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Calcium is Involved in Necroptosis Through the Expression of Phospho-MLKL in Skeletal Muscle Cell in Mice

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SUMMARY
Necroptosis is programmed cell death similar to necrosis, which is mediated by meticulous protein interactions such as those in apoptosis. Essentially, because it is a reaction to inflammation, necroptosis functions similarly to apoptosis and it is thought that necroptosis is an alternative cell death pathway when apoptosis cannot function for some reason. However, the research history of necroptosis is brief, and although there are various studies, many aspects are unknown. Here, we found that calcium was an essential factor for necroptosis in a muscle cell line. After verifying necroptosis was functional in mouse skeletal muscle cell line C2C12, we found suppression of necroptosis by controlling the amount of calcium in the culture medium. Furthermore, the reduced necroptosis was rescued by adding calcium to the culture medium. However, the recovery did not occur when magnesium or sodium was added to the medium. Next, we suppressed certain calcium transporters using inhibitors. Necroptosis was inhibited by reducing the amount of intracellular calcium. These results showed that necroptosis was controlled by the amount of calcium. This finding may facilitate development of new treatment methods for various muscle diseases that involve cell death.

KEY WORDS
Calcium; necroptosis; p-MLKL; C2C12; skeletal muscle.

INTRODUCTION

Cell death is a general and important biological function that is conserved widely among most organisms, in which inflamed or abnormal cells die spontaneously and are replaced by new normal cells. There are several kinds of cell death (1). Among them, apoptosis is the most thoroughly studied. Necroptosis occurs by breaking cell membranes. Pyroptosis is seen after infection by bacteria or viruses. Ferroptosis involves iron and autophagy is a "self-eating system" in which cell contents are transported to lysosomes for degradation.

In this study, we focused on necroptosis. Necroptosis is a type of necrosis, but the molecular mechanism is meticulously controlled by protein interactions and phosphorylation. It is cell death in a defense system against inflamed cells and is thought to function when apoptosis cannot operate. After inhibition of apoptosis, Caspase-8 recruits Receptor-interacting protein kinase (RIPK) 1 and necroptosis begins. RIPK1 binds to RIPK3, serine 231 and tyrosine 232 of RIPK3 are phosphorylated, and then necroptosis is activated. Phosphorylated RIPK3 binds to mixed lineage kinase domain-like protein (MLKL). After serine 345 in

mouse MLKL or serine 358 in human MLKL is phosphorylated, MLKL is activated by binding to Inositol phosphate (IP6) (2, 3). Activated MLKL is phosphorylated at tyrosine 363 by Tyro3/Axl/Mer receptor tyrosine (TAM) kinase and moves to the plasma membrane (4). MLKL oligomerizes at the plasma membrane to form pores. The cell then dies from cytosol escaping from the pores. Interestingly, protein synthesis continues in the dying cell. The cell produces proteins involved in inflammation and signals the condition to other cells, which is then targeted by macrophages (5).

In this study, we focused on the importance of calcium in necroptosis. Calcium is a representative second messenger with various roles in apoptosis and axon signaling between nerve cells (6, 7). Most general roles involve bone growth and muscle contraction by release from the sarcoplasmic reticulum. However, many aspects are unknown in the relationship between calcium and necroptosis. Although some studies have examined neuroblastoma and colon cancer (8, 9), few studies have investigated muscle cells. In this study, we found that calcium was involved in necroptosis of muscle cells. Calcium was indirectly involved by regulating the expression of phosphorylated MLKL (p-MLKL). This is a great success that we could understand the calcium-working area in necroptosis and make it narrow. Our results provide novel insights into muscle cells and may provide new approaches to identify causes of muscle diseases or discover drugs.

