AdMax system (Microbix). See the Online Data Supplement for details.

Immunofluorescence
NCM cultures infected with Ad-ßGal, Ad-UCP2, or no virus were stained with a rabbit ß anti-hUCP2 polyclonal antibody (Calbiochem, catalog no. 662047) followed by goat ß anti-rabbit IgG–horseradish peroxidase (Santa Cruz Biotechnology). UCP2 proteins were detected using a tyramide-based signal amplification kit (Perkin Elmer) and visualized using confocal microscopy. See the Online Data Supplement for details.

Cellular ATP Quantitation
Total cellular ATP levels were determined in NCM cultures with a luciferase-based assay (Sigma) carried out according to the manufacturer’s directions. See the Online Data Supplement for details.

Oxygen Consumption Assay
NCMs infected with Ad-ßGal or Ad-UCP2 were collected, suspended in ECM extracellular buffer and then transferred to a water-jacketed (37°C) and magnetically stirred microchamber. The rates of oxygen consumption were determined with a Clark-type electrode and the data were analyzed as previously described.

\[ \Delta \Psi_m, [Ca^{2+}]_i, [Ca^{2+}]_m \] Measurements
Imaging experiments were performed as previously described. \[ \Delta \Psi_m \] was evaluated after equilibrating NCMs with 4 mmol/L of the voltage-sensitive fluorescent dye tetracyanomethylenol renal ester (TMRE) using epifluorescence microscopy. For \([Ca^{2+}]_i\) measurements, NCMs were incubated with 2 \(\mu\)mol/L rhod-2/AM and permeabilized with digitonin, and \([Ca^{2+}]_m\) changes were measured by epifluorescence. \([Ca^{2+}]_i\) was measured by confocal microscopy in line scan mode using either fluo-5N/AM or fluo-3/AM. See the Online Data Supplement for details.

Results
Characterization of UCP2 Expression in NCMs
NCMs were infected for 48 hours with recombinant Ad-UCP2 or Ad-ßGal or no virus and relative UCP2 protein levels and subcellular distribution were evaluated with immunofluorescence (see Methods). Confocal images in Figure 1A demonstrate that endogenous, as well as overexpressed, UCP2 immunofluorescence appeared to be predominately mitochondrial with a punctate signal and perinuclear distribution. Ad-UCP2 infection led to a 6.3-fold increase in UCP2 immunofluorescence over both Ad-ßGal–infected and uninfected cells (Figure 1B).

The effects of UCP2 overexpression on \(\Delta \Psi_m\) were evaluated in permeabilized NCMs incubated in the presence of the membrane potential-sensitive dye, TMRE and fatty acids to stimulate UCP2 activity. Ad-UCP2–infected cells had...
Figure 1. UCp2 overexpression: immunocytochemistry and effects on mitochondrial function. A, Representative images demonstrating relative levels of UCp2 expression in control NCMs and cells infected with either Ad-βGal or Ad-UCp2. Control cells incubated without primary antibody are also depicted (No 1° IgG). B, Quantitation of relative fluorescence of UCp2. Data are means±SEM (n=3 coverslips from 2 separate preparations). *P<0.01 vs no virus and Ad-βGal. C, Effects of Ad-UCp2 with or without genipin on ΔΨ<sub>m</sub>. i, Trace of mean changes of TMRE fluorescence intensities±SEM (n=4 pairs of coverslips) in permeabilized NCMs infected with Ad-UCp2 (Green) or Ad-βGal (Red) in response to FCCP (5 μmol/L) plus oligomycin (5 μg/mL). ii, Quantitation of the change in TMRE fluorescence before and after treatment with FCCP plus oligomycin. Permeabilized cells were incubated 5 to 10 minutes with DMSO (0.1% vol/vol; control) or genipin (10 μmol/L) before TMRE fluorescence intensity measurements. Data are expressed as means±SEM (n=120 cells from 4 to 5 coverslips and 2 separate cell preparations). *P=0.05 vs Ad-βGal and Ad-UCp2 plus genipin. D, Effects of Ad-UCp2 with or without genipin on mitochondrial respiration. i, Sample trace demonstrating protocol followed for oxygen consumption measurements in intact cells. Oligomycin and FCCP were added at 5 μg/mL and 5 μmol/L, respectively. Dashed lines represent extensions of oxygen consumption rates before indicated additions to more clearly illustrate rate changes. ii, Ratio of the maximal rate of oxygen consumption in presence of oligomycin alone to the rate in presence of FCCP and oligomycin in intact cells. Where indicated, cells were incubated for 5 minutes with genipin (10 μmol/L) before oxygen measurements. Data are expressed as means±SEM (n=7 from 6 separate cell preparations). *P=0.01 vs Ad-βGal and Ad-UCp2 plus genipin.

protonophore-releasable TMRE fluorescence intensity values approximately 10% lower than that of paired pair-loaded Ad-βGal-infected cells (mean fluorescence values were 63±3 and 74±2 arbitrary units, respectively), demonstrating a mild depression of ΔΨ<sub>m</sub> by UCp2 overexpression (Figure 1C). To verify that the observed decrease in ΔΨ<sub>m</sub> was attributable to an increase in functional UCp2, permeabilized TMRE-loaded NCMs were pretreated with the UCp2-inhibitor genipin<sup>25</sup> (Wako Chemicals) for 5 to 10 minutes before measuring TMRE release. The data indicate that genipin treatment significantly increased TMRE uptake in Ad-UCp2 infected cells, but had no effect on dye accumulation in Ad-βGal-infected cultures (Figure 1C, ii). The rate of oxygen consumption in intact NCMs metabolizing glucose was measured using a Clark-type oxygen electrode to evaluate the impact of UCp2 overexpression on mitochondrial function (Figure 1D, i). Myocytes infected with Ad-UCp2 exhibited significantly elevated ratios of the pseudo-state 4 respiration rates in the presence of oligomycin compared to the maximal rates of respiration in the presence of the mitochondrial uncoupler, carbonyl cyanide 4-(trifluoromethoxy)-phenylhydrazone (FCCP) (Figure 1D, ii). Genipin treatment decreased the oligomycin to FCCP ratio in Ad-UCp2 infected NCMs, but did not alter respiratory parameters in Ad-βGal expressing cells. Taken together, these results suggest that Ad-UCp2 infection leads to the expression of a functional UCp2 protein that, in turn, enhances mitochondrial proton leak and increases basal oxygen utilization.

By increasing mitochondrial inner membrane proton leak and partially dissipating the proton motive force, UCp2 overexpression may compromise mitochondrial ATP formation. Thus, total cellular ATP levels under a variety of conditions were quantitated with a luciferase-based ATP assay (Online Table 1). The results demonstrate that cellular ATP levels were not affected by UCp2 overexpression relative to uninfected or Ad-βGal infected cultures (7.99±0.13 versus 7.87±0.28 or 8.34±0.26 nmol ATP/10<sup>6</sup> cells, respectively). Similarly, cells treated for five min with either oligomycin (5 μg/mL) or the weak protonophore, 2,4-dinitrophenol (DNP) sufficient to depolarize ΔΨ<sub>m</sub> similarly to UCp2 overexpression (Online Figure I, A) did not exhibit altered levels of ATP (Online Table 1). However, ATP levels were significantly lower than controls in cultures treated with FCCP (5 μmol/L), which caused complete collapse of ΔΨ<sub>m</sub>. These findings suggest that UCp2-mediated decreases in ΔΨ<sub>m</sub> do not significantly depress mitochondrial ATP synthesis, or the depression of oxidative phosphorylation that results from UCp2 overexpression is offset by a compensatory increase in glycolytic ATP generation.

