Mitochondrial calcium uniporter in Drosophila transfers calcium between the endoplasmic reticulum and mitochondria in oxidative stress-induced cell death

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Mitochondrial calcium plays critical roles in diverse cellular processes ranging from energy metabolism to cell death. Previous studies have demonstrated that mitochondrial calcium uptake is mainly mediated by the mitochondrial calcium uniporter (MCU) complex. However, the roles of the MCU complex in calcium transport, signaling, and dysregulation by oxidative stress still remain unclear. Here, we confirmed that Drosophila MCU contains evolutionarily conserved structures and requires essential MCU regulator (EMRE) for its calcium channel activities. We generated Drosophila MCU loss-of-function mutants, which lacked mitochondrial calcium uptake in response to caffeine stimulation. Basal metabolic activities were not significantly affected in these MCU mutants, as observed in examinations of body weight, food intake, body sugar level, and starvation-induced autophagy. However, oxidative stress-induced increases in mitochondrial calcium, mitochondrial membrane potential depolarization, and cell death were prevented in these mutants. We also found that inositol 1,4,5-trisphosphate receptor genetically interacts with Drosophila MCU and effectively modulates mitochondrial calcium uptake upon oxidative stress. Taken together, these results support the idea that Drosophila MCU is responsible for endoplasmic reticulum-to-mitochondrial calcium transfer and for cell death due to mitochondrial dysfunction under oxidative stress.

Mitochondrial Ca\(^{2+}\) is a key regulator for cellular metabolic functions by activating Krebs cycle dehydrogenases, metabolite shuttle systems, and ATP synthase. However, inappropriately high Ca\(^{2+}\) levels in the mitochondrial matrix threaten cell survival by increasing reactive oxygen species (ROS)\(^{6}\) production and triggering mitochondrial permeability transition (mPT). The main route for Ca\(^{2+}\) uptake into mitochondria is through mitochondrial calcium uniporter (MCU), a Ca\(^{2+}\)-selective ion channel located at the inner mitochondrial membrane (1), which was originally identified as CCDC109A (2, 3). Recent studies have demonstrated that MCU has a homopentameric structure in which the second transmembrane domain forms a hydrophilic pore across the membrane (4) and the N terminus domain modulates MCU activity by protein–protein interaction and binding divalent cations (5, 6). To counteract the Ca\(^{2+}\) influx via MCU, mitochondrial sodium–calcium exchanger (NCLX) provides mitochondrial Ca\(^{2+}\) efflux routes by exchanging Ca\(^{2+}\) for Na\(^{+}\) ions (7). Leucine zipper EF-hand-containing transmembrane protein 1 (LETM1) may also be involved in mitochondrial Ca\(^{2+}\) influx or efflux, which is still in a controversy (8–11).

Previous studies have identified multiple regulators of MCU activity, including mitochondrial calcium uptake 1 and 2 (MICU1/2), mitochondrial calcium uniporter regulator 1 (MCUR1), and essential MCU regulator (EMRE). EMRE is a 10-kilodalton mitochondrial inner membrane protein with a single transmembrane domain, which is an essential component that bridges MICU1/2 with MCU (12). The transmembrane helix of EMRE interacts with MCU, and the C terminus of EMRE binds to MICU1 (13). In addition, silencing of EMRE completely abolishes the channel activity of MCU (14). MICU1/2 are Ca\(^{2+}\)-binding EF-hand-containing proteins residing in the mitochondrial intermembrane space. They can modulate MCU activity, depending on cytoplasmic Ca\(^{2+}\) concentration.

6 The abbreviations used are: ROS, reactive oxygen species; MCU, mitochondrial calcium uniporter; IP\(_3\), inositol 1,4,5-trisphosphate receptor; mPT, mitochondrial permeability transition; MAM, mitochondria-associated ER membrane; [Ca\(^{2+}\)]\(_{\text{mito}}\), mitochondrial matrix Ca\(^{2+}\) concentration; MTRP, mitochondria-targeted ratio-pericam; tBuOOH, tert-butyl hydroperoxide; \(\Psi_{\text{mito}}\), mitochondrial membrane potential; MCU\(^{52}\), a null mutant of MCU; RPmit4.1, mitochondria-targeted ratio-pericam plasmid; ER, endoplasmic reticulum; DHE, dihydroethidium; RyR, ryanodine receptor; PFA, paraformaldehyde; Ex, excitation; Em, emission.
centration ([Ca^{2+}]_i) (15–17). MCU1 is an inner mitochondria membrane integral protein binding to MCU and regulates MCU-dependent mitochondrial Ca^{2+} uptake (18) and Ca^{2+} threshold for mPT (19).

Over the last few years, studies on the MCU complex have proposed diverse roles of MCU at the cellular and organism levels, using its genetic ablation and/or pharmacologic inhibition models. Although a loss-of-function mutation of MCU in mice exhibited negligible influence on metabolism and cell death (20), further studies revealed that MCU plays an essential role in heart rate acceleration during fight-or-flight response (21), ROS-mediated wound repair (22), and skeletal muscle trophy (23). In pancreatic β-cells, MCU silencing decreases mitochondrial ATP synthesis and impairs the metabolism-secretion coupling (11, 24). In neuronal cells, suppression of MCU relieves ischemia and reperfusion injuries as well as Ca^{2+} excitotoxicity (25, 26). In cardiac cells, however, a loss-of-function mutation of MCU did not protect from ischemic injury due to [Ca^{2+}] overload despite preserved mitochondrial membrane potential (ΔΨ_m) and reduced ROS formation (27).

The main source of mitochondrial Ca^{2+} is the endoplasmic reticulum (ER), which has a higher Ca^{2+} level (>400 μM) required for protein folding and Ca^{2+} signaling. Ca^{2+} release from the ER is taken up by mitochondria not only to avoid [Ca^{2+}] accumulation but also to stimulate mitochondrial energy metabolism (28). This ER–mitochondrial interaction is mediated by physical contacts between the two organelles via mitochondria-associated ER membrane (MAM) (29, 30). MAM is composed of several proteins, including inositol 1,4,5-trisphosphate receptor (IP5R), glucose-regulated protein 75 (grp75), porin, and MCU (31). The MAM formation can be expanded in pathologic conditions, such as obesity and diabetes, in which increased ER–mitochondrial Ca^{2+} connection exacerbates ER Ca^{2+} depletion and mitochondrial Ca^{2+} overload (32).