MATERIALS AND METHODS

Cell culture and differentiation

Mouse skeletal muscle cell line C2C12 was prepared as described previously (10). DMEM (Thermo Fisher Scientific, Waltham, MA) with 10% FBS (Biosera, Kansas, MO) and 1% penicillin/streptomycin (Wako, Osaka, Japan) was used as culture medium. For differentiation, DMEM or DMEM without calcium (Thermo Fisher Scientific) with 2% horse serum (Thermo Fisher Scientific) was used. One day after passaging cells, the culture medium was changed to medium with horse serum. Three days after changing the medium, the medium was renewed. Six days after passaging, reagents that referred in Figure Legends were added to the medium. One day after adding reagents, the cells were harvested and used for experiments.

Western blotting

Western blotting was performed as described previously (11) using C2C12 cell lysates treated with reagents that referred in figure legends. To detect phospho-proteins, Immobilon Signal Enhancer (Merck, Darmstadt, Germany) was used. Anti-rabbit-p-MLKL (S345) (1:1000) (Abcam, Cambridge, UK), anti-rabbit-p-CaMKII (1:500) (ABclonal, Wuhan, China), and anti-rabbit-GAPDH (1:2000) (Eurofin genomics, Tokyo, Japan) antibodies were used as primary antibodies and GAPDH was detected as a loading control. An anti-rabbit IgG antibody (1:1000) (CST, Danvers, MA) was used as a secondary antibody. Detection was performed by a LAS 4000mini (GE Healthcare, Chicago, IL).

PI assay

A propidium iodide (PI) assay was performed as described previously (12). PI was purchased from Nacalai Tesque (Kyoto, Japan). Fluorescence was detected by a microplate reader (Synergy H1, BioTek, Winooski, VT).

Intracellular calcium measurement

Intracellular calcium measurement was performed using a Calcium Kit-Fluo4 (Dojindo, Kumamoto, Japan) in accordance with the manufacturer's protocol. After differentiation, reagents that referred in figure legends were added to cells. One day later, the cells were re-plated in a 96-well plate. One day after re-plating, calcium measurement was performed. Fluorescence was detected by the microplate reader (Synergy H1, BioTek).

RESULTS

Necroptosis induction in C2C12 cells

To confirm that necroptosis occurs in muscle cells, we performed two experiments. First, we detected expression of p-MLKL, a typical marker of necroptosis. Because necroptosis is an alternative cell death pathway when apoptosis is inhibited, inhibition of Caspase expression is needed for necroptosis to occur (13). To induce inflammation in cells for cell death induction, cells were treated with Tumor necrosis factor α (TNFα) and zVAD-fmk (zVAD), a pan-caspase inhibitor. When cells were treated with TNFα only, p-MLKL expression was low. However, when cells were treated with both TNFα and zVAD, p-MLKL expression was high (**figure 1B**). The difference in expression was significant (**figure 1C**). Apoptotic cells die from DNA breakages, whereas necroptotic cells die from breakage of their plasma membrane and cytosol escape (14). Next, we measured the fluorescence intensity of propidium iodide (PI) that is used to evaluate necrosis-like cell death because PI enters cells through the broken plasma membrane. Compared with cells treated with TNFα only, significantly more cells had a damaged plasma membrane when treated with both TNFα and zVAD (**figure 1A**). Thus, we induced necroptosis in differentiated C2C12 cells.

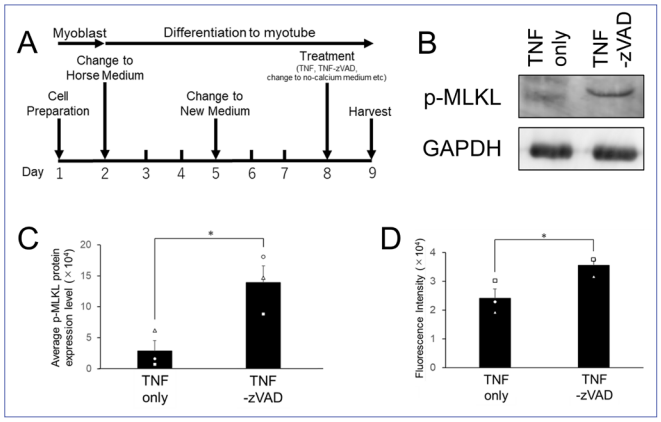

Figure 1. Necroptosis induction in C2C12 cells.

