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Ca\(^{2+}\)/calmodulin-dependent protein kinase kinase is involved in AMP-activated protein kinase activation by \(\alpha\)-lipoic acid in C2C12 myotubes

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Shen QW, Zhu MJ, Tong J, Ren J, Du M. \(\text{Ca}^{2+}/\text{calmodulin-dependent protein kinase kinase is involved in AMP-activated protein kinase activation by }\alpha\)-lipoic acid in C2C12 myotubes. Am J Physiol Cell Physiol 293: C1395–C1403, 2007. First published August 8, 2007; doi:10.1152/ajpcell.00115.2007.—\(\alpha\)-Lipoic acid (ALA) widely exists in foods and is an antidiabetic agent. ALA stimulates glucose uptake and increases insulin sensitivity by the activation of AMP-activated protein kinase (AMPK) in skeletal muscle, but the underlying mechanism for AMPK activation is unknown. Here, we investigated the mechanism through which ALA activates AMPK in C2C12 myotubes. Incubation of C2C12 myotubes with 200 and 500 \(\mu\)M ALA increased the activity and phosphorylation of the AMPK \(\alpha\)-subunit at Thr\(^{172}\). Phosphorylation of the AMPK substrate, acetyl CoA carboxylase (ACC), at Ser\(^{79}\) was also increased. No difference in ATP, AMP, and the calculated AMP-to-ATP ratio was observed among the different treatment groups. Since the upstream AMPK kinase, LKB1, requires an alteration of the AMP-to-ATP ratio to activate AMPK, this data showed that LKB1 might not be involved in the activation of AMPK induced by ALA. Treatment of ALA increased the intracellular Ca\(^{2+}\) concentration measured by fura-2 fluorescent microscopy (\(P < 0.05\)), showing that ALA may activate AMPK through enhancing Ca\(^{2+}/\text{calmodulin-dependent protein kinase kinase (CaMKK)}\) signaling. Indeed, chelation of intracellular free Ca\(^{2+}\) by loading cells with 25 \(\mu\)M BAPTA-AM for 30 min abolished the ALA-induced activation of AMPK and, in turn, phosphorylation of ACC at Ser\(^{79}\). Furthermore, inhibition of CaMKK using its selective inhibitor, STO-609, abolished ALA-stimulated AMPK activation, with an accompanied reduction of ACC phosphorylation at Ser\(^{79}\). In addition, ALA treatment increased the association of AMPK with CaMKK. To further show the role of CaMKK in AMPK activation, short interfering RNA was used to silence CaMKK, which abolished the ALA-induced AMPK activation. These data show that CaMKK is the kinase responsible for ALA-induced AMPK activation in C2C12 myotubes.

Skeletal muscle

\(\alpha\)-Lipoic acid (ALA) is a naturally occurring short-chain fatty acid with a powerful antioxidant capacity, which widely exists in foods (34). ALA has long been used as an antidiabetic agent (53, 54). A huge body of experiments has showed that ALA improves glucose uptake in skeletal muscle, reduces blood glucose level (5, 6, 23, 42), and increases insulin sensitivity in individuals with Type 2 diabetes (7, 20–22). Recently, it has been reported that ALA increases insulin sensitivity by activating AMP-activated protein kinase (AMPK) in skeletal muscle (28) and also in \(\beta\)-cells (43). ALA also activates AMPK in endothelial cells (27). However, the mechanism by which ALA activates AMPK remains undefined.

AMPK has three subunits, a catalytic \(\alpha\)-subunit and regulatory \(\beta\)- and \(\gamma\)-subunits (2). It is well established that the key function of AMPK is to regulate the energy balance within the cell. AMPK is activated in response to ATP depletion, which causes a concomitant increase in the AMP-to-ATP ratio. Once activated, AMPK phosphorylates downstream substrates, the overall effect of which is to switch off ATP-consuming pathways (e.g., fatty acid synthesis and cholesterol synthesis) and to switch on catabolic pathways that generate ATP (e.g., fatty acid oxidation and glycolysis) (15). Activation of AMPK requires the phosphorylation of Thr\(^{172}\) in the activation loop of the \(\alpha\)-subunit by upstream AMPK kinase (AMPKK) (18). Presently, two upstream AMPK kinases have been discovered: LKB1 and Ca\(^{2+}/\text{calmodulin-dependent protein kinase kinase (CaMKK)}\). LKB1 was originally recognized in humans as a tumor suppressor and exists in complex with two accessory subunits: STRAD and MO25 (44, 48). LKB1 is constantly active within cells (30, 35). Phosphorylation and activation of AMPK by LKB1 requires an increase in AMP or AMP mimetic agents in both cultured cells (16) and skeletal muscle (36). Binding of AMP to the AMPK \(\gamma\)-subunit alters the conformation of AMPK, making it a better substrate for LKB1 and leading to activation of AMPK (16). Recently, CaMKK has also been identified as an upstream AMPKK (17, 19, 49). CaMKK phosphorylates and activates AMPK in an AMP-independent manner, which is triggered instead by a rise in the intracellular Ca\(^{2+}\) concentration (17, 19, 49). The discovery that CaMKK acts as an AMPKK indicates that in addition to an increase of the AMP-to-ATP ratio, AMPK may also be activated by a rise in the intracellular Ca\(^{2+}\) concentration in response to nutrients, drugs, or physiological stimulation.