Measurement of [Ca<sup>2+</sup>]<sub>m</sub> Using Rhod-2 in Permeabilized NCMs

To make direct measurements of [Ca<sup>2+</sup>]<sub>m</sub> independent of cytosolic signals, rhod-2-loaded NCMs were permeabilized
with digitonin to release extramitochondrial rhod-2, and [Ca\textsuperscript{2+}]\textsubscript{m} measurements made thereafter, essentially as we have described previously.\textsuperscript{5,26} In permeabilized NCMs, the rhod-2 fluorescent fluorescence signal displayed a mitochondrial-like morphology (Online Figure II, A). When the extramitochondrial free Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{ex}) was increased above a threshold, oscillatory fluctuations in the rhod-2 fluorescence intensity were observed in both Ad-βGal and Ad-UCP2 infected NCMs (Online Figure III). Cells treated with either FCCP plus oligomycin (to collapse ΔΨ\textsubscript{m} and indirectly block [Ca\textsuperscript{2+}]\textsubscript{m} uptake) or ruthenium red (to inhibit the mitochondrial Ca\textsuperscript{2+} uniporter) no longer displayed changes in rhod-2 fluorescence (Figure 2A). Furthermore, treatment with the mitochondrial Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger inhibitor CGP-37157, caused step-wise increases in [Ca\textsuperscript{2+}]\textsubscript{m} with each consecutive spike (Online Figure II, B). These results indicate that rhod-2 monitors fluctuations in [Ca\textsuperscript{2+}]\textsubscript{m} in permeabilized NCMs. Increasing [Ca\textsuperscript{2+}]\textsubscript{ex} from 130 to 350 nmol/L caused a concentration-dependent increase in the frequency of [Ca\textsuperscript{2+}]\textsubscript{m} oscillations (Figure 2A, i). Treatment with thapsigargin abolished fluctuations in [Ca\textsuperscript{2+}]\textsubscript{m} (Figure 2A, iv), demonstrating the SR dependence of these [Ca\textsuperscript{2+}]\textsubscript{m} oscillations. Taken together, these data indicate that the close coupling between SR Ca\textsuperscript{2+} release and mitochondrial Ca\textsuperscript{2+} uptake sites are not disrupted by digitonin treatment nor significantly affected by UCP2 overexpression. Moreover, the dependency of the [Ca\textsuperscript{2+}]\textsubscript{m} spikes on medium Ca\textsuperscript{2+} levels suggests that SR Ca\textsuperscript{2+} load or Ca\textsuperscript{2+}-dependent activation of RyRs (i.e., CICR) may control the onset and frequency of [Ca\textsuperscript{2+}]\textsubscript{m} fluctuations.

**Effect of UCP2 Expression on Mitochondrial Ca\textsuperscript{2+} Uptake**

To evaluate UCP2-mediated changes in mitochondrial Ca\textsuperscript{2+} uptake in the absence of CICR or possible variations in the SR Ca\textsuperscript{2+} load, we treated Ad-βGal- and Ad-UCP2–infected cells with thapsigargin to deplete internal Ca\textsuperscript{2+} stores before elevating [Ca\textsuperscript{2+}]\textsubscript{ex}. The data indicate that UCP2 expression significantly impairs the initial rate of Ca\textsuperscript{2+} rise and the magnitude of the Ca\textsuperscript{2+} increase in the mitochondrial matrix (Figure 2C). Genipin treatment did not alter the rate or magnitude of Ca\textsuperscript{2+} uptake in the control Ad-βGal infected cultures (Figure 2B), but largely reversed the effects of UCP2 on [Ca\textsuperscript{2+}]\textsubscript{m} accumulation (Figure 2C). These data suggest that the effects of Ad-UCP2 infection on [Ca\textsuperscript{2+}]\textsubscript{m} uptake is attributable to UCP2 expression and activity.

NCM cultures were treated with DNP to mimic the effects of UCP2 expression on ΔΨ\textsubscript{m}. Permeabilized cells equilibrated with TMRE were incubated with increasing concentrations of DNP and the effect on TMRE fluorescence intensity evaluated. A 3-minute incubation with 150 μmol/L DNP caused a 8.7%±2.8% decrease in TMRE fluorescence intensity (Online Figure I), closely mimicking the effects of UCP2 overexpression on TMRE accumulation (Figure 1C). We evaluated the effects of this concentration of DNP on [Ca\textsuperscript{2+}]\textsubscript{m} uptake. Figure 2D illustrates that treating NCM cultures with DNP greatly attenuated [Ca\textsuperscript{2+}]\textsubscript{m} uptake and this effect was not reversed by pretreatment with genipin. These results demonstrate that a mild depression of ΔΨ\textsubscript{m} can have profound effects on both the rate and magnitude of mitochondrial Ca\textsuperscript{2+} uptake. Moreover, because genipin does not alter the effects of DNP on Ca\textsuperscript{2+} uptake, these data support the conclusion that genipin acts specifically on UCP2 activity.

**Overexpression of UCP2 Causes Dysregulation of Electrically Evoked [Ca\textsuperscript{2+}]\textsubscript{m} Transients**

The effects of UCP2-mediated mitochondrial uncoupling on electrically evoked [Ca\textsuperscript{2+}]\textsubscript{m} transients were examined in intact NCMs using confocal microscopy in line scan mode.\textsuperscript{27} These experiments were carried out with fluo-5N (K\textsubscript{d}=~90 μmol/L), because this indicator exhibited a large dynamic range, good signal-to-noise ratio, and minimal [Ca\textsuperscript{2+}]\textsubscript{m} buffering.\textsuperscript{28} We found that UCP2 overexpression altered the kinetics of electrically evoked [Ca\textsuperscript{2+}]\textsubscript{m} transients, causing significant prolongation of the declining phase of the [Ca\textsuperscript{2+}]\textsubscript{m} transient (Figure 3A, iii) and an increased propensity for spontaneous secondary [Ca\textsuperscript{2+}]\textsubscript{m} transients (Figure 3A, ii). Ad-UCP2–infected cells showed a nearly three-fold increase in the time...
required to recover to 90% of basal \([\text{Ca}^{2+}]_{c}\) (Figure 3B). The slower decay rate of the \(\text{Ca}^{2+}\) transient could not be attributed to alterations in basal \([\text{Ca}^{2+}]_{c}\), nor to the size of the thapsigargin-releasable SR \(\text{Ca}^{2+}\) pool (Online Figure IV).