(A) Scheme of experiment; **(B)** Western blotting was performed using C2C12 cells. The cells were treated with 1.5 nM TNFα with or without 18 μM zVAD. P-MLKL was detected and GAPDH was used as a loading control; **(C)** Western blotting in **(A)** was quantified by ImageJ. The experiment was repeated three times (n = 3). Error bars are the standard error and significance was evaluated by Student's t-test (*$p < 0.05$). Experiments were performed in triplicate. Circle dots show 1st trial, triangle dots show 2nd trial and square dots show 3rd trial; **(D)** Fluorescence of propidium iodide (PI) was measured in C2C12 treated with 1.5 nM TNFα with or without 18 μM zVAD. The experiment was repeated three times (n = 3). Error bars are the standard error and significance was evaluated by Student's t-test (*$p < 0.05$). Experiments were performed in triplicate. Circle dots show 1st trial, triangle dots show 2nd trial and square dots show 3rd trial.

Calcium is involved in expression of p-MLKL, a major factor in necroptosis

Calcium is involved in almost the entire process of necroptosis and acts after phosphorylation of RIPK1, a necrosome factor (8, 9). Therefore, we detected necroptosis after the calcium amount was controlled by treating the cells with various reagents. First, we trapped metal ions in culture medium using chelators, namely EDTA and EGTA. As a result, when EDTA was used as a chelator, p-MLKL expression was unchanged. However, when EGTA was used, p-MLKL expression was decreased drastically (**figure 2A**) and the difference was significant (**figure 2B**). Next, we used culture medium without calcium. As a result, there was low p-MLKL expression in cells cultured in the medium without calcium (**figure 2C**). Next, we confirmed whether this effect was rescued by adding calcium to the medium. we added 4 mM $CaCl_2$ to the medium and found that the decreased p-MLKL expression was rescued by supplementing calcium (**figure 2D**). To examine whether the decreased p-MLKL expression was rescued by other metal ions, we added 4 mM $MgCl_2$ and 4 mM NaCl to the medium without calcium and then measured p-MLKL expression. As a result, magnesium and sodium could not rescue the decreased p-MLKL expression (**figure 2E**). Therefore, calcium was involved in necroptosis.

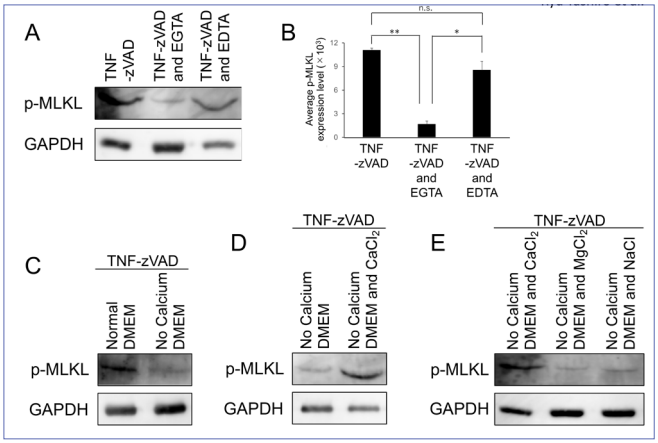

Figure 2. Calcium is involved in necroptosis by controlling p-MLKL expression.