The aim of our study was to investigate the mechanism leading to AMPK activation by ALA in muscle cells. The results we obtained reveal that ALA increased the intracellular Ca\(^{2+}\) concentration in C2C12 myotubes. AMPK is activated by ALA in an AMP-independent manner, and CaMKK is the upstream AMPKK responsible for the activation of AMPK induced by ALA.

MATERIALS AND METHODS

Materials. Antibodies reacting with AMPK-\(\alpha\) (rabbit, polyclonal), phospho-AMPK-\(\alpha\) (Thr\(^{172}\); rabbit, polyclonal), acetyl CoA carboxylase (ACC; rabbit, polyclonal), phospho-ACC (Ser\(^{79}\); rabbit, polyclonal), and phospho-ACC (Thr\(^{210}\); rabbit, polyclonal) antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-AMPK-\(\alpha\)-subunit (rabbit, polyclonal), phospho-ACC (Ser\(^{79}\); rabbit, polyclonal), and phospho-ACC (Thr\(^{210}\); rabbit, polyclonal) were purchased from Cell Signaling Technologies (Beverly, MA). 

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clonal), and anti-rabbit and anti-mouse IgG were all ordered from Cell Signaling Technologies (Danvers, MA). An antibody against β-actin (mouse, monoclonal) was purchased from Cell Signaling Technologies (Danvers, MA). An antibody against /H9252/H9252-actin (mouse, monoclonal) was purchased from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). ALA was purchased from Sigma-Aldrich (St. Louis, MO), and STO-609 acetate was from Tocris Bioscience (Ellisville, MO). BAPTA-AM was obtained from Calbiochem (San Diego, CA). Cell culture reagents and other chemicals were all purchased from Sigma-Aldrich.

Cell culture. C2C12 myoblasts were cultured in DMEM containing 10% (vol/vol) FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 95% air and 5% CO2 at 37°C for ~48 h. After cells reached confluence, the medium was changed by replacing 10% FBS with 2% horse serum to induce differentiation. The medium was replaced every other day during differentiation. After 5 days, differentiation was complete, and experimental procedures were initiated. In all experiments, C2C12 myotubes were exposed to ALA or STO-609 for 6 h in serum-free DMEM containing the antibiotics described above. All controls were incubated with equal amounts of the vehicles used for ALA and STO-609 treatments. C2C12 myotubes were then rinsed briefly with and harvested in 0.5 ml of ice-cold lysis buffer [50 mM Tris-HCl (pH 7.4), 137 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 10% (vol/vol) glycerol, 2% (wt/vol) SDS, 1% (vol/vol) Triton X-100, 2.5 mM EDTA, 100 mM NaF, 2 mM Na3VO4, and 1% proteinase inhibitor cocktail].

AMPK assay. Cell lysates obtained were used for analyses of AMPK. AMPK activity was assayed using the incorporation of 32P into a SAMS peptide (His-Met-Ser-Ala-Met-Ser-Gly-Leu-His-Leu-Val-Lys-Arg-Arg) as previously described (4, 40).

Immunoprecipitation. Immunoprecipitation was conducted as previously described (13). To decrease nonspecific immunoprecipitation, cell lysates obtained were preincubated with 40 μl of a 50% dilution of protein A-Sepharose in PBS for 2 h at 4°C. Samples were then centrifuged, and supernatants were used for immunoprecipitation. AMPK, CaMKK, or LKB1 were immunoprecipitated from cell lysates (200 μg protein) by the addition of 3 μg of respective antibodies and an incubation for 6 h at 4°C followed by the addition of 30 μl of a 50% dilution of protein A-Sepharose in PBS. Mixtures were incubated overnight at 4°C, and immunoprecipitates were used for immunoblot analyses (13).

