Uncoupling protein 2 modulates cell viability in adult rat cardiomyocytes

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Bodyak N, Rigor DL, Chen Y-S, Han Y, Bisping E, Pu WT, Kang PM. Uncoupling protein 2 modulates cell viability in adult rat cardiomyocytes. Am J Physiol Heart Circ Physiol 293: H829–H835, 2007. First published April 27, 2007; doi:10.1152/ajpheart.01409.2006.—Uncoupling protein 2 (UCP2) is an inner mitochondrial membrane proton carrier that couples ATP synthesis. The aim of this study was to determine whether UCP2 plays a role in survival of adult rat cardiac myocytes. We first studied the effects of UCP2 overexpression in vitro. Overexpression of UCP2 in primary cardiomyocytes led to a significant decline in ATP level and the development of acidosis but had no observable effect on cell survival. When cardiomyocytes were challenged with hypoxia-reoxygenation, cells overexpressing UCP2 survived significantly less compared with control. This finding was associated with upregulation of proapoptotic proteins Bcl-2 and 19-kDa interacting protein 3 (BNIP3). Furthermore, UCP2 short interfering RNA prevented both the increase in cell death and BNIP3 expression. To examine the in vivo role of UCP2 in the heart, we used the Dahl salt-sensitive rat heart-failure model. Northern blot analysis revealed that UCP2 mRNA level was significantly upregulated in rat heart failure along with BNIP3 protein level. In conclusion, UCP2 increases sensitivity of adult rat cardiac myocytes to hypoxia-reoxygenation by way of ATP depletion and acidosis, which in turn causes accumulation of prodeath protein BNIP3.

The loss of cardiomyocytes through apoptosis has been shown to contribute to numerous cardiovascular disorders such as progressive left-ventricular dysfunction in heart failure, ischemia-reperfusion, and dilated cardiomyopathy. Various cellular factors and signaling pathways that are known to promote cell survival or cell death are of great interest as possible therapeutic targets. The aim of the present study was, therefore, to investigate the effects of UCP2 overexpression in vitro on cell viability in cardiomyocytes under normal conditions and under hypoxia-reoxygenation, a clinically relevant model of several heart-disease states. We also analyzed UCP2 mRNA expression level in vivo in the heart in the Dahl salt-sensitive (DSS) rat model of heart failure. The present study describes a significant deleterious effect of UCP2 overexpression in vitro on adult rat cardiomyocyte survival in a hypoxia-reoxygenation setting and reports an accumulation of prodeath protein Bcl-2 and 19-kDa interacting protein 3 (BNIP3) in UCP2-overexpressing adult rat cardiomyocytes. In vivo results show that UCP2 is upregulated along with BNIP3 and suggest its deleterious effect in heart failure.

MATERIALS AND METHODS

Primary culture of adult cardiac myocytes and induction of apoptosis by hypoxia-reoxygenation. Adult rat cardiac myocyte cultures were obtained from the hearts of female Sprague-Dawley rats by using techniques consistent with a previously published protocol (19, 20). Briefly, the hearts were excised and perfused for 45–60 min with 0.3% collagenase (Worthington Biochemical). Cell death was induced by hypoxia (applied for 9 h) followed by reoxygenation (18 h), as previously described (19, 20).

Generation of recombinant adenovirus. Mouse UCP2 cDNA was cloned into pShuttle-CMV vector (Invitrogen). Recombinant adenovirus expressing UCP2 (AdUCP2) was generated by using the AdEasy adenoviral vector system (Stratagene) according to the manufacturer’s protocol. Clonal stock of recombinant adenovirus was amplified in HEK-293 cells and then purified by CsCl. The viral titer was determined with the Tissue Culture Infectious Dose 50 method (Qbiogene). Data were collected from myocytes infected with a multiplicity of infection of 1–50 plaque-forming units.

