Cardioprotective Role of the Mitochondrial ATP-Binding Cassette Protein 1
Hossein Ardehali, Brian O’Rourke, Eduardo Marbán

The mechanism by which mitochondria exert protection against oxidant stress is not clear. We recently showed that a purified mitochondrial fraction containing 5 coimmunoprecipitating proteins (succinate dehydrogenase, adenine nucleotide translocator, ATP synthase, inorganic phosphate carrier, and mitochondrial ATP-binding cassette protein 1 or mABC1) displayed mitochondrial ATP-sensitive K⁺-channel activity. mABC1, a member of the ABC family of proteins, is the only protein in this complex whose function is not known. A yeast homologue of mABC1 protein, Mdl1p, was recently identified to have a novel role for induction of cellular resistance to oxidant stress. Based on these observations, we hypothesized that mABC1 plays a key role in protection of myocardial cells against oxidant stress. We studied the function of mABC1 by modulating the levels of this protein in neonatal rat cardiomyocytes using various molecular techniques, followed by assessment of cell viability and measurement of mitochondrial membrane potential.

RNA interference resulted in reduced mABC1 mRNA and protein levels and was associated with significantly attenuated loss of tetramethylrhodamine ethyl ester fluorescence under basal conditions and an increase in trypan blue stained cells. In contrast, adenovirally mediated expression of mABC1 resulted in protection against oxidant stress loss of mitochondrial membrane potential. These results support the notion that mABC1 protein plays a major role in cellular protection against oxidant stress, identifying mABC1 as a novel target for cardioprotective therapeutics.

Mammalian ATP-binding cassette (ABC) family has been isolated from many organisms. They are membrane proteins that generally use the energy from ATP hydrolysis to transport various substrates, such as amino acids, steroids, proteins, and phospholipids. So far, only 3 yeast and 4 mammalian ABC proteins have been identified in the mitochondria. Of these mitochondrial proteins, only the function ATM1p, ABC7, and ABCme has been characterized. They are thought to be involved in Fe/S transport and maturation of cytosolic Fe/S proteins. The cDNA for a mammalian ABC protein, mitochondrial ABC1 (mABC1), was recently characterized; however, the function of this protein is unknown. mABC1 displays more homology with yeast Mdl1 and Mdl2 proteins than other ABC proteins. A recent study reported that Mdl1 may confer cellular resistance to oxidant stress. Another study suggested a role for Mdl1 in intracellular peptide transport; however, this function of Mdl1 would not fully explain its role in protection against oxidant stress.

Mitochondrial ATP-sensitive K⁺ channel (mitoKATP) is shown to play a key role in the process of ischemic preconditioning and protection against apoptosis; however, its structure remains unclear. We recently undertook studies to identify the molecular structure of mitoKATP. Using coimmunoprecipitation and yeast 2-hybrid techniques, we showed that a complex of at least 5 proteins, including mABC1, succinate dehydrogenase, inorganic phosphate carrier, adenine nucleotide translocator, and ATP synthase, form a macromolecular supercomplex in the mitochondrial inner membrane. A highly purified fraction of the inner-mitochondrial membrane, containing all 5 members of this supercomplex, was then isolated and shown to have mitoKATP-channel activity.

The observations that mABC1 is part of a complex with mitoKATP activity and that the yeast homologue of this protein confers resistance against oxidants stress suggest that mABC1 may either directly or indirectly influence cellular protection against ischemia and oxidant stress. To address this issue, contemporary molecular approaches were used here to modulate mABC1 expression. We show that small interfering RNA (siRNA)-mediated downregulation of mABC1 protein resulted in a significant reduction in mitochondrial membrane potential and a decrease in the number of viable cells. In contrast, adenoviral vector-mediated overexpression of the protein resulted in the attenuation of oxidant stress-induced loss of mitochondrial membrane potential.