Protein phosphatase 2C assay. The activity of protein phosphatase 2C (PP2C) was assessed using 4-nitrophenyl phosphate as a substrate (31). Briefly, cell lysates (50 μl) were added into an assay buffer (200 μl) containing 50 mM Tris (pH 7.5), 0.5 mM DTT, and 60 mM MgCl2. The mixture was incubated at 30°C. The liberation of p-nitrophenol was determined spectrophotometrically at 405 nm at different durations (31).

RNA interference. Short interfering (si)RNA oligonucleotides designed against mouse CaMKK-α (sc-29905) were obtained from...

Fig. 1. Effects of α-lipoic acid (ALA) on AMP-activated protein kinase (AMPK) and acetyl CoA carboxylase (ACC) phosphorylation in C2C12 myotubes. C2C12 myotubes were incubated with different concentrations of ALA for 6 h (A–C) or with 200 μM ALA for various durations (D and E). AMPK phosphorylation at Thr172 (phospho-AMPK (p-AMPK)) and ACC phosphorylation at Ser79 (phospho-ACC (p-ACC)) were determined by immunoblot analysis. Representative blots (top) and densitometric analyses (bottom) are shown. Values are mean ± SE from 4 independent measurements. *P < 0.05 vs. controls (0 μM ALA at time 0).
Santa Cruz Biotechnology (Santa Cruz, CA) and used to inhibit CaMKK expression in C2C12 cells. The CaMKK-α siRNA is a pool of three target-specific 20–25 nt siRNA targeted against all three transcript variants. As a control, a nonspecific siRNA with a scrambled sequence was purchased from Santa Cruz Biotechnology (sc-37007) and used to transfect cells at the same time. Transfection was carried out using TransIT-TKO Transfection Reagent (Mirus Bio, Madison, WI) according to the manufacturer’s instructions. Briefly, C2C12 cells were plated in 12-well plates the night before the experiment. Transfection was initiated when cell confluence was 50–60%. After transfection, cells were incubated for 48 h and then treated with or without ALA as described above.

**Immunoblot analysis.** Cell lysates obtained above were used to analyze AMPK and ACC concentration and phosphorylation and also the concentration of CaMKK-α when appropriate. Briefly, after a separation by SDS-PAGE, proteins were transferred to nitrocellulose membranes, blocked, and immunoblotted with primary antibodies overnight. After an incubation with a horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature, proteins were visualized using the ECL technique and quantified by using an Imager Scanner II and ImageQuant TL software as previously described (39, 52).

**Adenine nucleotide analysis.** Cell lysates (200 μl) were added with 55% perchloric acid to a final concentration of 5%, set for 30 min on ice, and then neutralized to pH 6–8 with 2 M KOH. After being centrifuged at 13,000 g for 10 min at 4°C to remove KClO4, the supernatant was passed through a 0.2-μm filter and was ready for HPLC analysis (Beckman Instruments, Fullerton, CA). HPLC conditions were the same as those previously reported (40). Lysate protein contents were determined using a protein assay kit (Bio-Rad, Hercules, CA). Adenine contents are expressed as micromoles per gram of protein.

**Intracellular Ca2+ measurement.** Intracellular Ca2+ concentrations were measured using the fura-2 technique. Monolayer C2C12 myotubes grown on glass coverslips were loaded with 5 μM fura-2 AM for 10 min in the dark at room temperature. After an incubation, glass coverslips were then transferred to a Sykes-Moore perfusion chamber. The intracellular Ca2+ fluorescence measurements were performed at room temperature using a spectrofluorometer (Spek, Edison, NJ). Fura-2 was excited alternatively at 340 and 380 nm, and emissions at 510 nm were recorded. Intracellular Ca2+ concentrations (in nM) were calculated using the following equation: intracellular Ca2+ concentration = Kd[(R – Rmin)/(Rmax – R)]β (12). R is the measured ratio of the fluorescence excited at 340 nm divided by that excited at 380 nm. Rmin and Rmax were taken after the addition of 5 mM EGTA (zero Ca2+) and 10 μM ionomycin (Ca2+ saturation), respectively. β is the ratio of emission intensities at 380-nm excitation under these two sets of conditions. Kd is the effective dissociation constant for fura-2 + Ca2+, which was assumed to be 224 nM (12).