Oxidative stress disrupts cellular Ca^{2+} homeostasis and consequently induces cytotoxicity. Oxidative stress activates IP5R, stimulates ER Ca^{2+} release, intensifies ER stress, and leads to apoptosis (33, 34). More importantly, oxidative stress plays significant roles in the pathogenesis of various chronic diseases, including neurodegeneration (35). However, the pathophysiological role of Ca^{2+} dysregulation by oxidative stress still remains elusive.

In this study, we generated Drosophila MCU loss-of-function mutants for the first time and characterized their phenotypes on metabolism, Ca^{2+} handling, and cell death. We demonstrated that attenuated Ca^{2+} transport from the ER to mitochondria in MCU mutants prevents ROS-induced mitochondrial dysfunction and cell death. Because the Drosophila system provides powerful tools for genetic studies, our loss-of-function mutants and transgenic flies for MCU and other components of MCU complex would provide crucial information for understanding the regulatory mechanism of mitochondrial Ca^{2+} homeostasis.

**Results**

**Drosophila MCU null mutant is viable**

According to a recent report, CG18769 is homologous to mammalian MCU (36). Human and mouse MCU protein sequences are similar to the protein sequence encoded by CG18769 (supplemental Fig. S1A). CG18769-encoded protein localizes to mitochondria, and silencing CG18769 decreases mitochondrial Ca^{2+} entry (36, 37). To assess the in vivo role of MCU, Drosophila MCU loss-of-function mutants were generated by P-element imprecise excision in the 5′-untranslated region of CG18769 using the P(GSV6)GS11565 fly line (Fig. 1A). From the 200 excision alleles obtained, we found MCU-F2 mutant, which lacked 1,476 bp (3R14578001–14580477) that encoded the transcription start site and the first exon of MCU (Fig. 1, A and B). In the mutant, MCU mRNA and protein were not detected by quantitative RT-PCR (Fig. 1C) and immunoblotting (Fig. 1D). Wild-type MCU protein was expressed weakly in embryo stage but strongly in larva, pupa, and adult stages (Fig. 1E). MCU-F2 mutant flies were viable and showed a similar survival rate compared with wild-type ones (Fig. 1E).

**MCU-F2 mutant does not show significant metabolic phenotypes**

To find the exclusive role of MCU in Drosophila physiology, we investigated metabolic phenotypes of MCU-F2 mutant. First, the body weight of MCU-F2 mutants was not different from that of wild-type flies in both sexes (Fig. 1F). The amount of food intake of MCU-F2 mutant was also similar to that of the wild-type fly (Fig. 1G). The concentration of circulating sugars in the hemolymph of MCU-F2 mutants was not significantly different from that in wild-type flies (Fig. 1H). We also did not observe any detectable changes in starvation-induced autophagy (Fig. 1I). Collectively, these results showed that loss of MCU does not alter basal metabolism in Drosophila.

**The Ca^{2+} channel activity of MCU is conserved in Drosophila**

To assess mitochondrial Ca^{2+} uptake in a physiological context, we measured mitochondrial matrix Ca^{2+} concentration ([Ca^{2+}]_{mito}) in a larval muscle expressing mitochondria-targeted ratio-pericam (MTRP), a genetically encoded Ca^{2+} indicator targeted to the mitochondrial matrix (38). MTRP was specifically expressed in muscle tissues by Mef-Gal4 and UAS-MTRP (Fig. 2A). The localization of expressed MTRP to mitochondria was confirmed with two mitochondria markers, streptavidin (Fig. 2B) and ATP5a (supplemental Fig. S2A).

To confirm that Drosophila MCU is a functional mitochondrial Ca^{2+} uptake route, we compared [Ca^{2+}]_{mito} increase in muscle tissues of control, MCU-F2 mutant, and MCU-F2 mutant expressing exogenous Drosophila wild-type MCU (Fig. 2C). Caffeine was used to stimulate Ca^{2+} release from the ER, inducing elevation of cytosolic (supplemental Fig. S3A) and mitochondrial Ca^{2+} levels (Fig. 2D). In control larvae, [Ca^{2+}]_{mito} was increased sharply after caffeine stimulation and slowly returned to the basal level (Fig. 2D). However, although caffeine-induced [Ca^{2+}]_{mito} changes were not significantly different between control and MCU-F2 mutant larvae (supplemental Fig. S3A), MCU-F2 mutant failed to elicit any [Ca^{2+}]_{mito} change upon caffeine stimulation (Fig. 2, D and E). Furthermore, when exogenous Drosophila MCU was overexpressed in MCU-F2 mutant, the absence of caffeine-induced [Ca^{2+}]_{mito} response in the mutant was completely rescued (Fig. 2, D and E). These results consistently demonstrate the indispensable role of Drosophila MCU in mitochondrial Ca^{2+} uptake.
To test whether *Drosophila* MCU is functionally equivalent to the mammalian MCU, we expressed human MCU in the muscle of *MCU52* mutants (Fig. 2F). Similar to the results with *Drosophila* MCU, overexpression of human MCU in *MCU52* mutant larvae resulted in full recovery of [Ca\(^{2+}\)]\(_{\text{mito}}\) response upon caffeine stimulation (Fig. 2, G and H). These results demonstrate that *Drosophila* MCU (encoded by CG18769) is a genuine orthologue of human MCU.

MCU has a mitochondrial targeting sequence at its N terminus, two coiled-coil domains, two transmembrane domains, and the DIME motif. Previous studies showed that substitution of two acidic amino acids within the DIME motif resulted in a dominant-negative effect on the uniporter activity of mammalian MCU (39, 40). To test whether the DIME motif in *Drosophila* MCU is also critical for its Ca\(^{2+}\) channel activity, we generated a mutant of MCU in the DIME motif (MCUNIMQ) (Fig. 2I).

Transgenic expression of wild-type MCU in the muscle of *MCU52* mutant resulted in full recovery of mitochondrial Ca\(^{2+}\) uptake, as shown (Fig. 2, D and E). In contrast, expression of MCUNIMQ failed to rescue the defective [Ca\(^{2+}\)]\(_{\text{mito}}\) response of *MCU52* mutant (Fig. 2, J and K). Therefore, the DIME motif is essential for its Ca\(^{2+}\) transport activity in *Drosophila* MCU.