Materials and Methods

Neonatal rat cardiomyocytes (NRCMs) were prepared as described before and are detailed in the online data supplement available at http://circres.ahajournals.org. siRNA duplexes were transfected into NRCM using the TransMessenger Kit (Qiagen). The level of mABC inhibition was assessed using RT-PCR and Western blot analysis. A recombinant adenoviral vector encoding green fluorescent protein and human mABC1 cDNA was constructed and transduced into NRCMs. Experimental procedures are described in detail in the online data supplement.

Results and Discussion

To reduce the baseline levels of the protein, we transfected NRCMs with a rhodamine-labeled mABC1 siRNA. As shown in Figure 1A, the transfected cells displayed a characteristic punctuate fluorescence around their nuclei, indicative of siRNA in the RNA, silencing complex. The level of mABC1 mRNA was then measured using RT-PCR in mABC1 siRNA-transfected and sham-transfected cells. There was a significant reduction in the levels of mABC1 mRNA in siRNA-transfected cells (Figure 1B). Western blot analysis also showed a significant reduction in the levels of mABC1 protein in mABC1 siRNA-transfected cells versus nonsense control siRNA-transfected cells (Figure 1C).
Cells transfected with mABC1 SiRNA and sham-transfected cells were then subjected to flow cytometry 48 hours after transfection. We have previously demonstrated 3 distinct phases of membrane potential changes in NRCMs as they undergo oxidant stress–induced cell death. As shown in Figure 1D, treatment of cells with mABC1 SiRNA resulted in a loss of membrane potential, as indicated by a reduction in the peak of high-fluorescence intensity (102) cells, and a significant increase in the number of depolarized and dying cells in the peaks of lower intensity (102). Figure 1E represents a summary of our experiments with mABC1 SiRNA. Treatment of cells with the SiRNA resulted in a 40% reduction in high-fluorescence cells and a significant decrease in the number of cells with lower fluorescence (<102). It should be noted that at baseline, we noted a lower proportion of cells with high fluorescence than reported previously. This is likely to be attributable to the addition of the transfection media and incubation of the cells in serum-free media for 24 hours.

Because treatment of cells with SiRNA can have nonspecific effects, we performed additional experiments in which we compared mABC1 SiRNA-treated cells to nonsilencing control SiRNA-treated cells. In accordance with previous results, we saw a significantly greater decrease in high-fluorescence cells as compared with SiRNA control (percentage of reduction of 22.0±1.3); however, this was slightly lower than the difference we had observed with sham-transfected cells.

We then used trypan blue exclusion studies as an additional measure of cell viability. Treatment of NRCMs with mABC1 SiRNA resulted in a significant increase in the number of stained cells (ie, dead cells) compared with cells treated with nonsilencing SiRNA (percentage of dead cells of 36.6±2.7 versus 73±1.5, respectively; P<0.05).

To better evaluate the potential role of mABC1 in cellular protection, we overexpressed the protein in NRCMs using an adenoviral expression system. AdCIG-mABC1, an adenovirus containing mABC1 cDNA and green fluorescent protein, was added to NRCMs, and cells were evaluated after 48 hours under confocal microscopy. As shown in Figure 2A, as low as 0.5×109 plaque-forming units of the adenovirus yielded green fluorescence in 90% of the cells. Extracts of the cells were then obtained and probed with mABC1 antibody after they were run on an SDS-
PAGE gel. There was a significant increase in mABC1 protein expression, as shown in Figure 2B.

We then studied the response of AdCIG-mABC1–transduced cells to oxidative stress by H2O2 exposure. Cells were first treated for 48 hours with AdCIG-mABC1, followed by the addition of H2O2. As shown in Figure 2C, treatment with AdCIG-mABC1 significantly preserved the number of cells in the high-fluorescence peak. Control experiments with ADCIG-only adenovirus did not result in protection against cell death (percentage of cells in high-fluorescence peak in AdCIG-treated cells versus not treated cells of 27.8 and 27.9, respectively; \(P=0.95\)). These results suggest that mABC1 protects cells against H2O2–induced mitochondrial dysfunction.