**Fatty acid oxidation.** Fatty acid oxidation was measured as previously described (45). Briefly, myotubes grown in six-well plates were incubated in DMEM with or without 200 μl ALA for 6 h. Then, 2% BSA and [14C]palmitate (0.5 mM, 1 μCi/ml; solution A) were added, and the incubation was continued for 1 h. To prepare solution A, the proper amount of BSA needed to prepare a 2% solution, unlabeled palmitic acid to achieve a final concentration 0.5 mM, and [14C]palmitic acid to make a 1 μCi/ml solution were weighed, incubated in a water bath to melt the palmitic acid, and then used for cell treatments. The release of 14CO2 and acid-soluble metabolites were collected for scintillation counting as previously reported (45).

**In vitro soleus muscle assay.** All procedures of animal handling were approved by the University of Wyoming Animal Care and Use Committee. Mouse soleus muscles were isolated from 2-mo-old C57BL/6J mice and pinned at a natural length in a rubber support. Soleus muscles were incubated in DMEM containing 0 or 200 μM ALA and subjected to BAPTA-AM and STO-609 treatments as described for C2C12 myotubes. Following 6-h treatments, the soleus muscle was collected, homogenized, and used for immunoblot analysis as described for C2C12 myotubes.

**Statistics.** All data are given as means ± SE of three or more independent experiments. Comparisons between multiple groups were made by ANOVA. The minimum level of significance was set at P < 0.05.

**RESULTS**

**ALA activates AMPK in C2C12 myotubes.** Incubation of C2C12 myotubes with ALA (100–500 μM for 6 h or 200 μM for 1–12 h) resulted in a dose- and time-dependent phosphorylation of AMPK-α at Thr172 (Fig. 1A). This phosphorylation was correlated with AMPK activity (Fig. 1). At the same time, we also measured the phosphorylation of ACC at Ser79, a site phosphorylated by AMPK. Phosphorylation of ACC at Ser79 is an indicator of AMPK activity. Consistent with AMPK activation, dose- and time-dependent increases of ACC phosphorylation at Ser79 by ALA treatment were also observed, showing increased AMPK activity (Fig. 1). The AMPK activation reached its maximum at 6 h following ALA treatment, and 6-h ALA treatments were used for further experiments. We also analyzed the phosphorylation of AMPK and ACC at 5, 15, and 30 min after ALA treatment, but no increased phosphorylation was observed (data not shown).

| Table 1. Effects of ALA on C2C12 adenine nucleotide contents |
|-----------------|-----------------|-----------------|-----------------|
| ALA, μM         | ATP, μmol/g protein | AMP, μmol/g protein | AMP/ATP          |
| 0               | 2.64 ± 0.336     | 1.07 ± 0.12     | 0.42 ± 0.002    |
| 100             | 2.45 ± 0.365     | 1.07 ± 0.17     | 0.44 ± 0.003    |
| 200             | 2.56 ± 0.256     | 1.01 ± 0.09     | 0.40 ± 0.001    |
| 500             | 2.37 ± 0.296     | 0.95 ± 0.05     | 0.40 ± 0.003    |

Values are means ± SE from 4 independent measurements. ALA, α-lipoic acid.
ALA does not change intracellular energy status. Since AMPK is the intracellular sensor and an increase in the AMP-to-ATP ratio can lead to AMPK activation, we measured the intracellular ATP, ADP, and AMP concentrations along with AMPK and ACC phosphorylation. Treatment of C2C12 myotubes with ALA for 6 h did not change intracellular ATP, ADP, and AMP levels or the calculated AMP-to-ATP ratio (Table 1). Since LKB1 activates AMPK in an AMP-dependent manner, we further analyzed the association between LKB1 and AMPK following 0 and 200 μM ALA treatments by immunoprecipitation. No differences in their association were observed between treatments (Fig. 2A). These data showed that ALA activates AMPK in an AMP-independent manner. Also, no alteration of PP2C activity was detected in myotubes with and without ALA treatment (Fig. 2B).