(A) Western blotting was performed using C2C12 cells. The cells were treated with 1.5 nM TNFα and 18 μM zVAD with or without 10 mM EGTA or EDTA. P-MLKL was detected and GAPDH was used as a loading control; **(B)** Western blotting in **(A)** was quantified by ImageJ. The experiment was repeated three times (n = 3). Error bars are the standard error and significance was evaluated by Student's t-test (n.s. = no significance, *$p < 0.05$, **$p < 0.01$); **(C)** Western blotting was performed using C2C12 cells. The cells were treated with 1.5 nM TNFα and 18 μM zVAD in DMEM with or without calcium. P-MLKL was detected and GAPDH was used as a loading control; **(D)** Western blotting was performed using C2C12 cells. The cells were treated with 1.5 nM TNFα and 18 μM zVAD in calcium-free DMEM with or without 4 mM $CaCl_2$. P-MLKL was detected and GAPDH was used as a loading control; **(E)** Western blotting was performed using C2C12 cells. The cells were treated with 1.5 nM TNFα and 18 μM zVAD in calcium-free DMEM with or without 4 mM $CaCl_2$, 4 mM $MgCl_2$, and 4 mM NaCl. P-MLKL was detected and GAPDH was used as a loading control.

Intracellular calcium is not increased during necroptosis

Calcium was involved in necroptosis, and therefore we measured intracellular calcium in variously treated cells. Interestingly, intracellular calcium after treating cells with both TNFα and zVAD was significantly decreased compared with that in cells treated with TNF only (**figure 3A**). However, because cells die from plasma membrane breakage during necroptosis, it was highly possible that calcium had escaped from the pores in the plasma membrane. we hypothesized that lost calcium was restored by calcium outside of the cells. Therefore, we added 4 mM $CaCl_2$ to the medium of cells treated with both TNFα and zVAD. Unexpectedly, there was no difference in intracellular calcium after adding calcium (**figure 3B**). Additionally, western blotting showed no change in p-MLKL expression with or without calcium in cells treated with both TNFα and zVAD (**figure 3C**).

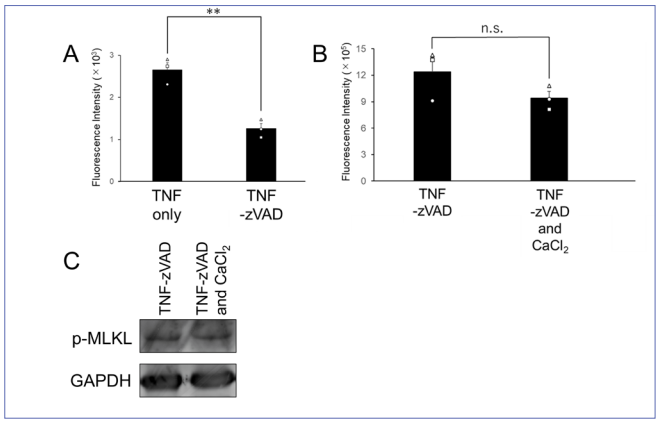

Figure 3. Intracellular calcium does not increase during necroptosis.

(A) Intracellular calcium was measured in C2C12 cells treated with 1.5 nM TNFα with or without 18 μM zVAD. The experiment was repeated three times (n = 3). Error bars are the standard error and significance was evaluated by Student's t-test (**p < 0.01). Experiments were performed in triplicate. Circle dots show 1st trial, triangle dots show 2nd trial and square dots show 3rd trial; **(B)** Intracellular calcium was measured in C2C12 cells treated with 1.5 nM TNFα and 18 μM zVAD with or without 4 mM $CaCl_2$. The experiment was repeated three times (n = 3). Error bars are the standard error and significance was evaluated by Student's t-test (n.s. = no significance). Experiments were performed in triplicate. Circle dots show 1st trial, triangle dots show 2nd trial and square dots show 3rd trial; **(C)** Western blotting was performed using the samples in **(B)**. C2C12 cells were treated with 1.5 nM TNFα and 18 μM zVAD with or without 4 mM $CaCl_2$. P-MLKL was detected and GAPDH was used as a loading control.