We previously argued that mABC1 is part of a mitochondrial macromolecular complex with mitoKATP-channel activity. Thus, the question may arise as to how this protective effect of mABC1 may be related to the mitoKATP-channel activity. To address this question, we treated AdCIG-mABC1 treated cells with mitoKATP inhibitors, 5-hydroxydecanoate and glybenclamide, followed by the addition of H2O2 and flow cytometry. Addition of these drugs did not cause any change in the pattern of tetramethylrhodamine ethyl ester (TMRE) uptake (percentage of change in high-fluorescence cells with the addition of 5-hydroxydecanoate and glybenclamide of −1.4 \(P=0.91\) and +16.6% \(P=0.40\), respectively). These results suggest that mABC1 may exert its protective effects through a novel mechanism and independent of mitoKATP. Alternatively, mABC1 overexpression may render cells refractory to the effects of pharmacological mitoKATP inhibitors, eg, through allosteric or stoichiometric effects on the channel complex.

In this article, we proposed that mABC1 plays a role in cellular protection against oxidant stress. This hypothesis was based on our previous studies showing that mABC1 is part of a complex that displays mitoKATP-channel activity, which plays a central role in cardioprotection, and on studies on yeast homolog of mABC1 (Mdl1p), which has been shown to play a novel role in induction of cellular resistance to oxidant stress.4 To address this question, we downregulated the levels of mABC1 protein using SiRNA technique and assessed TMRE uptake by mitochondria, in addition to trypan blue exclusion studies. We demonstrated that cells with lower mABC1 levels displayed reduced membrane potential and an increase in trypan blue stain at basal levels, suggesting that mABC1 protein is essential for viability of cells under basal conditions. We then overexpressed mABC1 protein in NRCMs using an adenovirus. Overexpression of mABC1 significantly attenuated mitochondrial membrane potential loss induced by hydrogen peroxide. These results suggest that mABC1 plays a significant role in cellular viability under basal conditions and protects cells against oxidant stress.

The mechanism by which mABC1 exerts its cardioprotective effects is not clear at this point. mABC1, based on its homology with other mitochondrial ABC proteins, may play a role in the mitochondrial iron homeostasis. Changes in the levels of this protein can, therefore, result in an increase in the cellular oxidative stress induced by metal ions. mABC1 may also exert cardioprotective effects by increasing the turnover of damaged mitochondrial membrane proteins induced by oxidant stress. Further insight into these possibilities will require functional characterization of mABC1 protein, which is currently the subject of our studies.

Acknowledgments

This study was supported by National Institutes of Health (NIH) grant R37 HL36957. E.M. holds the Michel Mirowski, MD, Professorship in Cardiology of The Johns Hopkins University. H.A. is supported by a grant from GlaxoSmithKline Research & Education Foundation for Cardiovascular Disease and NIH grant K08 HL79387.

References


Key Words: apoptosis □ mitochondria □ ATP-binding cassette proteins □ adenovirus □ RNA interference
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Circ Res. 2005;97:740-742; originally published online September 15, 2005;
doi: 10.1161/01.RES.0000186277.12336.11
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

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://circres.ahajournals.org/content/97/8/740

Data Supplement (unedited) at:
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Materials and Methods:

Cell Culture

NRCMs were prepared from 1- to 2-day-old Sprague-Dawley rats and cultured, as described. In brief, the hearts were removed, ventricles minced into small fragments, and cells dissociated by trypsinization. Cells were preplated for 0.5 h to enrich for myocytes in the non-adherent fraction. Myocytes were plated in medium consisting of Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 5% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 mg/ml), and 2 µg/ml vitamin B12. Cells were maintained at 37°C in the presence of 5% CO₂ in a humidified incubator.