Intracellular Ca\(^{2+}\) is the trigger for ALA-stimulated AMPK activation. Incubation of C2C12 myotubes with ALA for 6 h increased the intracellular free Ca\(^{2+}\) concentration in a dose-dependent manner (Fig. 3), consistent with AMPK activation and ACC phosphorylation at Ser\(^{79}\) induced by ALA. The intracellular Ca\(^{2+}\) concentration increased from 104 nM within C2C12 myotubes without ALA (control) to 281 nM when myotubes were incubated with 500 μM ALA for 6 h (Fig. 3). To further confirm the role of intracellular Ca\(^{2+}\) in the ALA-induced AMPK activation, we used a cell membrane-permeable Ca\(^{2+}\) chelator, BAPTA-AM, to eliminate intracellular free Ca\(^{2+}\). Incubation of C2C12 myotubes with 25 μM BAPTA-AM for 30 min before treatment of cells with ALA completely abolished the ALA-stimulated AMPK activation and ACC phosphorylation at Ser\(^{79}\) (Fig. 4, A and B), which was correlated with the absence of a Ca\(^{2+}\) surge in myotubes receiving both ALA and BAPTA-AM (Fig. 4C). These data show that intracellular Ca\(^{2+}\) is necessary for AMPK activation stimulated by ALA.

CaMKK is the upstream AMPKK mediating ALA-induced AMPK activation. LKB1 and CaMKK are two upstream AMPKKs that phosphorylate and activate AMPK. ALA stimulation did not change the intracellular AMP-to-ATP ratio within C2C12 myotubes, indicating that LKB1 was unlikely involved in the activation of AMPK by ALA. On the other hand, ALA incubation increased the intracellular Ca\(^{2+}\) concentration.
centration, suggesting that CaMKK might be the upstream kinase because CaMKK is activated by Ca^{2+}/calmodulin binding. To test our hypothesis, STO-609, a selective CaMKK inhibitor, was employed. Inhibition of CaMKK by STO-609 reduced ALA-stimulated AMPK activation and ACC phosphorylation at Ser79 (Fig. 5). This reduction was dose dependent, with doses above 5 µg/ml STO-609 totally eliminating ALA-stimulated AMPK activation and ACC phosphorylation. Furthermore, incubation of C2C12 myotubes with STO-609 in the absence of ALA also led to a decrease in AMPK activity and a reduction of ACC phosphorylation at Ser79.

To actively phosphorylate AMPK, the association between CaMKK and AMPK needed to be enhanced following ALA treatment. Indeed, following 200 µM ALA treatment, enhanced CaMKK and AMPK association was detected by immunoprecipitation with both AMPK and CaMKK antibodies (Fig. 6).

Next, we used siRNA transfection to silence CaMKK-α expression in C2C12 cells. Silence of CaMKK-α was verified by immunoblot analysis (Fig. 7). Compared with the control without siRNA and the control with siRNA of the scrambled sequence, incubation of C2C12 cells with specific CaMKK-α siRNA decreased CaMKK-α content within cells by ∼85%. Protein expression of CaMKK-α within cells transfected with the specific siRNA became almost undetectable (Fig. 7A). Silence of CaMKK-α also reduced the basal phosphorylation of AMPK and ACC (Fig. 7, B and C).

Consistent with the downregulation of CaMKK-α, preincubation of C2C12 cells with CaMKK-α siRNA inhibited ALA-stimulated AMPK activation (Fig. 8). Incubation of cells with 200 µM ALA for 6 h significantly (P < 0.05) increased AMPK phosphorylation in cells preincubated with transfection reagent only or siRNA with a scrambled sequence compared with untreated cells. For cells preincubated with CaMKK-α siRNA, incubation of cells with 200 µM ALA for 6 h did not increase AMPK phosphorylation. Correlating with AMPK activity, preincubation of C2C12 cells with CaMKK-α siRNA also abolished ALA-stimulated ACC phosphorylation (Fig. 8). All these results show that CaMKK-α is the upstream AMPKK that mediates ALA-stimulated AMPK activation in C2C12 cells.

To further show the role of CaMKK in AMPK activation, we used isolated soleus muscle, which was incubated in 200 µM ALA with or without BAPTA-AM and STO-609. Consistent with the results in myotubes, ALA increased AMPK phosphorylation (Fig. 9). In addition, this phosphorylation was inhibited by BAPTA-AM, which removed intercellular Ca^{2+}, and STO-609, which inhibited CaMKK activity (Fig. 9), showing that CaMKK is the AMPKK leading to AMPK activation induced by ALA.