Inhibiting calcium transporters inhibited necroptosis of cells

It was highly possible that necroptosis was caused by calcium from outside of the cells. Although a large amount of calcium had escaped from the cells, calcium may have also simultaneously entered the cells. Therefore, we determined whether calcium entered the cells during necroptosis. A change in p-MLKL expression was examined when each calcium channel in the plasma membrane was inhibited. Three kinds of calcium channel inhibitors were added to the medium: benidipine, an L- and T-type calcium blocker (15), YM-58483 that suppresses Transient receptor potential channel activity (16), and MRS1845, an inhibitor of Orai1 that acts with STIM1 to detect a shortage of intracellular calcium in the endoplasmic reticulum (17). These channel proteins are all located in the plasma membrane. Therefore, all inhibitors we checked this time decreased p-MLKL expression (**figure 4A**). The decreases were significant with all three inhibitors (**figure 4B**). Next, we used 2,5-di-tert-butylhydroquinone, a SERCA inhibitor that detects calcium in the endoplasmic reticulum membrane and controls the intracellular calcium amount (18, 19). As expected, p-MLKL expression was decreased (**figure 4C**) and not rescued by 4 mM $CaCl_2$ in cells treated with MRS1845, YM58483, or 2,5-di-tert-butylhydroquinone (**figure 4D**). Thus, to induce necroptosis, controlling the amount of intracellular calcium is important, but we could not identify specific calcium transporters that are involved in necroptosis.

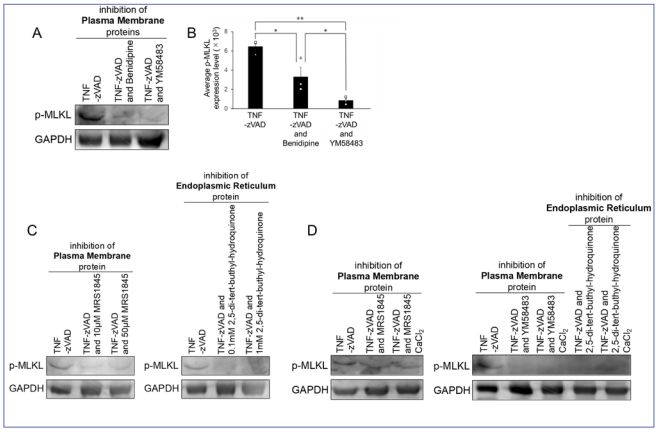

Figure 4. Four specific calcium transporters that have different mechanism could inhibit necroptosis.

(A) Western blotting was performed using C2C12 cells. The cells were treated with 1.5 nM TNFα, 18 μM zVAD, and 500 μM benidipine or 10 μM YM58483. P-MLKL was detected and GAPDH was used as a loading control; **(B)** Western blotting in **(A)** was quantified by ImageJ. The experiment was repeated three times (n = 3). Error bars are the standard error and significance was evaluated by Student's t-test (*p < 0.05, **p < 0.01). Experiments were performed in triplicate. Circle dots show 1st trial, triangle dots show 2nd trial and square dots show 3rd trial; **(C)** Western blotting was performed using C2C12 cells. The cells were treated with 1.5 nM TNFα, 18 μM zVAD, 1, 10, or 50 μM MRS1845, and 0.1 or 1 mM 2,5-di-tert-buthyl-hydroquinone. P-MLKL was detected and GAPDH was used as a loading control; **(D)** Western blotting was performed using C2C12 cells. The cells were treated with 1.5 nM TNFα, 18 μM zVAD, and 50 μM MRS1845, 10 μM YM58483, or 0.1 mM 2,5-di-tert-buthyl-hydroquinone with or without 4 mM $CaCl_2$. P-MLKL was detected and GAPDH was used as a loading control.

p-MLKL expression is synchronized to p-CaMKII expression

Calcium is involved in necroptosis through p-RIPK1 in cancer cells and its kinase is CaMKII (8). Next, I verified this in skeletal muscle cells. Phospho-CaMKII expression was also decreased in cells cultured without calcium and its expression was also rescued by adding $CaCl_2$ to the medium (**figure 5**). These results indicated that, at least in skeletal muscle cells and as shown in neuroblastoma (8), CaMKII may be the most upstream factor to induce necroptosis by calcium (**figure 6**).