**EMRE is required for MCU activity in Drosophila**

Eye-specific expression of MCU using Gmr-Gal4 led to glazed phenotypes in the eye with partial loss of pigmentation.
and irregular ommatidial array (Fig. 3A). The activity of MCU appeared critical for these effects because knockdown of MCU completely suppressed the eye phenotype (Fig. 3A). Consistently, a higher MCU expression with two copies of UAS-MCU transgene resulted in more severe phenotypes (Fig. 3A). Overexpression of EMRE, an essential component of the MCU complex, alone did not elicit defective phenotypes (Fig. 3A). Unexpectedly, co-expression of MCU and EMRE led to lethality. However, when those flies were reared at 23 °C instead of 25 °C, few managed to survive with defective eyes marked with black mass tissues, indicating that MCU and EMRE have a strong genetic interaction in vivo (Fig. 3A).

To confirm the role of EMRE in Drosophila MCU activity, we used the UAS-EMRE RNAi fly line to knock down EMRE expression. Silencing of EMRE led to impairment of mitochondrial Ca²⁺ uptake (Fig. 3, B and C), closely resembling the result obtained from MCU² mutant (Fig. 2, D and E), implying that EMRE is required for mitochondrial Ca²⁺ uptake. Furthermore, expression of MCU in EMRE knockdown fly failed to increase the caffeine-induced [Ca²⁺]mito response (Fig. 3, B and D).
address whether mitochondrial Ca\(^{2+}\) uptake via MCU has any pathogenic role in inducing cell death under oxidative stress conditions, we first investigated the survival rates of wild-type fly and MCU\(^{52}\) mutant fed on hydrogen peroxide (H\(_2\)O\(_2\))-containing food. Dihydroethidium (DHE) staining was used to detect the level of ROS in the thorax of flies. Elevated level of ROS was detected in the flies fed on 1% H\(_2\)O\(_2\)-containing food for the past 72 h, indicating that feeding H\(_2\)O\(_2\) induced oxidative stress in the fly (supplemental Fig. S4A). Interestingly, MCU\(^{52}\) mutant flies survived significantly longer than wild-type flies when fed on 1% H\(_2\)O\(_2\)-containing food, whereas normal food caused the mutants to survive slightly less than wild-type flies. These results suggest that MCU\(^{52}\) mutant flies are more resistant to oxidative stress than wild-type flies (Fig. 4A).

To demonstrate whether ROS-induced apoptosis was attenuated by loss-of-function mutations of MCU, we performed a TUNEL assay under oxidative stress conditions. Wild-type flies showed strong TUNEL signals by 2% H\(_2\)O\(_2\) treatment for 3 h, which was markedly reduced in MCU\(^{52}\) mutants (Fig. 4B). Additionally, we checked hid\(^{5F}\)-WT-GFP reporter expression and cleaved caspase-3 as a marker of early and late phases of apoptosis, respectively (41). Wild-type flies showed up-regulated GFP reporter expression from hid\(^{5F}\)-WT enhancer and increased cleaved caspase-3 staining, but MCU\(^{52}\) mutants displayed markedly reduced signals for both apoptotic markers (Fig. 4 (C and D) and supplemental Fig. S4 (B and C)). Consistently, we also confirmed the reduction of H\(_2\)O\(_2\)-induced apoptotic cell death by knockdown of MCU in Drosophila S2 cells (Fig. 4, E and F). Taken together, these results indicate that loss of MCU endows resistance to oxidative stress.

**MCU-dependent Ca\(^{2+}\) uptake contributes to mitochondrial dysfunction by oxidative stress**

To more clearly demonstrate whether oxidative stress can increase [Ca\(^{2+}\)]\(_{\text{mito}}\) in Drosophila, we applied tert-butyldihydroperoxide (t-BuOOH) to larval muscle and applied H\(_2\)O\(_2\) to S2 cells. First, in control larval muscle in vivo, [Ca\(^{2+}\)]\(_{\text{mito}}\) was increased after treatment of t-BuOOH from 1 to 100 mM in a dose-dependent manner, suggesting that oxidative stress can increase [Ca\(^{2+}\)]\(_{\text{mito}}\) (supplemental Fig. S5A). Compared with in vitro experiments, a higher dose of t-BuOOH is required to induce rapid oxidative stress on inner muscle tissue under the cuticle. Treatment of t-BuOOH (35 mM) on control flies

**Drosophila MCU in ROS-induced cell death**

![Figure 2. MCU regulates rapid mitochondrial calcium uptake.](image)