Even though we have found no effect of UCP2 overexpression on total cellular ATP levels, a decrease in local \([\text{ATP}]\) could potentially contribute to the observed effects of UCP2 overexpression on the electrically evoked \([\text{Ca}^{2+}]_{c}\) transients. To test this possibility, cells were acutely treated with the ATP synthase inhibitor oligomycin in the absence or presence of a synthetic antioxidant, 1,4-phenylenediamine (DPPD). DPPD was included in these experiments to prevent possible increases in mitochondrial ROS formation associated with elevated \(\varDelta \Psi_{m}\) induced by oligomycin treatment, which could alter \(\text{Ca}^{2+}\) homeostasis independent of ATP levels. These experiments showed that oligomycin-treatment slowed the decay phase of the \([\text{Ca}^{2+}]_{c}\) transient, but this effect was completely abrogated by DPPD treatment (Online Figure V, A and B). These results suggest that the actions of oligomycin on \(\text{Ca}^{2+}\) transients are mediated through increased ROS formation and probably not through the depletion of local ATP levels. As an alternative approach, we also examined the effects of depletion near-mitochondrial ATP by inhibiting the mitochondrial adenine nucleotide translocator with atracyloside. As shown in Online Figure V (B), atracyloside treatment had no effect on electrically evoked \([\text{Ca}^{2+}]_{c}\) transients. Importantly, the prolongation of the decay phase in UCP2 expressing NCMS was unaffected by DPPD treatment (Online Figure V, C). These experiments support the hypothesis that the UCP2-induced effects on electrically evoked \([\text{Ca}^{2+}]_{c}\) transients are not mediated through local ATP depletion or ROS augmentation.

To test the hypothesis that the observed effects of UCP2 overexpression on electrically evoked \([\text{Ca}^{2+}]_{c}\) transients were attributable to impaired mitochondrial \(\text{Ca}^{2+}\) uptake, we examined the effects of DNP and Ru360, a cell permeant inhibitor of the mitochondrial \(\text{Ca}^{2+}\) uniporter (Figure 3C). Acute treatment with both DNP and Ru360 produced a prolongation of the \([\text{Ca}^{2+}]_{c}\) transient similar to that observed in UCP2 expressing cells (Figure 3D). Taken together, these data indicate that inhibition of mitochondrial \(\text{Ca}^{2+}\) uptake leads to abnormal \([\text{Ca}^{2+}]_{c}\) transients in NCMS.

**UCP2 Overexpression Increases Spontaneous \([\text{Ca}^{2+}]_{c}\) Spark Activity and Can Be Mimicked by DNP**

CICR, resulting from the close apposition of the RyR with L-type \(\text{Ca}^{2+}\) channels, is thought to play an important role in normal EC coupling: the action potential depolarizes the myocyte and causes the synchronous activation of L-type \(\text{Ca}^{2+}\) channels and RyRs leading to a global \(\text{Ca}^{2+}\) transient. However, in resting isolated cells, activation of single \(\text{Ca}^{2+}\) release units from individual junctional complexes can be observed in the form of \(\text{Ca}^{2+}\) “sparks.” Sparks are spontaneous local increases in \([\text{Ca}^{2+}]_{c}\) which average 3 to 5 \(\mu\text{m}\) in diameter and last 30 to 100 ms. Most \([\text{Ca}^{2+}]_{c}\) sparks in the resting myocyte remain as discrete events and do not propagate across the cell. However, during EC coupling sparks are thought to be the building blocks of the \(\text{Ca}^{2+}\) transient, with the spatiotemporal summation of discrete \(\text{Ca}^{2+}\) release units underlying global events. We were interested in understanding how UCP2-induced changes in \(\varDelta \Psi_{m}\) may impact spontaneous \([\text{Ca}^{2+}]_{c}\) events and if this may contribute to the observed changes in EC coupling described in the previous section.

Cells infected with either Ad-\(\beta\text{Gal}\) or Ad-UCP2 were loaded with the high affinity \(\text{Ca}^{2+}\) indicator, fluo-3, and line scans of spontaneous \([\text{Ca}^{2+}]_{c}\) activity acquired with confocal microscopy. NCMS overexpressing UCP2 displayed an increase in spontaneous \([\text{Ca}^{2+}]_{c}\) sparks compared with Ad-\(\beta\text{Gal}\)-infected controls (Figure 4). We found that: (1) a greater percentage of Ad-UCP2–infected cells demonstrated some degree of spontaneous \([\text{Ca}^{2+}]_{c}\) activity (51% versus 89% for Ad-\(\beta\text{Gal}\) and Ad-UCP2–infected cells, respectively; Figure 4B); (2) \([\text{Ca}^{2+}]_{c}\) sparks had a much greater tendency to propagate as \([\text{Ca}^{2+}]_{c}\) waves (Figure 4A); and (3) Ad-UCP2–infected cells with \([\text{Ca}^{2+}]_{c}\) sparks had nearly a 3.5-fold higher frequency of global \(\text{Ca}^{2+}\) events (average of 21 versus 73 events per min for Ad-\(\beta\text{Gal}\) and Ad-UCP2–infected cells, respectively; Figure 4C).

Figure 4D illustrates line scans of the same Ad-\(\beta\text{Gal}\) and Ad-UCP2 cells analyzed in Figure 4A, but this time while...
being electrically paced. The Ad-βGal cell demonstrates regular [Ca^{2+}] sparks and loses nearly all [Ca^{2+}] sparks. Thus, there are no global [Ca^{2+}] transients out of phase with the electric pacing, and the intervening [Ca^{2+}] sparks that were observed under spontaneous conditions are essentially eliminated. By contrast, the Ad-UCP2–infected cell maintained a dysfunctional [Ca^{2+}] phenotype with a high level of spontaneous activity even while being paced. Both premature global transients and skipped beats were commonly observed with UCP2 overexpression. Furthermore, localized [Ca^{2+}] sparks and propagating [Ca^{2+}] waves were frequently seen between global transients. Thus, the high level of spontaneous activity observed in Ad-UCP2 overexpressing NCMs underlies the significant lengthening of the [Ca^{2+}] transients in paced cells. DNP was again used to mimic the effects of UCP2 on [Ca^{2+}] homeostasis. For these experiments, spontaneous [Ca^{2+}] changes were monitored before and after treatment with 150 μmol/L DNP. We observed many of the same changes that were commonly seen in cells expressing UCP2. Specifically, DNP treatment increased the frequency of spontaneous global [Ca^{2+}] transients, which were accompanied by more frequent and spatially longer local [Ca^{2+}] events (Figures 4E and 5C). To better understand the changes in [Ca^{2+}] sparks and related spontaneous [Ca^{2+}] sparks and related spontaneous [Ca^{2+}] events resulting from UCP2 overexpression, representative surface plots of local [Ca^{2+}] events were generated for cells from both groups. Figure 5A depicts the topology of a typical [Ca^{2+}] spark from a control Ad-βGal–infected cell. In NCMs overexpressing UCP2, the rising phase of [Ca^{2+}] sparks is very similar to that seen in Ad-βGal cells, suggesting the process of [Ca^{2+}] release is unaffected (Figure 5B). However, with UCP2 overexpression, [Ca^{2+}] sparks are not as easily contained to the Ca^{2+} release site. Instead, [Ca^{2+}] changes propagate spatially, as demonstrated by the bidirectional branching and multiple peaks seen in Figure 5B. In Figure 5C, we monitored typical [Ca^{2+}] sparks from a single spatial location and tracked the changes resulting from treatment with DNP. As with UCP2 overexpression, DNP administration commonly led to the spatiotemporal propagation of [Ca^{2+}] sparks as illustrated in Figure 5C. The mean spatial and temporal dimensions of the [Ca^{2+}] sparks are shown in Figure 5D (see the Online Data Supplement for details on the quantitation of [Ca^{2+}] sparks). These results further support the hypothesis that mitochondrial Ca^{2+} uptake serves to “buffer” local [Ca^{2+}] increases, thereby suppressing excitability and protecting against aberrant propagation of [Ca^{2+}] sparks.