Generation of short interfering RNA adenovirus. Single-stranded sense short interfering RNA (siRNA) and antisense siRNA oligo sequences were designed with siRNA Target Finder (Ambion) and were cloned downstream of the H1 RNA polymerase III promoter into modified pENTR-H1/Zeo, an RNA interference (RNAi) expression vector in which the RNAi expression cassette is flanked by recognition sequences for the Gateway recombination system (Invitrogen). Correct recombinants were conveniently selected by Zeocin resistance, and LR clonase was used to transfer the RNAi expression cassette into pAdDest, a derivative of Adeno-X (Clontech) containing

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a Gateway destination cloning cassette. The recombinant adenovirus containing the siRNA expression cassette was transfected into HEK-293 cells. In total, three UCP2 siRNA adenoviruses were constructed. Scrambled siRNA with no match to any rat or mouse genome sequences was used as a control.

**ATP-level assay.** ATP levels were measured with a bioluminescence assay kit (Molecular Probes division of Invitrogen). Cardiomyocytes were washed with PBS, then covered with lysis buffer containing 0.8% Triton X-100. Samples were centrifuged at 5,000 RPM and 4°C for 15 min, and the supernatant was retrieved for use in the ATP assay according to the manufacturer’s instructions. Luciferase activity was measured by using a luminometer (TD-20/20; Turner Design). Protein content was also measured by using the collected supernatant.

**Cell survival and cell-death analysis.** Cell survival in vitro was measured by the widely used 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer’s instructions (Sigma, St. Louis, MO). Briefly, cells were treated with MTT for 3 h. Formazan crystals were dissolved with MTT solvent while plates were agitated on shaker for 30 min. Absorbance was measured as a ratio of a wavelength of 570–690 nm. MTT assay can no longer be considered strictly a mitochondrial assay, because MTT reduction is associated not only with mitochondria but also with the cytoplasm and with nonmitochondrial membranes (3).

Western blotting. UCP2 (Santa Cruz), BNIP3 (Sigma), GAPDH (RDI), and COX IV (Cell Signaling) protein levels were examined in cell or heart tissue lysates by using Western blotting. Samples were run on 15% SDS gels and were transferred to PVDF membranes. Antibodies were diluted as follows: UCP2 (1:100), BNIP3 (1:1,000), GAPDH (1:5,000), COX IV (1:1,000).

**Northern blotting.** Total RNA was extracted from rat hearts by using Trizol Reagent (Invitrogen). A 15-μg aliquot of total RNA was electrophoresed in 1.3% denaturing formaldehyde agarose gels and was blotted onto Hybond N (Amersham). Membranes were hybridized with UCP2 (whole mouse cDNA fragment), atrial natriuretic peptide (ANP), and GAPDH probes labeled with [α-32P]CTP (random primers DNA labeling system; Invitrogen). Details of the probes were previously described (28).

**DSS rat heart-failure model.** DSS rat heart-failure model was generated as described previously (19, 20). Briefly, female DSS rats (Harlan Sprague Dawley) were fed a high-salt diet (6% NaCl) beginning at 6 wk of age. Only rats that showed overt clinical evidence of heart failure were included in the study, with an average age of 20 wk. Control age-matched rats were fed normal chow.

**Statistical analysis.** All data were expressed as means ± SE. Between-group and among-group comparisons were conducted with unpaired Student’s t-tests and ANOVA, respectively. Probability (P) values of <0.05 were considered significant.

**RESULTS**

UCP2 overexpression leads to ATP depletion and acidosis but does not affect cardiomyocyte viability at baseline. First, to study specific effects of UCP2, we designed siRNA against UCP2 to block UCP2 expression at both mRNA and protein levels. To deliver siRNA into cardiomyocytes, we constructed three separate siRNA adenoviruses (AdsiRNA), differing only in cDNA sequences. Although all constructed AdsiRNAs effectively blocked adenovirus-mediated UCP2 overexpression at the mRNA and protein levels in H9c2 cells and cardiomyocytes, respectively (Fig. 1), construct 3 (siUCP2 #3), with the sequence 5’-GCCACCTCATTCTGCTTGG-3’, was found to be most efficient and therefore was used for all subsequent studies. Overexpression of UCP2 did not affect mitochondria level in cardiomyocytes, because COX IV protein level remained unchanged (Fig. 1B).