A, schematic representation of the muscle region of a dissected Drosophila larva to measure mitochondrial Ca\(^{2+}\) level. MTRP was expressed in larval muscle in Mef\(\rightarrow\)MTRP. Fluorescence intensity was recorded from a well-focused region of body wall muscles 6, 7, 15, 16, and 17 of abdomen segments A2–A6. The central nervous system is marked with solid black. For simplicity, not all muscles and segments are shown. Fluorescence intensity from MTRP expressed in muscle was measured upon stimulation with 10 mM caffeine. B, mitochondrial localization of MTRP. Green, subcellular localization of MTRP in larval muscle. Hoescht (blue) and streptavidin (red) were used to mark DNA and mitochondria, respectively. The genotype is Mef-Gal4/UAS-MTRP. Scale bar, 10 μm. C, immunoblot analyses of endogenous MCU (red) and exogenously expressed FLAG-tagged MCU (Flag). D–E, averaged traces (D) and quantitative analysis (E) of [Ca\(^{2+}\)]\(_{\text{mito}}\) signals after treatment of 10 mM caffeine from wild-type, MCU\(^{52}\), and MCU\(^{52}\) mutant with exogenous Drosophila wild-type MCU expression (n = 5–7). Genotypes are as follows: Mef-Gal4/UAS-MTRP (Mef\(\rightarrow\)), Mef-Gal4/UAS-MTRP/MCU\(^{52}\) (MUCU\(^{52}\)–Mef\(\rightarrow\)), and UAS-MCU-FLAG/Mef-Gal4/UAS-MTRP/MCU\(^{52}\) (MUCU\(^{52}\)–Mef\(\rightarrow\)). F, immunoblot analyses of endogenous MCU (red) and exogenously expressed FLAG-tagged MCU (Flag). G and H, averaged traces (G) and quantitative analysis (H) of [Ca\(^{2+}\)]\(_{\text{mito}}\) signals after treatment with 10 μM caffeine from wild-type, MCU\(^{52}\), and MCU\(^{52}\) mutant with human MCU expression (n = 3–6). Genotypes are as follows: Mef-Gal4/UAS-MTRP (Mef\(\rightarrow\)), Mef-Gal4/MCU\(^{52}\)-UAS-MTRP/MCU\(^{52}\) (MUCU\(^{52}\)-Mef\(\rightarrow\)), and UAS-MCU-FLAG/Mef-Gal4/UAS-MTRP/MCU\(^{52}\) (MUCU\(^{52}\)-Mef\(\rightarrow\)). I, a schematic representation of Drosophila wild-type MCU and MCU\(^{52}\)-Mef\(\rightarrow\) knockout (KO) constructs, MTS, mitochondrial targeting sequence; CC, coiled-coil domain; TM, transmembrane domain; DIME, DIME motif. Immunoblot analyses of endogenous MCU (red) and exogenously expressed FLAG-tagged MCU (Flag) are shown in the bottom panels. J and K, averaged traces (J) and quantitative analysis (K) of [Ca\(^{2+}\)]\(_{\text{mito}}\) signals after 10 min caffeine treatment from wild type, MCU\(^{52}\), MCU\(^{52}\) mutant with Drosophila wild-type MCU overexpression, and MCU\(^{52}\) mutant with Drosophila wild-type MCU overexpression (n = 4–10). Genotypes are as follows: Mef-Gal4/UAS-MTRP (Mef\(\rightarrow\)), Mef-Gal4/MCU\(^{52}\)-UAS-MTRP/MCU\(^{52}\) (MUCU\(^{52}\)-Mef\(\rightarrow\)), UAS-MCU-FLAG/Mef-Gal4/UAS-MTRP/MCU\(^{52}\) (MUCU\(^{52}\)-Mef\(\rightarrow\)), and UAS-MCU\(^{52}\)-FLAG/Mef-Gal4/UAS-MTRP/MCU\(^{52}\) (MUCU\(^{52}\)-Mef\(\rightarrow\)). J. Biol. Chem. (2017) 292(35) 14473–14485

**Figure 2.** MCU regulates rapid mitochondrial calcium uptake. A, schematic representation of the muscle region of a dissected Drosophila larva to measure mitochondrial Ca\(^{2+}\) level. MTRP was expressed in larval muscle in Mef\(\rightarrow\)MTRP. Fluorescence intensity was recorded from a well-focused region of body wall muscles 6, 7, 15, 16, and 17 of abdomen segments A2–A6. The central nervous system is marked with solid black. For simplicity, not all muscles and segments are shown. Fluorescence intensity from MTRP expressed in muscle was measured upon stimulation with 10 mM caffeine. B, mitochondrial localization of MTRP. Green, subcellular localization of MTRP in larval muscle. Hoescht (blue) and streptavidin (red) were used to mark DNA and mitochondria, respectively. The genotype is Mef-Gal4/UAS-MTRP. Scale bar, 10 μm. C, immunoblot analyses of endogenous MCU (red) and exogenously expressed FLAG-tagged MCU (Flag). D–E, averaged traces (D) and quantitative analysis (E) of [Ca\(^{2+}\)]\(_{\text{mito}}\) signals after treatment of 10 mM caffeine from wild-type, MCU\(^{52}\), and MCU\(^{52}\) mutant with exogenous Drosophila wild-type MCU expression (n = 5–7). Genotypes are as follows: Mef-Gal4/UAS-MTRP (Mef\(\rightarrow\)), Mef-Gal4/UAS-MTRP/MCU\(^{52}\) (MUCU\(^{52}\)-Mef\(\rightarrow\)), UAS-MCU-FLAG/Mef-Gal4/UAS-MTRP/MCU\(^{52}\) (MUCU\(^{52}\)-Mef\(\rightarrow\)), and Mef-Gal4/UAS-MTRP/MCU\(^{52}\) (MUCU\(^{52}\)-Mef\(\rightarrow\)). F, immunoblot analyses of endogenous MCU (red) and exogenously expressed FLAG-tagged MCU (Flag). G and H, averaged traces (G) and quantitative analysis (H) of [Ca\(^{2+}\)]\(_{\text{mito}}\) signals after treatment with 10 μM caffeine from wild-type, MCU\(^{52}\), and MCU\(^{52}\) mutant with human MCU expression (n = 3–6). Genotypes are as follows: Mef-Gal4/UAS-MTRP (Mef\(\rightarrow\)), Mef-Gal4/MCU\(^{52}\)-UAS-MTRP/MCU\(^{52}\) (MUCU\(^{52}\)-Mef\(\rightarrow\)), and UAS-MCU-FLAG/Mef-Gal4/UAS-MTRP/MCU\(^{52}\) (MUCU\(^{52}\)-Mef\(\rightarrow\)). I, a schematic representation of Drosophila wild-type MCU and MCU\(^{52}\)-Mef\(\rightarrow\) knockout (KO) constructs, MTS, mitochondrial targeting sequence; CC, coiled-coil domain; TM, transmembrane domain; DIME, DIME motif. Immunoblot analyses of endogenous MCU (red) and exogenously expressed FLAG-tagged MCU (Flag) are shown in the bottom panels. J and K, averaged traces (J) and quantitative analysis (K) of [Ca\(^{2+}\)]\(_{\text{mito}}\) signals after 10 min caffeine treatment from wild type, MCU\(^{52}\), MCU\(^{52}\) mutant with Drosophila wild-type MCU overexpression, and MCU\(^{52}\) mutant with Drosophila wild-type MCU overexpression (n = 4–10). Genotypes are as follows: Mef-Gal4/UAS-MTRP (Mef\(\rightarrow\)), Mef-Gal4/MCU\(^{52}\)-UAS-MTRP/MCU\(^{52}\) (MUCU\(^{52}\)-Mef\(\rightarrow\)), UAS-MCU-FLAG/Mef-Gal4/UAS-MTRP/MCU\(^{52}\) (MUCU\(^{52}\)-Mef\(\rightarrow\)), and UAS-MCU\(^{52}\)-FLAG/Mef-Gal4/UAS-MTRP/MCU\(^{52}\) (MUCU\(^{52}\)-Mef\(\rightarrow\)). J. Biol. Chem. (2017) 292(35) 14473–14485.
Mef/H11022 markedly increased \([\text{Ca}^{2+}]_{\text{mito}}\) (Fig. 5A). As expected, the \([\text{Ca}^{2+}]_{\text{mito}}\) response induced by \(t\)-BuOOH was abolished in \(MCU^{52}\) mutant (MCL1\(^{52},\text{Mef}^{>}\)), demonstrating the critical role of MCU in oxidative stress-induced \([\text{Ca}^{2+}]_{\text{mito}}\) increase (Fig. 5, A and B). Transgenic expression of \(Drosophila\ MCU\) (MCU\(^{52},\text{Mef}^{>}\)) rescued the abolished \([\text{Ca}^{2+}]_{\text{mito}}\) response in \(MCU^{52}\) mutant (MCU\(^{52},\text{Mef}^{>}\)) (Fig. 5, A and B). By contrast, the \([\text{Ca}^{2+}]_{\text{mito}}\) changes induced by \(t\)-BuOOH were not significantly different between control (Mef\(^{>}\)) and MCL1\(^{52}\) mutant (supplemental Fig. S5B). We also detected \([\text{Ca}^{2+}]_{\text{mito}}\) in ROS-induced cell death (Drosophila MCU in ROS-induced cell death).
rise in S2 cells upon H$_2$O$_2$ stimulation (Fig. 5C). Consistent with the in vivo muscle data, MCU dsRNA-treated S2 cells showed reduced H$_2$O$_2$-induced mitochondrial Ca$^{2+}$ uptake (−63%) in comparison with control (Luciferase dsRNA-treated) (Fig. 5C, and D). To estimate the functional deterioration of mitochondria as a result of [Ca$^{2+}$]$_{mito}$ overload, we monitored mitochondrial membrane potential ($\Psi_{mito}$) by using potential-sensitive JC-1 dye. Oxidative stress by H$_2$O$_2$ treatment (3 mM) elicited depolarization of $\Psi_{mito}$ in S2 cells (Fig. 5E). Intriguingly, knockdown of MCU strongly prevented H$_2$O$_2$-induced $\Delta$Ψ$_{mito}$ collapse in S2 cells (Fig. 5, E and F). Based on these results, we suggest that mitochondrial dysfunction and apoptotic cell death induced by oxidative stress are related to MCU-mediated [Ca$^{2+}$]$_{mito}$ overload.