**Discussion**

The ability of cardiomyocytes to effectively regulate the large and rapid fluxes of [Ca^{2+}] that underlie EC coupling is vital for proper cardiac function. However, despite our understanding of many of the central players in generating [Ca^{2+}] transients, the importance of [Ca^{2+}]_\text{mt} uptake in EC coupling is not well appreciated. Ultrastructural analysis has revealed that mitochondria are positioned in close apposition to myocardial junctional complexes. In combination with estimates of perimitochondrial [Ca^{2+}] peaks ranging from 30 to 600 μmol/L, this morphology supports a possible role for the mitochondria in shaping [Ca^{2+}] transients. Seguchi et al. used the mitochondrial Ca^{2+} uniporter inhibitor Ru360 to show that mitochondrial Ca^{2+} uptake limits the propagation of local [Ca^{2+}]_k increases isolated ventricular cardiomyocytes. Similarly, active mitochondrial Ca^{2+} uptake has been shown to act as a barrier in pancreatic acinar cells to limit agonist-evoked [Ca^{2+}]_k increases to the secretory pole. Moreover, and others have shown that treating nonexcitable cells with FCCP (plus oligomycin) to dissipate ΔΨ\text{m} and prevent mitochondrial Ca^{2+} uptake markedly slows the declining phase of [Ca^{2+}]_k spikes and increases the propagation rate of cytosolic Ca^{2+} waves following hormone stimulation. These data indicate that mitochondrial Ca^{2+} uptake can buffer local [Ca^{2+}] increases and regulate the extent of Ca^{2+} wave propagation in both excitable and nonexcitable cells.

The ability of mitochondria to track [Ca^{2+}]_k transients on a beat-to-beat basis in adult cardiomyocytes is still an open question. It has been estimated that the quantity of cytosolic Ca^{2+} entering the mitochondria is minimal during E-C coupling with mitochondria only able to slowly accumulate...
Ca^{2+} ions over many contractile cycles.\textsuperscript{38–40} On the other hand, our data in permeabilized NCMS (Figure 2) and H9c2 cardiomyocytes,\textsuperscript{4} as well as the work of Sheu colleagues in “skinned” adult cardiomyocytes,\textsuperscript{5} have demonstrated a tight coupling of SR Ca^{2+} release to mitochondrial Ca^{2+} uptake. Single-cell imaging studies of rhod-2 or TMRE fluorescence intensity changes during a contractile cycle have also indicated that [Ca^{2+}]\textsubscript{c} is rapidly transferred to the mitochondria.\textsuperscript{2,3,5,26,41–44} Moreover, recent studies using Ca^{2+}-sensitive photoproteins ectopically expressed in the mitochondria have revealed large [Ca^{2+}]\textsubscript{m} transients in both neonatal\textsuperscript{4} and adult cardiomyocytes\textsuperscript{45} preparations, which mirror the changes in [Ca^{2+}]\textsubscript{c} on a beat-to-beat basis. These data indicate that mitochondrial Ca^{2+} transport is fast enough to follow [Ca^{2+}]\textsubscript{c} transients and modulate Ca^{2+} handling during EC coupling.

UCP2 is expressed in mammalian myocardium under normal conditions and has been found to be upregulated in pathological conditions.\textsuperscript{15,16} It is thought that UCP2 is capable of modulating ROS generation through the dissipation of ΔΨ\textsubscript{m} and that increased UCP2 expression protects cardiomyocytes against oxidative stress and ischemia reperfusion injury.\textsuperscript{17,46,47} However, the beneficial adaptive effects of upregulating UCP2 expression to limit excessive ROS formation\textsuperscript{47} may also have detrimental costs on mitochondrial ATP synthesis\textsuperscript{16} and, thus, EC coupling. Our data indicate that biologically relevant increases in UCP2 expression\textsuperscript{48} can mildly depress ΔΨ\textsubscript{m} with significantly altering total cellular ATP levels. Nevertheless, the expression of UCP2 markedly alters [Ca^{2+}]\textsubscript{m} uptake and caused a dysregulation of beat-to-beat [Ca^{2+}]\textsubscript{c} handling. Specifically, we found that UCP2 overexpression significantly lengthens the decay phase of [Ca^{2+}]\textsubscript{c} transients in paced cells, causes increases in spontaneous [Ca^{2+}]\textsubscript{c} sparks, and leads to spatiotemporal propagation of [Ca^{2+}]\textsubscript{c} sparks into waves. Moreover, using the acute application of oligomycin (in the presence of DPPD) or atractyloside, we showed that near-mitochondrial ATP depletion is not responsible for the observed beat-to-beat changes in [Ca^{2+}]\textsubscript{c}, that result from UCP2 overexpression. These results are contrary to the study by Trenker et al.\textsuperscript{18} who proposed that UCP2 and UCP3 are fundamental for mitochondrial Ca^{2+} uniporter-mediated [Ca^{2+}]\textsubscript{m} uptake. They show that UCP2 and UCP3 overexpression in HeLa and endothelial cells increased the capacity of mitochondria for Ca^{2+} uptake, which augmented agonist-stimulated mitochondrial ATP production. By contrast, in cardiomyocytes we have demonstrated a significant UCP2-induced depression of [Ca^{2+}]\textsubscript{m} uptake in permeabilized cardiomyocytes. To better understand these discrepancies, we examined the effect of UCP2 overexpression on [Ca^{2+}]\textsubscript{m} uptake in permeabilized HeLa cells. Overexpression of UCP2 in HeLa cells did not augment mitochondrial Ca^{2+} uptake (Online Figure VI) as previously reported by Trenker et al. These results are in line with Brooks et al.\textsuperscript{19} who refute the proposed role for UCP2 (and UCP3) in mitochondrial Ca^{2+} uniporter-mediated [Ca^{2+}]\textsubscript{m} uptake.

Based on our results, we propose that normally functioning mitochondria are essential for effectively “containing” local, spontaneous changes in [Ca^{2+}]\textsubscript{c} through mitochondrial Ca^{2+} uptake. With increased UCP2 expression, the resultant depression of ΔΨ\textsubscript{m} compromises the ability of mitochondria to buffer local [Ca^{2+}]\textsubscript{c} changes, facilitating [Ca^{2+}]\textsubscript{c} spark generation and allowing [Ca^{2+}]\textsubscript{c} release to spread to neighboring junctional complexes and elicit aberrant propagation of RyR-mediated CICR. This can lead to asynchronous [Ca^{2+}]\textsubscript{c} events that give rise to out of phase [Ca^{2+}]\textsubscript{c} transients and waves, prolongation of [Ca^{2+}]\textsubscript{c} transient decay and [Ca^{2+}]\textsubscript{c} spikes during the relaxation phase. Thus, the compensatory expression of UCP2 during disease processes associated with the generation of ROS, such as heart failure, could exacerbate defects in EC coupling and cause the loss of contractile function and enhance arrhythmogenic potential.
Acknowledgments

We thank Dr J. Sadoshima and colleagues for invaluable assistance with the isolation of NCMs. We also thank Dr M.D. Brand for generously providing the plasmid containing hUCP2 for our generation of Ad-UCP2 and Dr A. Palmer for the 4mt-D3-cvp plasmid.