Fig. 1. Effective silencing of uncoupling protein 2 (UCP2) overexpression by coinfection with short interfering (siRNA) adenoviruses. A: Northern blot was performed on H9c2 cardiac myoblasts 4 days after infection with UCP2 alone (AdUCP2) and coinfection with 3 different siRNA adenoviruses. Construct 3 (siUCP2 #3) was most effective in silencing UCP2 expression. UCP2 and GAPDH mRNA levels are represented. B: Western blot was performed on cardiomyocytes infected with UCP2 alone and coinfection with scrambled (Scr) siRNA and the most effective UCP2 siRNA adenovirus, construct 3. Samples were run on a 15% SDS gel and were probed for UCP2, GAPDH, or COX IV.

To investigate the effects of UCP2 overexpression specifically in adult cardiomyocytes at baseline, we initially focused on the protein’s uncoupling abilities. One of the suggested functions of UCP2 is the regulation of ATP biosynthesis. Consistent with the findings of others (43, 44) showing that UCP2 leads to ATP depletion in various cell types, an ATP determination assay revealed that ATP level was down significantly in cardiomyocytes after UCP2 gene transfer (Fig. 2A). ATP depletion observed in UCP2-overexpressing cardiomyocytes was rescued by simultaneous infection with AdsiRNA, whereas AdsiRNA alone did not affect ATP content. During the course of the experiments using UCP2 adenoviral transfer, we observed that the pH of extracellular medium with the cardiomyocytes overexpressing UCP2 was shifted into the acidic range, and although AdsiRNA prevented this pH shift, AdsiRNA alone did not affect it (Fig. 2B).

We then assessed whether UCP2 overexpression (or depletion) has an effect on cardiomyocyte survival. At baseline, there was no significant difference in cell survival between cardiomyocytes infected with AdUCP2 or AdsiUCP2 compared with the LacZ control (AdLacZ) (Fig. 2C). A significant drop of ATP level did not seem to affect cardiomyocyte survival at baseline during the period of experiments, although it is very likely that there may be long-term consequences of severe ATP depletion. These data are in agreement with other findings showing that complete loss of cell ATP in the case of anaerobic cold storage of cardiomyocytes did not interfere with the ability of cells to restore normal baseline function under the appropriate rewarming conditions (15). Thus UCP2 overexpression in adult rat cardiomyocytes leads to ATP depletion and the development of acidosis but does not affect cell survival at baseline. UCP2 depletion did not have a significant effect on cell survival.
UCP2 overexpression results in increased cell death after hypoxia-reoxygenation. The next set of experiments was carried out to determine whether UCP2 overexpression (or depletion) has a protective or deleterious effect on cardiomyocytes under stress conditions such as hypoxia-reoxygenation. Hypoxia causes a switch from oxidative to glycolytic energy generation, with increased glucose consumption, lactic acid production, and lower pH (9, 34, 48). Impaired mitochondrial ATP formation is the key stress of anoxic and ischemic injury, and necrotic cell death after ischemia-reperfusion is directly linked to ATP depletion. When cardiomyocytes were challenged with hypoxia-reoxygenation, cells overexpressing UCP2 were most affected and survived 38% less than control cardiomyocytes infected with LacZ adenovirus. Moreover, siRNA adenovirus against UCP2 successfully blocked this effect and rescued cardiomyocytes (Fig. 3). Compared with the control, siRNA alone did not have a significant effect on cell survival in response to hypoxia-reoxygenation. Thus these experiments have demonstrated that UCP2 overexpression in cardiomyocytes leads to cell death under hypoxia-reoxygenation conditions.