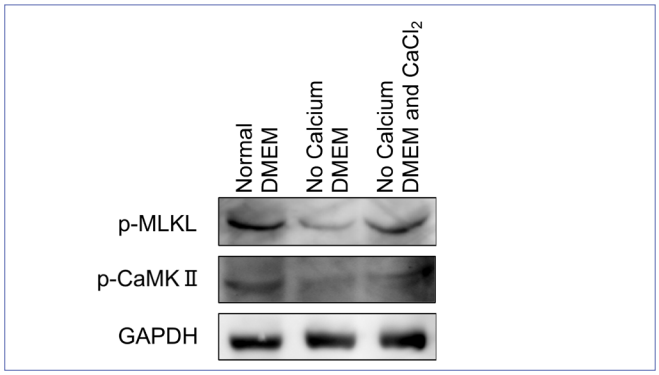

Figure 5. CaMKII might be involved in necroptosis of mouse skeletal muscle cells.

Western blotting was performed using C2C12 cells. The cells were treated with 1.5 nM TNFα and 18 μM zVAD in normal or calcium-free DMEM with or without 4 mM $CaCl_2$. P-MLKL and CaMKII were detected and GAPDH was used as a loading control.

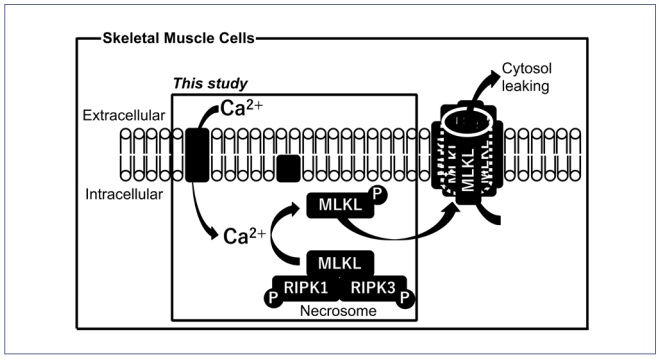

Figure 6. Proposed model in this study.

DISCUSSION

In this study, we found that calcium was needed for necroptosis in skeletal muscle. Previous studies have obtained similar results, but they all examined cancer cells (8, 9). This study focused on muscle cells. Although previous studies were performed using biochemical assays, the position where calcium is involved in necroptosis remained controversial. Here, from calcium in the cytosol to necroptosis, I found that calcium was involved in p-MLKL expression in skeletal muscle cells. This result showed that, at least in skeletal muscle cells, calcium is involved in the process before formation of the necrosome, the complex formed by RIPK1, RIPK3, and MLKL.

As shown in **figure 1**, we evaluated how necroptosis functions in C2C12 cells. Because few studies have examined p-MLKL expression by western blotting in C2C12 cells (20), this result showed that C2C12 cells are useful to study cell death mechanisms. Accordingly, few studies have focused on the relationships between muscle and necroptosis. In muscle cell research, it is common to perform *in vivo* analysis using mice. However, to analyze detailed molecular mechanisms, it is important to perform studies at the protein or cellular level. Our study showed the importance of using cultured cells for preliminary research and its significance for necroptosis.

As shown in **figure 2**, we revealed the importance of calcium in necroptosis. Reducing or eliminating calcium clearly decreased p-MLKL expression. This decreased expression was rescued by adding $CaCl_2$ to the medium. However, magnesium and sodium could not rescue the phenotype. These results showed that calcium was involved in necroptosis by phosphorylation of MLKL. Calcium is involved in necroptosis of cancer cells. However, the two studies of neuroblastoma and colon cancer presented different results on where calcium was involved. At least in skeletal muscle cells, we showed that calcium was involved in phosphorylation of MLKL, which demonstrated how calcium works throughout the whole necroptosis process.