Sources of Funding

This work was supported by NIH grants R01-AA014980 (to A.P.T.) and R01-AA017752 (to L.D.G.) and NIH predoctoral MD/PhD fellowship F30-AA015004 (to J.D.T.).

Disclosures

None.

References


**Novelty and Significance**

**What Is Known?**

- UCP2 expression is elevated in human heart diseases.
- Increased UCP2 expression can protect against oxidative stress.
- It has been proposed that UCP2 is an intrinsic component of the electrogenic mitochondrial Ca\textsuperscript{2+} uptake pathway.

**What New Information Does This Article Contribute?**

- UCP2 is not an essential component of the mitochondrial Ca\textsuperscript{2+} uptake pathway in cardiomyocytes but depresses mitochondria Ca\textsuperscript{2+} accumulation by lowering the mitochondrial membrane potential.
- Increased UCP2 expression causes abnormal cytosolic Ca\textsuperscript{2+} transients during excitation–contraction coupling and enhances spontaneous Ca\textsuperscript{2+} release (Ca\textsuperscript{2+} sparks) in the diastolic phase.
- UCP2 upregulation is a double-edged sword, offering protection against oxidative stress but disruption of excitation–contraction coupling, and may therefore contribute to the pathogenesis of heart disease.

Uncoupling proteins (UCPs) are integral mitochondrial membrane proteins that share structural similarities with metabolite transporters. UCP1 mediates a proton leak that uncouples substrate oxidation from ATP synthesis, and is involved in thermogenesis in brown adipose tissue. However, the function of UCP2-5 has not been fully elucidated. UCP2 is expressed in mammalian myocardium and is upregulated in heart disease. The increase in UCP2 is thought to protect against oxidative stress by dissipating the mitochondrial membrane potential, thereby reducing the generation of reactive oxygen species. However, by lowering mitochondrial membrane potential, UCP2 could also interfere with other bioenergetic functions, including ATP synthesis and Ca\textsuperscript{2+} uptake. Our data show that overexpression of UCP2 in cardiomyocytes does not alter cellular ATP levels but markedly inhibits mitochondrial Ca\textsuperscript{2+} uptake. This leads to disruption of the cytosolic Ca\textsuperscript{2+} transients underlying excitation-contraction coupling. Specifically, UCP2 overexpression prolongs the decay phase of Ca\textsuperscript{2+} transients in electrically paced cardiomyocytes, enhances spontaneous Ca\textsuperscript{2+} spark activity and increases the probability that Ca\textsuperscript{2+} sparks propagate into Ca\textsuperscript{2+} waves. We conclude that the adaptive increases in UCP2 expression that protect against oxidative stress also have deleterious effects on beat-to-beat Ca\textsuperscript{2+} handling, which could exacerbate defects in excitation–contraction coupling and contribute to the progression of heart disease.
Uncoupling Protein-2 Modulates Myocardial Excitation-Contraction Coupling
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*Circ Res.* 2010;106:730-738; originally published online January 7, 2010;
doi: 10.1161/CIRCRESAHA.109.206631

*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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World Wide Web at:
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Data Supplement (unedited) at:
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SUPPLEMENT MATERIAL

Uncoupling protein-2 modulates myocardial excitation-contraction coupling

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ONLINE METHODS

Cardiomyocyte isolation and culture.
Neonatal rats (1 day) were sacrificed by cervical dislocation, chest cavities opened, and cardiac ventricular tissue excised and minced into small fragments. Ventricular myocytes were dissociated by serial digestions at 37°C with collagenase type IV, 0.1% trypsin, and 15 µg/ml DNase I (Sigma). Cell suspensions were purified on a discontinuous Percoll gradient as previously described. The myocyte layer was removed from the Percoll gradient and cells resuspended in NCM culture medium comprised of DMEM/F-12 supplemented with 5% (v/v) horse serum, 4 µg/ml transferrin, 0.7 ng/ml sodium selenite (Life Technologies, Inc.), 2 g/liter bovine serum albumin (fraction V), 3 mmol/L pyruvic acid, 15 mmol/L HEPES, 100 µmol/L ascorbic acid, 100 µg/ml ampicillin, 5 µg/ml linoleic acid, and 100 µmol/L 5-bromo-2'-deoxyuridine (Sigma), and plated at 1.25 x 10⁶ cells/cm² on either 25 mm coverglass or tissue culture-treated polystyrene plates coated with a solution of 0.1% (w/v) gelatin and 12.5 µg/mL fibronectin (Sigma). Culture media were changed to serum-free after 24 hrs and myocytes were cultured under serum-free conditions for a minimum of 48 hrs before experimentation.

Adenovirus constructs.
For the synthesis of UCP2 adenovirus (Ad-UCP2), human UCP2 cDNA was amplified from a previously constructed pET21b-hUCP2 vector, digested, and ligated into adenovirus shuttle vector pDC316 (Microbix) at dephosphorylated EcoRI and Sall sites using DNA Ligation Kit 2.1 (Takara) for 3 min at 25°C according to the manufacturer’s instructions. The ligated vector was amplified, isolated, and cotransfected with the replication-deficient adenoviral genomic plasmid pBHGlox(Δ)E1,3Cre (Microbix) into HEK293 cells using Lipofectamine 2000 (Invitrogen). After allowing cells to recover from the transfection for 24 hrs, cells were overlaid with DMEM + 0.65% agarose. Plates were then screened for viral plaques over the following 7-14 days. Isolated viral plaques were picked and further amplified with HEK293 cells. Cells were resuspended in adenovirus stabilizing buffer (20 mmol/L Tris, 25 mmol/L NaCl, 2.5% Glycerol, pH 8.0) and viral lysate prepared with 3 freeze/thaw cycles in a bath of liquid nitrogen and 37°C water bath. The solution was centrifuged to remove cellular debris and lysates were aliquoted and stored at -80°C. A stock of β-galactosidase adenovirus (Ad-βGal) was provided as a gift from Timothy R. Billiar (University of Pittsburg). Ad-βGal was propagated, prepared, and stored...
in the same manner as described above for Ad-UCP2. A plaque assay was performed to calculate the viral titer (in plaque forming units (pfu)/mL) for both Ad-βGal and Ad-UCP2 lysates using serial dilutions of viral stock and DMEM/0.65% agarose overlay. A multiplicity of infection (MOI) of 10 (10 pfu/myocyte) was used for all experiments.