BNIP3 is upregulated in vitro. Next, we examined possible molecular mechanisms that may be involved in UCP2- and hypoxia-mediated cell death. It has been shown that a prodeath member of the BH-3 only subfamily of Bcl-2 proteins, BNIP3, is a key regulator of cell death of ventricular myocytes during hypoxia (37). BNIP3 is implicated in the control of both apoptotic and necrotic cell death, as well as in guarding mitochondrial integrity. Hypoxia alone induces BNIP3, but BNIP3 requires activation, such as acidosis, to trigger death program (13). Acidic pH also leads to BNIP3 accumulation and increases half-life of BNIP3 dimers and monomers (11). BNIP3 activates the death program in cardiac myocytes when hypoxia coincides with acidosis (11, 13, 37). In this regard, we focused on BNIP3 and found that BNIP3 protein level, as expected, was upregulated in cardiomyocytes subjected to hypoxia-reoxygenation (Fig. 4A). In addition, UCP2 overexpression alone in cardiomyocytes resulted in upregulation of BNIP3 protein level. When cardiomyocytes were coinfected with AdsiRNA against UCP2, BNIP3 increase was effectively blocked (Fig. 4B). These results are concordant with our cell-survival data as well as with known mechanisms of BNIP3 activation. Thus UCP2 overexpression alone causes acidosis, which in turn leads to accumulation of BNIP3, causing cells to become more susceptible to death. At this point cardiomyocyte survival is not yet compromised, but BNIP3 activation may trigger cell death. Indeed, when cells were challenged with apoptotic stimuli, such as hypoxia-reoxygenation, they survived significantly less than control cardiomyocytes. Simultaneous infection of cardiomyocytes with AdsiRNA against UCP2 was able to rescue loss of cells to a level comparable with control group under hypoxia-reoxygenation.

Upregulation of both UCP2 and BNIP3 is associated with heart failure in DSS rats. To analyze differential UCP2 expression in vivo, we used the DSS rat model of heart failure.
Rats were maintained on a high-salt diet and developed either stroke or progressive heart failure between 14 and 18 wk or 19 and 21 wk, respectively. All high-salt-diet rats died by 21–23 wk, and only rats that showed overt clinical evidence of heart failure were included in the study, with an average age of 20 wk (20). We have shown previously, using the gene chip array in this model, that among genes known to be expressed in heart failure, including biomarker ANF, there was a significant increase in UCP2 mRNA expression level (21). To confirm this data, we performed Northern blot analysis. RNA was isolated from the hearts of an independent group of rats to ensure that animals used for the Northern blot analysis were different from animals used for the microarray. Northern blot analysis confirmed that UCP2 mRNA level was significantly upregulated in failed hearts (approximately threefold) as was ANF (Fig. 5, A and B). Next, we checked for BNIP3 in this model and found an increase in BNIP3 protein-expression level in heart failure (Fig. 5, C and D). Thus we have found that UCP2 level is upregulated in the heart failure in DSS rats and that it coincides with increased BNIP3 expression.

DISCUSSION

In this study, we showed that the overexpression of UCP2 in adult cardiomyocytes does not affect cell survival at baseline but leads to significant ATP depletion, acidosis, and accumulation of prodeath protein BNIP3. Consequently, when cardiomyocytes overexpressing UCP2 were challenged with hypoxia-reoxygenation, they survived significantly less than control cells. Our results indicate that UCP2 overexpression has a deleterious effect on cell survival and, for the first time, show that UCP2 overexpression affects BNIP3. Controversial data, however, exist in the literature concerning the role of UCP2, as well as its protein-expression level. In vivo low expression of UCP2 contrasts with a high UCP2 mRNA level, and induction of UCP2 expression occurs without change in mRNA level, demonstrating a robust translational control (17). Several studies have shown the presence of the protein in the rat and human heart (27, 30, 32), whereas others do not observe UCP2 in rat or mouse heart (2, 38). Results may vary for a variety of reasons, such as species-specific variations, the quality of antibodies used, possible cross-reactivity with other UCP2 isoforms, limitations in detection systems, or intrinsic protein properties such as stability and a very short half-life, −30 min (39). Further clarification of this issue is still required.

Currently, the exact physiological role of UCP2 remains unknown, and this field is quite controversial. Some studies show that UCP2 overexpression can be beneficial for the cells (24, 45), notably in the protection against ROS in neuronal tissues (16, 26). In contrast, other studies support our findings and demonstrate that UCP2 overexpression can cause cell death (29, 44). One should keep in mind that any overexpression system could potentially produce an artificial biological phenomenon. To rule out possible artificial outcomes of UCP2 overexpression that may result from insertion of this protein into the inner mitochondrial membrane, Mills and coauthors (29) overexpressed another mitochondrial carrier protein, adenine nucleotide translocator-2, as a control to confirm specificity of the observed effects. Loss-of-function studies also yield quite divergent results. Simultaneous analysis of the depletion of UCP2 and UCP3 isoforms reveals a deleterious effect of UCP2 depletion in particular (27), whereas in animal
models, UCP2 deficiency resulted in increased resistance to cerebral ischemia with a significantly reduced infarct size in the brain of UCP2-knockout mice (7). Our findings show that UCP2 depletion in cardiomyocytes did not have a significant effect on cell survival.