Owing to the involvement of calcium and its import from outside cells, we determined how calcium entered the cells. There are various kinds of transporters to import calcium into cells. we inhibited single transporters and checked for downregulation of p-MLKL expression to determine whether the transporter was involved in necroptosis. Inhibitors of three transporters were employed and all of them reduced p-MLKL expression significantly. Therefore, to undergo necroptosis, it is essential to obtain calcium from outside cells, but the route to obtain calcium is irrelevant. Inhibition of SERCA, which is located at the endoplasmic reticulum and controls intracellular calcium, also decreased p-MLKL expression. In this experiment, calcium efflux occurred after TNFα and zVAD treatment and necroptosis (**figure 3A**). Additionally, calcium influx was not limited because there was no inhibition of calcium transporters in the plasma membrane. However, the endoplasmic reticulum stores calcium (21) and cells might not maintain the calcium concentration by SERCA inhibition. Accordingly, the intracellular calcium concentration may be reduced compared with the normal state. Therefore, the calcium involved in phosphorylation of MLKL was not obtained by cooperating with other proteins and calcium obtained extracellularly may be used.

There are two steps in MLKL phosphorylation. One is the phosphorylation introduced in this study and the other is phosphorylation by TAM kinase. The former is involved in necrosome formation, and the latter is involved in IP6 and polymerization, the last step to form pores in the plasma membrane. RIPK is a kinase, and when activated, it is involved in MLKL phosphorylation. On the basis of our results, calcium might be involved in the step from RIPK3 phosphorylation to MLKL phosphorylation. However, Nomura *et al.* (8) showed that the kinase of RIPK1 is CaMKII, which involves calcium. In their study, if intracellular calcium was limited, the cell survival rate increased and CaMKII activity decreased.

Additionally, because RIPK1 phosphorylation was reduced by ^{32}P in the *in vitro* kinase assay by inhibition of CaMKII, calcium was involved in RIPK1 phosphorylation. However, Sun *et al*. (9) showed that, when necroptosis was functional, intracellular calcium was increased, and when RIPK1 was inhibited, intracellular calcium was decreased. However, because they could not inhibit cell death, they believed that calcium acted at another position compared with the study of Nomura *et al.* (8). In this study, although we also found that calcium was involved in necroptosis, the cells were skeletal muscle and MLKL phosphorylation was induced by calcium obtained from outside of the cells during necroptosis. Zhou *et al*. (22) showed that, during necroptosis of smooth muscle, CaMKII acts downstream of MLKL. Although this study appears to demonstrate known facts, the details of necroptosis may differ in terms of the cell type and experimental conditions, which is why this research is novel.

Necroptosis has been indicated in various diseases, but there are few examples in muscle cells. For example, necroptosis is essential to regenerate muscle cells after inflammation (23). It is also involved in dermatomyositis and polymyositis (24), and Duchenne muscular dystrophy (25). Thus, necroptosis is a developing field. Although factors such as MLKL and RIPK are well known in necroptosis, how and where other proteins and molecules act are unknown, which require further study. This study may contribute to elucidating the mechanisms of skeletal muscle diseases and drug discovery.

CONCLUSIONS

This study showed the importance of calcium in necroptosis using mouse C2C12 cells biochemically. In addition to cancer and smooth muscle, skeletal muscle also requires calcium to perform necroptosis. However, because it was showed by mouse cell lines, new results that are performed *in vivo* or human cell/tissues will be needed in the future.

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DATA AVAILABILITY

Data are available under reasonable request to the corresponding author.

CONTRIBUTIONS

RY: conceptualization, data curation, formal analysis, Funding acquisition, investigation, methodology, project administration, resources, software, validation, visualization, writing – original draft, writing – review & editing. YM: supervision.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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