**Immunofluorescence.**

NCM cultures infected with Ad-βGal, Ad-UCP2, or no virus were fixed with 1% (w/v) paraformaldehyde, permeabilized with 95% ethanol/1% acetic acid (v/v) and endogenous peroxidase activity quenched with 1% (v/v) H₂O₂. Cells were stained with a rabbit anti-hUCP2 polyclonal antibody (1:500; Calbiochem, cat# 662047) diluted in PBS containing 5% (w/v) BSA followed by goat anti-rabbit IgG-HRP (1:1000; Santa Cruz). UCP2 proteins were detected using a tyramide-based signal amplification kit as described by the manufacturer (TSA-tetramethylrhodamine; Perkin Elmer). UCP2 immunofluorescence was excited with a HeNe laser (543 nm) and emitted light (> 570 nm) was collected on a BioRad Radiance 2000 laser scanning confocal microscope with a 60x water objective (NA 1.20). All UCP2 immunofluorescence images were acquired using the same gain and laser power settings. The mean fluorescence intensity was calculated from randomly chosen individual cells (n > 10 cells) after subtracting background levels and carried out in triplicate for each condition. The mean UCP2 immunofluorescence intensity for uninfected cells was set to 100% and the percent increase for Ad-βGal- and Ad-UCP2-infected cells was calculated based on these values. Statistical significance was evaluated with Student’s t-test.

**ATP bioluminescent assay.**

Total cellular ATP levels were quantitated with ATP bioluminescent assay (Sigma). In brief, media was removed from NCMs plated on 35 mm plates and cells were incubated with extracellular buffer (25 mmol/L HEPES, 121 mmol/L NaCl, 5 mmol/L NaHCO₃, 2.0 mmol/L CaCl₂, 10 mmol/L glucose, 4.7 mmol/L KCl, 1.2 mmol/L MgSO₄, 0.23 mmol/L KH₂PO₄, 0.97 mmol/L K₂HPO₄, 0.25% (w/v) BSA, pH 7.4) containing either no additives (groups 1-3), 150 μmol/L DNP (group 4), 5 μg/mL oligomycin (group 5), or 5 μmol/L FCCP (group 6) for 5 min at room temperature. The buffer was then removed, cells rinsed with PBS and lysed with 200 μL of CelLyticM (Sigma). Cells were quickly scraped and 20 μL of lysate combined with 80 μL sterile, tissue culture-grade distilled H₂O and 100 μL of ATP assay mix (diluted 1:25 with Assay Mix Dilution Buffer; Sigma) and photons liberated by the luciferase reaction immediately counted on a Optocompl luminometer (MGM Instruments) for 6 s. ATP levels were extrapolated from a standard curve constructed on the day of experiment.

**Imaging measurements in permeabilized cells.**

For [Ca²⁺]ᵢ measurements, NCMs were incubated for 10 min at room temperature in extracellular buffer supplemented with 2 μmol/L rhod-2/AM, 4 μmol/L fura-2/AM and 0.02% (w/v) Pluronic acid F-127 (Molecular Probes). For ∆Ψᵢ measurements, NCMs were only loaded with fura-2/AM. Dye-loaded cells were washed twice with Ca²⁺-free extracellular buffer, twice with intracellular-like medium (ICM) composed of: 130 mmol/L KCl, 10 mmol/L NaCl, 1.0 mmol/L KH₂PO₄, 20 mmol/L Tris-HEPES, 100 μmol/L K-EGTA, 50 μmol/L CaCl₂, pH 7.2, and then transferred to a thermostatically regulated microscope chamber. The cells were permeabilized with digitonin (20 μg/mL) and the dissipation of intracellular fura-2 fluorescence (340nm excitation/ 510nm emission) was used to monitor loss of plasmalemmal integrity. Cells were washed with fresh ICM then incubated for 20 min with ICM supplemented with glutamate (5 mmol/L), malate (1 mmol/L), ATP (2 mmol/L), MgCl₂ (2 mmol/L), creatine phosphate (5 mmol/L), creatine phosphokinase (5
units/mL), oleic acid (30 μmol/L) bound to BSA (15 μmol/L), and protease inhibitor cocktail (Sigma; diluted 1:100). For \( \Delta \Psi_m \) measurements, ICM also included 4 nmol/L tetramethylrhodamine ethyl ester (TMRE; Molecular Probes). To minimize coverslip-to-coverslip variations in [TMRE], coverslips with equal cell densities from each group were paired, and equilibrated with a single solution of TMRE-containing buffer. Rhod-2 and TMRE fluorescence images were acquired as previously described\(^2\). Rhod-2 fluorescence was normalized to the minimum fluorescence obtained in the presence of 10 μmol/L ionomycin plus 1.1 mmol/L EGTA and the maximum fluorescence obtained after further addition of 10 μmol/L cyclosporin A, 1 μmol/L FCCP, 5 μg/mL oligomycin and 1.2 mmol/L CaCl\(_2\). TMRE fluorescence was normalized by subtracting fluorescence values after treatment with FCCP and oligomycin.

**[Ca\(^{2+}\)]_c** measurements in intact NCMs.

To measure electrically-evoked [Ca\(^{2+}\)]_c transients, NCMs were loaded for 10 min at room temperature with 4 μmol/L fluo-5N/AM and 0.02% (w/v) Pluronic acid in ECM. Cells were paced at 1 Hz with 5 ms square-wave pulses using a Grass Stimulator (model SD9). For measurements of spontaneous [Ca\(^{2+}\)]_c activity, NCMs were loaded for 30 min at room temperature with 4 μmol/L fluo-3/AM and 0.02% (w/v) Pluronic acid. Line scans were acquired at 2 ms intervals on a BioRad Radiance 2000 laser scanning confocal microscope with a 20x objective (NA 0.75) or 40x oil objective (NA 1.30). Ca\(^{2+}\)-sensitive indicators were excited with an Ar laser (488 nm) and emitted light collected with an HQ 528/50 nm emission filter. Surface plots were generated using Interactive 3D Surface Plot v2.1 plugin for Image J. For the quantitation of local spontaneous [Ca\(^{2+}\)]_c events, the width and duration of [Ca\(^{2+}\)]_c sparks were quantitated based on the following exclusionary criteria: increase in fluorescence \( \geq \) 2 standard deviations above basal fluorescence, width \( \geq \) 2 μm and \( \leq \) 20 μm, and duration \( \geq \) 15 ms and \( \leq \) 200 ms. These exclusionary criteria were established to distinguish “local” events from more “global” occurrences. Events falling within these parameters in Ad-UCP2-infected cells were nearly two-fold greater in both width and duration, as compared with those in Ad-βGal-infected cells. It is important to note that these exclusionary parameters resulted in the omission of many of the most dramatic events seen with Ad-UCP2-infected cells and that the effects on the propagation of local [Ca\(^{2+}\)]_c may in fact be even more profound than suggested by these calculations.

**Calibration of [Ca\(^{2+}\)]_ex.**

NCMs seeded on 25 mm coverglass were rinsed with ICM and then permeabilized for 5 min with digitonin (20 μg/mL). The buffer was then replaced with fresh ICM supplemented with 4 μmol/L fura-2 free acid. Epifluorescence images were acquired at 3-s intervals at 37°C using a 20x objective (NA 0.75) with a cooled CCD camera under computer control and fura-2 fluorescence (excitation 340 and 380 nm) measured with successive additions of CaCl\(_2\). [Ca\(^{2+}\)]_ex was calculated using the following equation: [Ca\(^{2+}\)]_ex = K\(_D\) x \( \beta \) x \((R\_max-R\_min)/(R\_max-R)\). A K\(_D\) of 135 nmol/L was used\(^3\), R\(_min\), R\(_max\), and \( \beta \) were calculated from zero [Ca\(^{2+}\)]_ex (in presence of 1 mmol/L EGTA) and saturating [Ca\(^{2+}\)]_ex (excess of 1 mmol/L Ca\(^{2+}\)).