**IP$_3$R and MCU participate in oxidative stress-induced ER–mitochondria Ca$^{2+}$ transfer**

Oxidative stress is reported to activate IP$_3$R, a Ca$^{2+}$ channel in the ER, resulting in ER Ca$^{2+}$ release (33, 34). Released Ca$^{2+}$ from the ER provides high Ca$^{2+}$ level in microdomains of ER-mitochondrial junction called MAM. IP$_3$R is a component of tethering structure between the ER and mitochondria (31). Therefore, we investigated the involvement of IP$_3$R in ER-mitochondria Ca$^{2+}$ transfer under oxidative stress.

Muscle-specific expression of exogenous MCU using Mef-Gal4 was lethal in pupa stage of the transgenic fly (Fig. 6A). However, this lethality was blocked by knockdown of IP$_3$R, suggesting that MCU and IP$_3$R have a strong genetic interaction in vivo (Fig. 6A). To confirm the role of IP$_3$R in mitochondrial Ca$^{2+}$ uptake, we compared [Ca$^{2+}$]$_{mito}$ increase induced by oxidative stress between control (Mef>) and muscle-specific IP$_3$R knockdown flies (Mef>–IP$_3$R RNAi). The IP$_3$R knockdown flies showed significantly reduced mitochondrial Ca$^{2+}$ uptake (−41.2%) upon t-BuOOH stimulation in comparison with control (Mef>) (Fig. 6, B and C). Additionally, when we silenced IP$_3$R in MCU transgenic flies (Mef>–MCU, IP$_3$R RNAi), the [Ca$^{2+}$]$_{mito}$ induced by t-BuOOH was reduced by 34.7% when compared with that of Mef>–MCU flies (Fig. 6, B and C). This was consistent with our previous results, where knockdown of IP$_3$R blocked the lethality induced by MCU overexpression (Fig. 6A). In S2 cells, H$_2$O$_2$-induced [Ca$^{2+}$]$_{mito}$ increase was also strongly inhibited (−64.0%) by transfection of IP$_3$R dsRNA (Fig. 6, D and E). These results strongly indicate that both IP$_3$R and MCU are critical for the Ca$^{2+}$ transfer from the ER to mitochondria.

Finally, we examined the involvement of IP$_3$R and MCU in oxidative stress-induced toxicity using transgenic flies that overexpress or silence superoxide dismutase 1 (Sod1). First, the degenerated eye phenotype in the flies expressing MCU using Gmr-Gal4 driver, as shown in Fig. 3A, was restored by Sod1 expression but was exacerbated by Sod1 knockdown (Fig. 6F). As stated above, the lethality of MCU overexpression using Mef-Gal4 was prevented by the knockdown of IP$_3$R (Fig. 6A). However, interestingly, simultaneous knockdown of both Sod1 and IP$_3$R failed to rescue the lethal phenotype of MCU overexpression using Mef-Gal4 (Fig. 6G). These results suggest that endogenous ROS plays an imperative role in the IP$_3$R- and MCU-mediated mitochondrial Ca$^{2+}$ overload and toxicity.