RNA Interference Experiments

Search of the rat genome GenBank revealed a short fragment of the rat mABC1 cDNA. Using a web-based SiRNA design program (Qiagen, USA), two different sequences were designed. Nonsilencing SiRNA sequences from Qiagen (USA) and Dharmacon (USA) were used for control experiments. 3’-rhodamine labeled RNA duplexes were transfected into NRCM using the TransMessenger Kit (Qiagen, USA). About 70-80% of the cells displayed a characteristic punctuate fluorescence around the nuclei, when analyzed with confocal microscopy (UltraVIEW; PerkinElmer). The level of mABC inhibition was assessed using RT-PCR and Western blot analysis. Fluorescence-based kinetic real-time PCR was performed using a Perkin–Elmer Applied Biosystems Model 7900 sequence detection system, as described previously. Total RNA isolated from the cells and primers and probes were designed using the Primer Express software (applied Biosystems, USA). Ribosomal RNA was used as an internal control. Western blots were performed as described previously. mABC1 antibody was synthesized using a mABC1 peptide (spanning the C-terminal 35 aa), linked to keyhole limpet hemocyanin (Invitrogen, USA).

Adenoviral Vector Production

mABC1 cDNA was a kind gift from Dr. Victor Ling (BC Cancer Research Center, British Columbia, Canada). A recombinant adenoviral vector encoding GFP and
human mABC1 cDNA, separated by the polio virus internal ribosome entry site (IRES) element and under the transcriptional control of CMV promoter, was constructed. Briefly, mABC1 cDNA was cloned into BamHI and EcoRI sites of the adenovirus shuttle vector pAdCIG to make the vector designed pAdCIG-mABC1. A 25 cm² flask (T25; Sarstedt, Newton, NC) of CRE8 cells was cotransfected with 2.1 µg of ψ5 viral packaging plasmid and 2.1 µg of shuttle vector plasmid using Lipofectamine Plus (Invitrogen). Cells were incubated 5–9 days until cytopathic effects were observed. Cells and supernatant were collected and subjected to three cycles of freeze-thaw. Following centrifugation, 2 ml of the vector-containing supernatant was added to a 90% confluent T25 and returned to the incubator until cytopathic effects were observed. This procedure was repeated three to four times after which the vector was plaque purified and expanded. Large-scale vector preparations were purified on a cesium chloride gradient, as described previously. Virus titers were determined by plaque assays, and by absorbance at 260 nm on a DU640 spectrophotometer (Beckman Instruments, Fullerton, CA). The former was used for calculating multiplicity of infection (MOI), yielding concentrations of the order of 10⁹ plaque forming units (Pfu) per ml. The insertion of mABC1 cDNA was confirmed by sequencing the vector genome, and the vector was functionally validated by the expression of GFP and mABC1.

NRCMs were transduced on day 6 after isolation, and were imaged 2 days following transduction by a laser scanning confocal microscope (UltraVIEW; PerkinElmer), using a 403 water-immersion lens and X40 optical zoom. Western blot analysis was also performed to assess gene expression.

Flow Cytometry

Mitochondrial membrane potential (∆Ψₘ), which depolarizes rapidly at the onset of cell death, was used as a marker of cell injury. ∆Ψₘ was assessed using the fluorescence dye, tetramethylrhodamine ethyl ester (TMRE). NRCMs were transfected with SiRNA duplex and transduced with adenoviral vectors on day 6 and ∆Ψₘ assessed on day 8. No rhodamine labeled SiRNA was used for the flow cytometry studies since rhodamine may interfere with fluorescence measurement of TMRE. H₂O₂ was used to induce oxidant stress on target cells. This was applied for 60 minutes after which ∆Ψₘ
was measured. Glybenclamide and 5-HD at 10 and 500 µM concentrations were added about 30 minutes prior to the addition of hydrogen peroxide.

**Trypan Blue Studies**

For trypan blue studies, cells were treated with SiRNA and adenovirus for 48 hours. The media was removed, cells were detached dissolved in phosphate-buffered saline (PBS). Equal volume of trpan blue was then added, and the number of unstained (viable) and stained cells was counted using a hemocytometer. The percentage of viable cells was determined by calculating the percentage of unstained cells. Three different counts were performed in at least three independent experiments for each group.

**Data Analysis**

Data were presented as mean±SEM. Multiple comparisons among groups were carried out by one-way ANOVA test. A level of $P<0.05$ was considered as statistically significant.

**References**