**[Ca\(^{2+}\)]_m** measurements in HeLa cells.

HeLa cells were plated on 75 cm\(^2\) flasks or 25 mm glass coverslips and grown to 70%–80% confluency in DMEM (GIBCO-BRL) supplemented with 4.5 g/L glucose, 10% (v/v) FBS, and 1% (v/v) penicillin/streptomycin at 37°C and 5% CO\(_2\). Cells were transfected with a plasmid containing the mitochondrially-targeted cameleon, 4mt-D3-cpv,\(^4\) using FuGENE-HD transfection reagent (Roche), according to the manufacturer’s instructions. Cells were allowed to recover for 8 hours post-transfection, infected with either Ad-βGal or
Ad-UCP2 at 10 MOI (UCP2 expression confirmed with immunocytochemistry, as described above), and imaging experiments conducted 14–20 hours after the addition of virus. Epifluorescence images were acquired at 1-s intervals using a 440DF20 excitation filter, a 450 nm dichroic mirror, and two emission filters (475/40 nm for CFP and 535/25 nm for YFP). Prior to permeabilization, cells were washed twice with intracellular-like buffer supplemented with BAPTA (200 µmol/L), CaCl₂ (100 µmol/L), MgCl₂ (2 mmol/L). Cells were permeabilized in intracellular-like buffer supplemented with digitonin (30 µg/mL), BAPTA (1 mmol/L), CaCl₂ (0.4 mmol/L), glutamate (5 mmol/L), pyruvate (1 mmol/L) ATP (2 mmol/L), MgCl₂ (2 mmol/L), creatine phosphate (5 mmol/L), creatine phosphokinase (5 units/mL), and protease inhibitor cocktail III (A.G. Scientific, Inc.). SR Ca²⁺ was depleted with thapsigargin (2 µmol/L). Basal [Ca²⁺]ᵢ was established in intracellular-like buffer containing 100 nmol/L [Ca²⁺]ᵢ (calibration of [Ca²⁺]ᵢ was conducted as described above). After establishing basal [Ca²⁺]ᵢ levels, [Ca²⁺]ᵢ was rapidly increased to 1 µmol/L. Mean CFP and YFP fluorescence were calculated for a minimum of 10 cells per coverslip using custom software (Spectralyzer). YFP:CFP fluorescence ratios were calculated for each cell and the mean rate of the linear phase of calcium uptake was calculated. Significance was determined by student’s t-test.

ONLINE RESULTS AND DISCUSSION

UCP2 raises [Ca²⁺]ᵢ threshold for SR-mediated [Ca²⁺]ᵢ oscillations in permeabilized NCMs.
In permeabilized NCMs loaded with rhod-2, we examined UCP2-induced changes in SR-driven [Ca²⁺]ᵢ oscillations with step-wise increases in [Ca²⁺]ᵢ (Online Figure III). There was substantial cell-to-cell variability in the [Ca²⁺]ᵢ threshold and amplitude of [Ca²⁺]ᵢ oscillations that was observed in both the control and UCP2 groups. Therefore, we calculated the percentage of cells that began to display [Ca²⁺]ᵢ oscillations at each step of [Ca²⁺]ᵢ. With UCP2 overexpression, we found that a greater proportion of cells required higher levels of [Ca²⁺]ᵢ before [Ca²⁺]ᵢ oscillations were observed (Online Figure III C and D). In Ad-βGal-infected cells the majority of [Ca²⁺]ᵢ oscillations were initiated at 340 nM [Ca²⁺]ᵢ (30.1%), while 440 nM was required in the majority of Ad-UCP2-infected cells (30.6%; representative traces are illustrated). We did not observe any significant UCP2-mediated effects on the kinetics, frequency, or amplitude of the [Ca²⁺]ᵢ spikes. Based on these results, we conclude that mitochondrial UCP2 overexpression attenuates SR-driven [Ca²⁺]ᵢ entry by shifting the threshold to a requirement for higher [Ca²⁺] in permeabilized NCMs and hypothesize that UCP2-mediated depression of ΔΨᵢ is the underlying mechanism.

UCP2 overexpression does not alter SR Ca²⁺ content.
In order to evaluate UCP2-induced changes in SR Ca²⁺ content, we loaded NCMs with the ratiometric Ca²⁺ indicator, fura-2, depleted mitochondrial Ca²⁺ with 5 µmol/L FCCP plus 5 µg/mL oligomycin, and measured the release of SR Ca²⁺ after the addition of 2 µmol/L thapsigargin. As illustrated in Online Figure IV, we found no significant changes in SR Ca²⁺ content using this experimental protocol.

Near-mitochondrial ATP depletion does not alter [Ca²⁺]ᵢ transients.
In order to test whether a decrease in local [ATP] may contribute to the observed effects of UCP2 overexpression on [Ca²⁺]ᵢ transients, cells were acutely treated with the ATP synthase inhibitor oligomycin (Online Figure V Ai). Results indicated an increase in the decay phase of the [Ca²⁺]ᵢ transient, suggesting a potential role for the depression of
mitochondrial ATP generation in the observed UCP2-induced changes in \([\text{Ca}^{2+}]_{c}\) transients. However, by inhibiting the primary route of proton transport back into the matrix, it has been demonstrated that oligomycin hyperpolarizes the mitochondrial membrane\(^5\) and consequently causes an increase in ROS production\(^6\)—two effects that are opposite of what is seen with increases in UCP2. Further, it is well-established that ROS can cause dramatic changes in \([\text{Ca}^{2+}]_{c}\) which could confound the oligomycin-induced effects on \([\text{Ca}^{2+}]_{c}\) transients. To test this hypothesis, we examined oligomycin-induced effects on \([\text{Ca}^{2+}]_{c}\) transients in the presence of the ROS scavenger, \(N,N'\)-Diphenyl-1,4-phenylenediamine (DPPD). We found that 20 \(\mu\)mol/L DPPD completely abrogated the oligomycin-induced changes in \([\text{Ca}^{2+}]_{c}\) transients (Online Figure V Aii). This experiment demonstrates that the oligomycin-induced effects are, in fact, mediated through ROS formation and not through the depletion of near-mitochondrial ATP. In order to confirm this finding, we also examined the effects of depleting near-mitochondrial ATP by inhibiting the adenine nucleotide translocator with atractylloside. We found that 20 \(\mu\)mol/L atractylloside had no effect on electrically-evoked \([\text{Ca}^{2+}]_{c}\) transients (Online Figure V B). Finally, we investigated the impact of the ROS scavenger, DPPD, on \([\text{Ca}^{2+}]_{c}\) transients in UCP2 overexpressing NCMs in order to evaluate whether ROS contributes to the UCP2-induced effects on \([\text{Ca}^{2+}]_{c}\) transients. We found that the prolongation of the decay phase was unaffected by the antioxidant DPPD (Online Figure V C). These experiments support the hypothesis that the effects of UCP2 are not mediated by ATP depletion or ROS generation.