**Discussion**

In this study, we established a *Drosophila* model system to understand functional roles of MCU in mitochondrial Ca$^{2+}$ homeostasis in vivo. By generating and characterizing a null mutant of MCU (MCU$^{122}$), we investigated the physiological roles of MCU in *Drosophila*. MCU$^{122}$ mutant did not show significant changes in body weight, metabolism, and autophagic flux compared with wild-type flies. However, caffeine-induced [Ca$^{2+}$]$_{mito}$ increase was abolished in MCU$^{122}$ mutant larval
muscle, which was completely rescued by transgenic expression of either human or Drosophila MCU. In addition, the DIME amino acid motif, which forms the ion selectivity filter of the MCU channel, was indispensable for the activity of Drosophila MCU, suggesting that MCU is evolutionarily highly conserved. In MCU52 mutant larval muscle and MCU-silenced S2 cells, exogenous ROS-induced [Ca\textsuperscript{2+}]\textsubscript{mito} increase, ΔΨ\textsubscript{mito} dissipation, and cell death were prevented. Suppression of IP\textsubscript{3}R, which is a Ca\textsuperscript{2+} release channel in the ER, also protects from ROS-mediated mitochondrial Ca\textsuperscript{2+} overload and cytotoxicity. These results demonstrate the critical role of Drosophila MCU in Ca\textsuperscript{2+} transfer from the ER to mitochondria, contributing to oxidative stress-induced mitochondrial dysfunction and apoptotic cell death.

Mitochondrial Ca\textsuperscript{2+} is a crucial regulator in energy metabolism, Ca\textsuperscript{2+} sequestration, and cell death. However, our Drosophila MCU loss-of-function mutant did not exhibit significant metabolic phenotypes. These unexpected results can be explained by undefined compensatory mechanisms for mitochondrial Ca\textsuperscript{2+} uptake, such as MCU-independent slow Ca\textsuperscript{2+}
channels or exchangers. It is also conceivable that rapid changes in \( [\text{Ca}^{2+}]_{\text{mito}} \) via MCU may not be required for maintaining basal metabolism and daily activities in *Drosophila* except for exogenous stress or emergency crisis. Mouse MCU knock-out models with outbred CD1 background also did not show any significant phenotypes except for reduced abilities to perform strenuous work (20). However, strangely, MCU deletion within a C57BL/6 background resulted in embryonic lethality (42). This discrepancy suggests that a compensatory mechanism that is absent in C57BL/6 background exist in CD1 mice and allows MCU-independent mitochondrial Ca\(^{2+}\) uptake during animal development. By contrast, up-regulation of MCU augments mitochondrial Ca\(^{2+}\) uptake, leading to aberrantly high \( [\text{Ca}^{2+}]_{\text{mito}} \) level, and this stress accelerates further superoxide production through activation of the electron transport chain or other mechanisms. Together with increased \( [\text{Ca}^{2+}]_{\text{mito}} \) and oxidative stress in mitochondrial matrix, this facilitates opening of mPT and cytochrome c release, leading to apoptotic cell death. In our study, ectopic overexpression of MCU in *Drosophila* muscle led to pupal lethality (Fig. 6A). Additionally, MCU overexpression in *Drosophila* eye using Gmr-Gal4 driver resulted in severely destroyed ommatidial array (Fig. 3A). These results imply that overexpression of *Drosophila* MCU accelerates mitochondrial Ca\(^{2+}\) overload that is detrimental to various tissues and ultimately impairs a viability of organism.

EMRE is an essential auxiliary subunit containing a mitochondrial targeting sequence at its N terminus, a single transmembrane domain located at inner mitochondrial membrane, and an aspartate-rich C terminus (12). The MCU complex requires EMRE for its reconstitution in mammalian cells, but not in *Dictostelium discoideum* (43). In mammalian cells, suppression of EMRE abrogates MCU-mediated Ca\(^{2+}\) currents demonstrated by mitoplast patch clamp experiment (12, 14). However, functional consequences of EMRE overexpression have not been studied yet (44). In our study, EMRE showed a strong genetic interaction with MCU in *Drosophila*. Co-expression of *Drosophila* EMRE and MCU in fly muscle led to lethality in larva stage. Furthermore, depletion of EMRE in larval muscle abolished caffeine-induced \( [\text{Ca}^{2+}]_{\text{mito}} \) increase regardless of the expression level of MCU, indicating that EMRE is required for MCU activity from human to *Drosophila*.

Recent studies reported that MCU is involved in Ca\(^{2+}\) excitotoxicity of cortical neurons (26) and oxidative stress-induced cell death of primary cerebellar granule neurons (45). Oxidative stress is proposed as the main causative factor for neurodegenerative, cardiovascular, and other mitochondria-related diseases (46). Previous studies reported that oxidative stress increases \( [\text{Ca}^{2+}]_{\text{mito}} \), although there are controversies whether the source of Ca\(^{2+}\) is from the extracellular environment or intracellular stores. We observed marked and sustained \( [\text{Ca}^{2+}]_{\text{mito}} \) rises by exogenous oxidative stress inducers, such as H\(_2\)O\(_2\) and t-BuOOH in *Drosophila*, which have not been investigated previously. Oxidative stress produced by endoplasmic reticulum oxidase 1α (ERO1α) can stimulate Ca\(^{2+}\) release from the ER by activating IP\(_{3}\)R (33). Loss of Ca\(^{2+}\) store in the ER by ROS induces ER stress due to impaired Ca\(^{2+}\) -sensitive chaperone activities and further ROS production by induction of C/EBP homologous protein (33). In addition, depletion of Ca\(^{2+}\) in the ER stimulates Ca\(^{2+}\) influx from outside of the cell via store-operated Ca\(^{2+}\) entry (50). Both Ca\(^{2+}\) release from the ER and influx from extracellular environment burden cells with the pathology of mitochondrial Ca\(^{2+}\) overload.

Our study clearly showed that loss-of-function mutation of MCU abrogated \( [\text{Ca}^{2+}]_{\text{mito}} \) increases triggered by t-BuOOH in fly muscle. Moreover, knockdown of IP\(_{3}\)R also significantly attenuated oxidative stress-induced \( [\text{Ca}^{2+}]_{\text{mito}} \) rises, which explains the protective roles of IP\(_{3}\)R RNAi against lethality in MCU-overexpressed flies (Fig. 6A). Oxidative stress can increase ER Ca\(^{2+}\) release by activating not only IP\(_{3}\)R but also ryanodine receptor (RyR), both of which are main Ca\(^{2+}\) release channels in the ER (33, 51). Although it has been known that RyR plays more important roles than IP\(_{3}\)R in Ca\(^{2+}\) release from the sarcoplasmic reticulum in skeletal muscle, the muscle tissues from larva, pupa, and adult *Drosophila* express IP\(_{3}\)R, which is critical for the muscle development (52). In this study, we could not investigate the interaction between RyR and MCU for oxidative stress-induced mitochondrial Ca\(^{2+}\) overload and lethality because all of the flies with muscle-specific knockdown of RyR died before larval stage. However, we still consider that RyR is another strong candidate involved in mitochondrial Ca\(^{2+}\) overload by oxidative stress in muscle.

