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Discussion

Unresolved questions from the analysis of mice lacking MCU expression

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ABSTRACT

Entry of mitochondrial calcium is believed to play an essential role in regulating bioenergetics and initiating cell death pathways. We have recently described a mouse model lacking MCU expression. Surprisingly, these mice are viable and the cells and tissues from these animals do not exhibit any marked protection from cell death. Here, we discuss our findings as well as potential explanations for some of the more unexpected results.

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We recently described the initial analysis of a mouse model that lacked expression of the mitochondrial calcium uniporter (MCU) [1]. The mouse embryonic stem cells used to create these animals used a gene trap approach to integrate a targeting vector within the first intron of the MCU (CCDC109A) locus. One caveat of our strategy is that gene trap approaches can sometime lead to reduced but not completely absent expression of the targeted gene. Nonetheless, by Western blot analysis we observed no detectable protein expression of MCU in cells or tissues derived from our $MCU^{-/-}$ mice. In addition, when we isolated mitochondria from these animals, we saw a complete abrogation of any Ru360-inhibitable calcium uptake. Thus, it would appear that these animals are truly MCU deficient.

Quite honestly, we were quite surprised that we were able to obtain viable mice that had a total body knockout of MCU. Given the central role of mitochondrial calcium in metabolic regulation, we assumed that these animals would probably die sometime during embryogenesis. Nonetheless, as we reported, these mice are indeed viable if the knockouts are maintained on a mixed genetic background. In our case, the mice were on the outbred CD1 background. We have tried to generate a pure inbred strain of $MCU^{-/-}$ mice within a C57BL/6 background. In this case, the mice do in fact die around E11.5–E13.5. We are not certain of the cause of death, although our best guess is that it involves the myocardium. Even for the case of our CD1 mice, when we breed $MCU^{+/-}$ mice, while we would expect that 25% of the offspring would have the $MCU^{-/-}$ genotype, our experience is that the number is closer to 12–15%. Thus, we suspect that there is still a significant amount of embryonic lethality, even within a mixed genetic background.

We are not completely sure why MCU deletion is viable in the outbred CD1 strain, while it results in embryonic lethality in the inbred C57BL/6 strain. One potential explanation is that in CD1 mice, there is a compensatory gene product that allows for mitochondrial Ca^{2+} uptake, and that this gene product is absent in the C57BL/6 strain. While we cannot formally exclude this possibility, this explanation is inconsistent with our functional data. In our study, mitochondria and cells from the viable CD1 $MCU^{-/-}$ mice seemed incapable of any rapid mitochondrial calcium uptake. Thus, there did not appear to be any evidence for any functional compensation in these animals. More likely, this represents a relatively common phenomenon in which the phenotype of a knockout is less severe in the context of an outbred strain [2–4]. This can perhaps be best understood as an example of hybrid vigor, wherein an outbred strain is simply better able to tolerate a stress (e.g. MCU deletion) than an inbred strain.

We were able to derive primary cell lines from our CD1 and C57BL/6 $MCU^{-/-}$ embryos. These mouse embryonic fibroblasts (MEFs) proliferated at an indistinguishable rate when compared to wild type MEFs. In contrast, in our own experience with a variety of immortalized cell lines, we found that there was often a profound slowing of cell growth following transient knockdown of MCU. Again, these results are a bit puzzling to us and suggest that there are either different requirements for MCU between primary and established cell lines, or more likely, there are significant differences between acute versus chronic MCU knockdown. This gets back to the notion of compensation or alternative pathways for the entry of mitochondrial calcium. As noted above, we did not see any functional compensation for the rapid entry of calcium into the mitochondrial matrix. Nonetheless, when we measured

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mitochondrial calcium levels they were reduced but not absent in the mitochondria derived from our $MCU^{-/-}$ mice. Thus, it is possible that there is some slow mechanism for calcium to enter the matrix of the mitochondria that is not dependent on MCU. We're not sure what this mechanism is although there has been evidence recently for other potential MCU-independent mitochondrial calcium channels [5]. It also remains possible, that in the absence of MCU, certain mitochondrial calcium exchangers might work in the opposite direction, bringing calcium into the matrix rather than exporting calcium.

Another puzzling finding was the lack of protection from cell death in the hearts lacking MCU [1]. A number of studies have suggested that calcium uptake into the mitochondria by MCU leads to activation of the mitochondrial permeability transition pore (mPTP). The opening of the mPTP has been suggested to play a major role in cell death in cardiac ischemia and reperfusion [6,7]. Studies have shown that loss or inhibition of cyclophilin D (CypD), an activator of the mPTP, reduces ischemia–reperfusion (I/R) injury [8,9]. In isolated cardiac mitochondria from mice lacking MCU, we found no calcium uptake into the mitochondria and, as expected, addition of large amounts of extramitochondrial calcium did not lead to mPTP activation [1]. Surprisingly, however, following global I/R in a Langendorff perfused heart model, infarct size was indistinguishable between WT hearts and hearts from $MCU^{-/-}$ mice. To further complicate the picture, cyclosporine A, an inhibitor of the mPTP, reduced infarct size in WT hearts, but not in $MCU^{-/-}$hearts.

What are some possible explanations for these data? Perhaps it is an elevation in cytosolic/sarcoplasmic reticulum calcium that is primarily responsible for initiating cell death. It has been suggested that sarcoplasmic reticulum calcium cycling and hypercontracture lead to depletion of ATP and plasma membrane rupture [10]. During ischemia, $MCU^{-/-}$ hearts might have a higher cytosolic calcium than WT hearts, since the knockout mice would be incapable of using their mitochondria as a storage depot for calcium. However, during early reperfusion and activation of the mPTP, the calcium accumulated in WT mitochondria would be released and the level of calcium in the cytosol (and thus the trigger for injury) would be the same in the WT and $MCU^{-/-}$ hearts. Inhibition of mPTP would block the release of mitochondrial calcium from the WT hearts, which would result in a lower cytosolic calcium and reduced injury. This scenario might explain why the degree of injury appears similar between WT and $MCU^{-/-}$ hearts, while also explaining why only the WT hearts appear protected by cyclosporine A treatment.

An alternative explanation is that certain mPTP-independent modes of death are upregulated in the $MCU^{-/-}$ hearts. For instance, a recent study using a model of kidney ischemia showed that there are at least two independent modes of necrosis [11]. This includes a pathway involving the protein kinases RIP1 and RIP3 (often called necroptosis), as well as a pathway involving mPTP. In this recent study, mice deficient for RIPK3 and mice lacking CypD both had improved survival compared to WT mice, although mice lacking RIPK3 had better survival than the CypD-KO mice. However, a RIPK3-CypD double knockout showed synergistic protection, suggesting that both of these pathways contribute to death in renal I/R. It is therefore conceivable that WT hearts activate both of these separate pathways, while $MCU^{-/-}$ hearts are only capable of activating the RIP1/RIP3 pathway. This scenario might provide another explanation for the lack of effect of cyclosporine A in the knockout animals. Finally, the $MCU^{-/-}$mice should also be useful in potentially addressing the controversy regarding the relative role of matrix calcium versus reactive oxygen species (ROS) in activating mPTP during ischemia and reperfusion [12,13]. As such, it will be of considerable interest to determine whether mPTP opening actually occurs in the $MCU^{-/-}$ hearts during I/R.

Thus, although our initial characterization clearly confirmed the requirement of MCU for rapid calcium uptake into the mitochondria, as well as the role of MCU for calcium activation of mPTP in isolated mitochondria, this study clearly raised a number of interesting and unresolved questions for future studies.

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