There are several in vitro and in vivo pieces of evidence that demonstrate that UCP2 upregulation may be deleterious and downregulation may be beneficial. Increased UCP2 expression in primary cultured mesencephalic cells markedly enhanced necrosis in response to cyanide exposure (36) and led to a reduction in a number of hypocretin neurons in mice (6). In cortical cells treated with cyanide, transfection with UCP2 switched the death mode from apoptosis to necrosis, whereas UCP2 RNAi and a dominant negative mutant blocked the necrotic response (25). Also, an increase in UCP2 expression correlated with parenchymal necrosis and the severity of symptoms in models of acute experimental pancreatitis in rats and in mice (41). Furthermore, in mice with a spontaneous mutation in the leptin gene, protection against ischemia-reperfusion injury by treatment with cerulenin, an inhibitor of protein acetylation, was associated with a decrease in UCP2 expression and an increase in ATP (5). From these studies, it would appear that UCP2 can function either as a death or survival factor, depending on the initiation stimulus and death pathway executed.

Our in vivo observation that UCP2 is upregulated in the DSS rat heart failure model leads to a suggestion that UCP2 may play an important role in cardiomyocyte survival by affecting energy homeostasis. This is supported by our in vitro data showing an inverse relationship between UCP2 and ATP level. In failing hearts, there is an increase in energy demand, and maintaining a high ATP supply is critically important for maintaining cardiac performance. In the failing myocardium, ATP level has been shown to progressively decrease to ∼70–75% of normal values (18, 42), whereas the level of UCP2 has been shown to be increased (32). In fact, the adverse role of UCP2 in the heart is also supported by various animal models of heart failure. In a rat model of aortic regurgitation, UCP2 mRNA level was upregulated, whereas the creatine phosphate content was significantly decreased (35). In the same rat model, treatment with angiotensin-converting enzyme inhibitor perindopril improved cardiac performance and myocardial energy efficiency but also suppressed UCP2 overexpression, lending support to the hypothesis that overexpression of UCP2 may alter energy efficiency in the progression of heart failure (31). Increased UCP2 expression is also found in Gα, β1-adrenergic receptor-, β2-adrenergic receptor-, or protein kinase A-induced cardiomyopathy (12), as well as in pathological hypertrophy resulting from prolonged thyroid-hormone treatment (8).

Concomitant with ATP depletion and acidosis, UCP2 overexpression in adult cardiomyocytes also led to the upregulation of BNIP3. It is known that acidosis is further exacerbated when ATP levels begin to decline (1, 34). BNIP3 protein is induced by hypoxia and accumulates more rapidly under acidic pH (13). Acidosis may provide a “death” signal that activates BNIP3 in cardiac myocytes (14, 49). Low pH activates proapoptotic functions of BNIP3 by stimulating its intracellular translocation and integration into mitochondrial membranes, where BNIP3 stimulates mitochondrial permeability transition pore opening. This process is an integral part of the cardiac myocyte death pathway (47, 49) and is referred to as an atypical form of cell death with features of both apoptosis and necrosis (49). These data support our finding that UCP2 overexpression leads to significant cell death in cardiomyocytes challenged with hypoxia-reoxygenation via acidosis and BNIP3. In vivo, a significant increase in BNIP3 expression was detected in adult rat hearts with chronic heart failure (37). Our results are in agreement with these data and show that BNIP3 is upregulated...
in the DSS rat model of heart failure, along with UCP2. A direct relationship between UCP2 and BNIP3 is unknown and is yet to be determined.

Existing controversial results regarding the role of UCP2 led to the suggestion that UCP2 function may vary depending on the cell or organ type, as well as the stimulus applied. Also, it is possible that UCP2 may have a protective effect under certain conditions but may lead to negative consequences at another. Most likely up- or downregulation of UCP2 is a fine-tuned balance. It can be protective by decreasing mitochondrial ROS production, or it could cause a deleterious effect on cell survival and organ function by resulting in ATP depletion and acidosis. Additional studies are required to shed light on the function of UCP2.

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