In summary, we have established a *Drosophila* system to study the MCU complex in vivo, demonstrating that oxidative stress induces Ca\(^{2+}\) release from the ER and mitochondrial Ca\(^{2+}\) uptake via MCU, resulting in mitochondrial dysfunction and cell death in vivo. Intriguingly, a recent study showed that ROS modifies MCU directly by S-glutathionylation and consequently influences the channel activity of MCU (53). These pieces of evidence indicate both direct and indirect regulation of the Ca\(^{2+}\) channel activity of MCU by ROS. Further genetic studies would enable us to discover novel relationships connecting mitochondrial Ca\(^{2+}\) homeostasis and other cellular activities. In addition, studying the in vivo role of MCU and its related genes in *Drosophila* will further extend our knowledge of their pathophysiological significance.

**Experimental procedures**

**Fly strains**

MCU\(^{+/–}\) mutant and a revertant were generated using P-element excision of the GS11565 line obtained from the Kyoto Stock Center (Kyoto Institute of Technology, Kyoto, Japan). The revertant with a precise P-element excision was used as a wild-type control. The P-element excisions in MCU\(^{+/–}\) and the revertant flies were confirmed by PCR using a primer set of the following sequences: 5’-GACGGAAATTCGATGGAAGAATTC-3’ and 5’-GGGAAAAATCCATTACTAGT-3’. The MCU cDNA clone LD26402 and the EMRE cDNA clone RE55001 were purchased from the *Drosophila* Genomics Resource Center (Indiana University, Bloomington, IN). UAS-MCU-FLAG, UAS-hMCU-FLAG, UAS-MCU\(^{N\text{IMO}}\)-FLAG, and UAS-EMRE-FLAG were generated by microinjection of pUAST vector-cloned DNA into w\(^{1118}\) embryos. Cg-Gal4, Mef-Gal4, Gmr-Gal4, and UAS-GCaMP3.T were obtained from the Bloomington *Drosophila* Stock Center (Indiana University, Bloomington, IN).
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Bloomington, IN). UAS-MCU RNAi (v9501), UAS-IP₃R RNAi (v6484), and UAS-EMRE RNAi (v104493) were provided by the Vienna Drosophila RNAi Center (Vienna, Austria). Other flies were provided with generosity: UAS-MTRP from Dr. G. T. Macleod (University of Texas Health Science Center, San Antonio, TX); UAS-mCherry-ATG8a from Dr. T. P. Neufeld (University of Minnesota, Minneapolis, MN); UAS-MCU-Myc from Dr. S. B. Lee (DGIST, Daegu, Korea); and hid5′-F-WT-GFP from Dr. W. Du (University of Chicago).

**Quantitative RT-PCR**

Wandering larvae were collected, and total RNA was extracted using TRIzol reagent (Invitrogen). Extracted RNAs were reverse transcribed by Moloney murine leukemia virus reverse transcriptase (Promega) and amplified by PCR with primer sets of the following sequences: 5′-GTCTCGCCCT-GCTTTGG-3′ and 5′-CGAAGCTTCTGTGCTGCTG-3′ for MCU and 5′-GCCTCTTGGAGGAGACGCCG-3′ and 5′-GCTTCAATGACCATCGCCC-3′ for RP49. MCU mRNA levels were normalized by RP49 mRNA levels.

**Immunoblotting analysis**

For immunoblot samples, tissues were homogenized in ice-cold lysis buffer (20 mM Tris-Cl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 2 mM EGTA, 1 mM Na₂VO₄, 50 mM β-glycerophosphate, 50 mM NaF, and 1% Triton X-100). Homogenized samples were incubated in ice for 15 min, centrifuged, and denatured. Rabbit anti-FLAG antibody (Cell Signaling Technology), mouse anti-Drosophila MCU polyclonal antibody (epitope: EDGETDKHKKPTTG) (AbClon), mouse anti-β tubulin antibody (DSHB), and anti-porin antibody (54) were used in immunoblot analyses.

**Food intake assay**

To measure feeding activity for 24 h, the food intake assay was conducted as described previously (55).

**Measurement of trehalose and glucose**

Trehalose and glucose in fly body fluid were measured as described previously (56). For each genotype, hemolymph from 5–7 wandering larvae was extracted by tearing up the cuticles. 1 µl of hemolymph was diluted with 99 µl of trehalose buffer (5 mM Tris, pH 6.6, 137 mM NaCl, and 2.7 mM KCl) and incubated at 70 °C for 5 min. Then 40 µl of diluted hemolymph was mixed with either 40 µl of trehalose buffer or 40 µl of trehalose solution. Trehalose solution was prepared by diluting 3 µl of porcine trehalase (Sigma) in 1 ml of trehalose buffer. Samples were incubated at 37 °C overnight, and glucose levels were measured using a glucose assay kit (Sigma-Aldrich).

**Starvation-induced autophagy assay**

Third instar larvae before wandering stage were rinsed with PBS and either starved in a double-distilled water–containing Petri dish or fed in a food-containing vial for 4 h. Then larvae were dissected and fixed in 4% paraformaldehyde. mCherry-ATG8a signals in larval fat body were observed under a confocal microscope.

**Survival curves on H₂O₂-containing food**

120–130 3–5-day-old males were starved 4–6 h in double-distilled water–containing vials and transferred to vials containing either 1% H₂O₂ or 5% sucrose. Surviving flies were counted every 12 h.