**UCP2 overexpression depresses the rate of \([\text{Ca}^{2+}]_{m}\) uptake in HeLa cells.**

In 2007, Trenker et al.\(^7\) reported that UCP2 is necessary for \([\text{Ca}^{2+}]_{m}\) uniport activity, and more specifically, that UCP2 overexpression augments \([\text{Ca}^{2+}]_{m}\) uptake—a finding that is contrary to the results described in this manuscript. In order to better understand these apparent differences, we examined the effect of UCP2 overexpression on \([\text{Ca}^{2+}]_{m}\) uptake in permeabilized HeLa cells (a cell type used by Trenker et al.) using mitochondrially-targeted cameleon \(^4\) to measure \([\text{Ca}^{2+}]_{m}\). Consistent with our results in NCMs, we found that UCP2 overexpression resulted in a decrease in the rate and amplitude of \([\text{Ca}^{2+}]_{m}\) uptake in HeLa cells (Online Figure VI). These data suggest that UCP2-mediated depression of \([\text{Ca}^{2+}]_{m}\) is not limited to neonatal cardiomyocytes, but that UCP2 overexpression likely has a similar impact in other cell types. These data are also in line with Brookes et al.\(^8\), who critiqued the findings reported by Trenker et al.

**ACKNOWLEDGEMENTS**

We would like to thank Dr. A. Palmer the generous gift of the 4mt-D3-cpv plasmid for \([\text{Ca}^{2+}]_{m}\) measurement in HeLa cells.
ONLINE REFERENCES


### Sample group nmol ATP/10^6 cells

<table>
<thead>
<tr>
<th>Sample group</th>
<th>nmol ATP/10^6 cells</th>
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<tr>
<td>No Virus</td>
<td>7.87 ± 0.28</td>
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<tr>
<td>Ad-βGal</td>
<td>8.34 ± 0.26</td>
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<tr>
<td>Ad-UCP2</td>
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<tr>
<td>DNP</td>
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<tr>
<td>Oligomycin</td>
<td>* 8.44 ± 0.41</td>
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<tr>
<td>FCCP</td>
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**Online Table 1. Cellular ATP levels.** ATP levels were quantified with a luciferase-based assay under the following conditions: No Virus, Ad-βGal infection, Ad-UCP2 infection, DNP (150 µmol/L), Oligomycin (5µg/mL), and FCCP (5 µmol/L). Drug treatment was for 5 min prior to ATP quantitation. n= 4 for each condition from two separate cell preparations. *P<0.05 vs No Virus.
Online Figure I. Effects of dinitrophenol on $\Delta \Psi_m$. This figure illustrates the effects of titration of DNP (50 $\mu$mol/L increments) on $\Delta \Psi_m$ in permeabilized NCM equilibrated with 4 nmol/L TMRE. DNP is used in subsequent experiments to mimic mild uncoupling exhibited by UCP2. A. Representative trace of TMRE fluorescence intensity changes in response to DNP additions. FCCP (5 $\mu$mol/L) and oligomycin (5 $\mu$g/mL) were added at the end of each experiment to fully depolarize the mitochondria. F/F0 represents fluorescence values normalized to initial fluorescence prior to DNP addition. B. Average change in TMRE fluorescence after 180 s incubation with 150 $\mu$mol/L DNP. Data are expressed as mean ± SEM (n = 140 cells from 3 coverslips and 3 separate cell preparations). *P<0.05 vs F0.
Online Figure II. Specificity of rhod-2 \([\text{Ca}^{2+}]_m\) signal in permeabilized NCMs. A. Representative image demonstrating the localization of rhod-2 signal to the mitochondria with perinuclear distribution and punctuate morphology. B. Treatment with the mitochondrial \(\text{Na}^+\)-\(\text{Ca}^{2+}\) exchanger inhibitor CGP-37157 (1 \(\mu\)mol/L) prevented the extrusion of \([\text{Ca}^{2+}]_m\). The inset illustrates the step-wise increases in \([\text{Ca}^{2+}]_m\) with each spike. Where indicated (Ca), a 220 nmol/L pulse of \(\text{Ca}^{2+}\) was added for a total of 350 nmol/L \([\text{Ca}^{2+}]_{ex}\). Each trace is representative of three experiments and two separate cell preparations.
Online Figure III. Effects of UCP2 overexpression on SR-driven \([\text{Ca}^{2+}]_m\) oscillations. NCMs were loaded with rhod-2/AM, permeabilized, and \([\text{Ca}^{2+}]_\text{ex}\) increased step-wise. A-B. Representative traces from Ad-\(\beta\)Gal- and Ad-UCP2-infected cells demonstrating the initiation of \([\text{Ca}^{2+}]_m\) oscillations with increasing steps of \([\text{Ca}^{2+}]_\text{ex}\). C-D. The percentage of total Ad-\(\beta\)Gal- and Ad-UCP2-infected cells, respectively, with \([\text{Ca}^{2+}]_m\) oscillations beginning at the indicated \([\text{Ca}^{2+}]_\text{ex}\). (\(\beta\)Gal \(n = 186\) cells, UCP2 \(n = 157\) cells, 3 pairs of coverslips).
Online Figure IV. Effects of UCP2 overexpression on SR Ca\(^{2+}\) content. NCMs infected with either Ad-βGal or Ad-UCP2 were loaded with fura-2/AM. Mitochondrial Ca\(^{2+}\) was depleted with 5 μmol/L FCCP plus 5 μg/mL oligomycin and SR Ca\(^{2+}\) release measured after the addition of 2 μmol/L thapsigargin. **A.** Mean traces of thapsigargin-induced SR Ca\(^{2+}\) release. **B.** Average fura-2 fluorescence before and after the addition of thapsigargin. Data are presented as mean ± SEM (Ad-βGal n=129 cells, Ad-UCP2 n=135 cells, from three pairs of coverslips).
Online Figure V. Effects of near-mitochondrial ATP depletion on $[\text{Ca}^{2+}]_c$ transients.
Representative confocal line scans of electrically-evoked $[\text{Ca}^{2+}]_c$ transients of Fluo-5N-loaded NCMs: 
A. $[\text{Ca}^{2+}]_c$ traces before and after treatment with: i. Oligomycin (5 µg/mL). ii. Oligomycin (5 µg/mL) after pretreatment with DPPD (20 µmol/L). B. $[\text{Ca}^{2+}]_c$ traces before and after treatment with atracyloside (20 µmol/L). C. $[\text{Ca}^{2+}]_c$ trace in Ad-UCP2-expressing cells pre-treated with DPPD. These traces are representative of a minimum of 12 cells and three experiments from two separate preparations.
Online Figure VI. Effect of UCP2 overexpression on $[\text{Ca}^{2+}]_m$ uptake in HeLa cells. $[\text{Ca}^{2+}]_m$ uptake was examined in permeabilized HeLa cells infected with either Ad-βGal or Ad-UCP2. Mitochondrially-targeted cameleon was used for $[\text{Ca}^{2+}]_m$ measurements. 

A. Representative mean traces of $[\text{Ca}^{2+}]_m$ uptake. $[\text{Ca}^{2+}]_{\text{ex}}$ was increased from 100 nmol/L to 1 µmol/L where indicated. $R/R_0$ represents the YFP:CFP fluorescence ratio normalized to the initial ratio prior to $[\text{Ca}^{2+}]_{\text{ex}}$ increase. 

B-C. The relative rate and amplitude of $[\text{Ca}^{2+}]_m$ uptake. Data are expressed as mean ± SEM. *P<0.01.