**TUNEL assay**

To detect H₂O₂-induced cell death, wandering larvae were dissected and incubated for 3 h in Schneider’s medium containing 2% H₂O₂. Dissected larvae were fixed in 4% paraformaldehyde (PFA) and washed with PBS. Samples were incubated in 0.1 M sodium citrate at 65 °C, and cell death was detected using an in situ cell death detection kit (Roche Applied Science). After TUNEL reaction, the samples were stained by phalloidin and Hoechst to detect filamentous actin and nucleus, respectively. To detect in S2 cells, S2 cells were incubated for 6 h in Schneider’s medium-containing 100 mM H₂O₂. After fixation with 4% PFA for 15 min and washing with PBS, cells were incubated in 0.1 M sodium citrate at room temperature for 10 min, and apoptosis was detected by using an in situ cell death detection kit (Roche Applied Science).

**Immunostaining**

Third instar larvae were dissected and incubated for 3 h in Schneider’s medium containing 2% H₂O₂. Then samples were fixed in 4% PFA PBST (0.1% Triton X-100 in PBS) for 30 min and washed with PBST. The samples were blocked with 5% FBS, 0.5% BSA PBST (blocking buffer) for 1 h and then incubated with primary antibodies overnight at 4 °C. After three washes for 10 min in blocking buffer, the samples were treated with secondary antibodies and Hoechst 33258 (Invitrogen) in blocking buffer for 45 min. After extensive washes, samples were placed in mounting medium. Primary antibodies used in this study were as follows: anti-GFP rabbit monoclonal antibody (1:500; Thermo Fisher Scientific, A11222) and anti-cleaved caspase-3 polyclonal antibody (1:200; Cell Signaling Technology, 9661). Alexa Fluor 568 streptavidin (Thermo Fisher Scientific) and anti-ATP5a mouse monoclonal antibody (Abcam, ab14748) were used to detect mitochondria. Fluorescein-conjugated F(ab′)$_2$ fragment goat anti-rabbit IgG (Jackson Immunoresearch, 111-096-144) and rhodamine red-X–conjugated F(ab′)$_2$ fragment goat anti-mouse IgG (Jackson Immunoresearch, 115-296-146) were used as secondary antibodies.

**DHE staining**

For ROS detection, adult fly thoraces were dissected in Schneider’s medium, incubated for 5 min with 30 µM DHE in the same medium, washed twice, fixed slightly with 4% PFA for 8 min, and rinsed with PBS.

**Drosophila S2 cell culture and dsRNA bathing**

Drosophila S2-DRSC cells were cultured and dsRNA bathing was conducted as described previously (57). For silencing mRNA expression of MCU and IP₃R, dsRNA was synthesized and bathed. The following primers were used for dsRNA synthesis: MCU dsRNA, 5′-TAATAGCATCTACTATAGGGTG-GAGGATGTAAGAATCGC-3′ and 5′-TAATAGCATCTACTATAGGGTG-GAGGATGTAAGAATCGC-3′.
CACTATAGGTTAATGCTCAACTTGTCGCT-3'; IP$_5$R dsRNA, 5'-TAATACGACTCACTATAGGGGCTTCCCTATGCAACA-3' and 5'-TAATACGACTCACTATAGGGGCTTCCCTATGCAACAG-3'; luciferase dsRNA, 5'-TAATACGACTCACTATAGGGGCTTCCCTATGCAACAG-3'; and 5'-TAATACGACTCACTATAGGGGCTTCCCTATGCAACAG-3'.

**Measurement of mitochondrial membrane potential**

To measure [Ca$^{2+}$]$_{mito}$ in larval muscle, MTRP was expressed by Mef-Gal4 and UAS-MTRP. Wandering third instar larvae were dissected as described previously (58) with some modifications. Larvae were perfused in 2 mM CaCl$_2$, 4 mM MgCl$_2$, 2 mM KCl, 2 mM NaCl, 5 mM HEPES, 35.5 mM sucrose, 7 mM d-glutamic acid, 7.3 mM, with NaOH) on a stereomicroscope and transferred to a confocal microscope. Transferred larvae were perfused with the same buffer, and fluorescence images were acquired by using an inverted microscope (IX-81, Olympus, Tokyo, Japan) with an array laser confocal spinning disk (CSU10, Yokogawa Electric Corp., Tokyo, Japan) and a cooled charge-coupled device camera (Cascade 512B, Photometrics, Tucson, AZ). Acquired fluorescence images from 435-nm excitation (Ex) and 535-nm emission (Em) were analyzed using Metafluor 6.3 software (Universal Imaging, Molecular Devices) (11). Cytosolic Ca$^{2+}$ level ([Ca$^{2+}$]$_c$) was measured using the confocal system (488-nm Ex/535-nm Em) in GCaMP3-expressed larval muscle (59). To measure [Ca$^{2+}$]$_{mito}$ in S2 cells, we used a mitochondria-targeted ratio-pericam plasmid (RPmit4.1), generously provided by Dr. Roger Tsien (University of California, San Diego, CA). Cells were transfected with siRNA for 24 h and then transfected with RPmit4.1 using X-tremeGENE (Roche Diagnostics GmbH, Mannheim, Germany). After 48 h of RPmit4.1 transfection, cells were perfused with KRP solution (140 mM NaCl, 3.6 mM KCl, 0.5 mM Na$_2$HPO$_4$, 0.5 mM MgSO$_4$, 1.5 mM CaCl$_2$, 10 mM HEPES, 2 mM NaHCO$_3$, 5.5 mM glucose, pH 7.4, titrated with NaOH), and fluorescence images (435-nm Ex/535-nm Em) were acquired.

**Calculation of [Ca$^{2+}$]$_{mito}$ increases**

In every scattered plot, the y axis represents area under the curve of the [Ca$^{2+}$]$_{mito}$ imaging result under caffeine, t-BuOOH, or H$_2$O$_2$ treatment.

**Measurement of mitochondrial membrane potential**

To measure the mitochondrial membrane potential (Ψ$_{mito}$), S2 cells seeded onto black-walled 96-well plates (1.2 × 10$^5$ cells/well) were loaded with a lipophilic cationic dye, JC-1 (1.5 μM), for 30 min. Cells were washed with KRP solution, and JC-1 fluorescence intensities of red (540-nm Ex/590-nm Em; J-aggregates) and green (490-nm Ex/540-nm Em; monomer) were measured ratiometrically at room temperature using a multiwell fluorescence reader (Flex-Station, Molecular Devices) (60).

**Statistics**

Values are presented as mean ± S.D., and n is the number of independent experiments. p values were obtained by Student’s t test or one-way analysis of variance, and <0.05 was considered to be significant.

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