ALA enhanced fatty acid oxidation in C2C12 myotubes. To show the function of ALA, the fatty acid oxidation in C2C12 myotubes was analyzed. Treatment with ALA dose dependently increased the fatty acid oxidation in C2C12 myotubes (Fig. 10).
ALA has been used for decades as an antidiabetic agent (7). A huge body of evidence has well defined that ALA increases glucose uptake in skeletal muscle, reduces blood glucose levels (5, 6, 23, 42), and increases insulin sensitivity in individuals with Type 2 diabetes (7, 20–22). Recently, it has been demonstrated that ALA activates AMPK, a kinase that has a key role in mediating energy metabolism in cells (10, 14, 25, 26, 51). In peripheral tissues, ALA improves insulin sensitivity and increases glucose uptake and fatty acid oxidation by activating AMPK in obese rats (28). ALA-stimulated AMPK activation has also been observed in the heart (29). ALA activation of AMPK in endothelial cells mitigated vascular dysfunction by normalizing lipid metabolism (27). Furthermore, our previous study (38) has showed that dietary ALA supplementation to mice increased AMPK activity in mouse skeletal muscle. In this study, ALA treatment enhanced fatty acid oxidation in C2C12 myotubes. Since fatty acid accumulation in muscle is known to induce insulin resistance, these data confirm the role of ALA as an antidiabetic agent. However, we observed that the stimulation effect of ALA on fatty acid oxidation peaked at 100 μM ALA without additional enhancement by further increasing ALA concentration. The possible reason may be due

**Fig. 7.** Effect of CaMKK-α silence on CaMKK expression and AMPK and ACC phosphorylation of C2C12 cells. C2C12 cells were incubated with transfection reagent only (control) or in the presence of non-specific short interfering (si)RNA (control siRNA) or synthetic RNA duplexes targeted against mouse CaMKK-α. After transfection, cells were grown in DMEM for 48 h. A: CaMKK-α concentration. B: AMPK phosphorylation at Thr172. C: ACC phosphorylation at Ser79. Representative blots (top) and densitometric analyses (bottom) are shown. Values are means ± SE from 3 independent measurements. *p < 0.05 vs. samples treated with no siRNA.

**Fig. 8.** Effect of CaMKK-α silence on ALA-stimulated AMPK activation. C2C12 cells were first preincubated with transfection reagent only (control) or in the presence of nonspecific siRNA (control siRNA) or synthetic RNA duplexes targeted against mouse CaMKK-α. After transfection, cells were grown in DMEM for 48 h and then treated with or without ALA for 6 h. AMPK phosphorylation (Thr172) and ACC phosphorylation (Ser79) were determined by immunoblot analysis. A: representative blots (top) and densitometric analysis (bottom) of AMPK. B: representative blots (top) and densitometric analysis (bottom) of ACC. Values are means ± SE from 3 independent measurements. *p < 0.05 vs. samples treated with no siRNA and no ALA.

**DISCUSSION**

ALA has been used for decades as an antidiabetic agent (7). A huge body of evidence has well defined that ALA increases glucose uptake in skeletal muscle, reduces blood glucose levels (5, 6, 23, 42), and increases insulin sensitivity in individuals with Type 2 diabetes (7, 20–22). Recently, it has been demonstrated that ALA activates AMPK, a kinase that has a key role in mediating energy metabolism in cells (10, 14, 25, 26, 51). In peripheral tissues, ALA improves insulin sensitivity and increases glucose uptake and fatty acid oxidation by activating AMPK in obese rats (28). ALA-stimulated AMPK activation has also been observed in the heart (29). ALA activation of AMPK in endothelial cells mitigated vascular dysfunction by normalizing lipid metabolism (27). Furthermore, our previous study (38) has showed that dietary ALA supplementation to mice increased AMPK activity in mouse skeletal muscle. In this study, ALA treatment enhanced fatty acid oxidation in C2C12 myotubes. Since fatty acid accumulation in muscle is known to induce insulin resistance, these data confirm the role of ALA as an antidiabetic agent. However, we observed that the stimulation effect of ALA on fatty acid oxidation peaked at 100 μM ALA without additional enhancement by further increasing ALA concentration. The possible reason may be due
to the competitive inhibition of fatty acid oxidation by ALA in cultured cells, because ALA has a very similar structure with palmitic acid, which may inhibit palmitate oxidation at high levels.

In agreement with the above reports, the present in vitro study shows that ALA activates AMPK and increases ACC phosphorylation at Ser\(^{79}\) in skeletal muscle cells. AMPK was activated in C2C12 myotubes after 3 h of incubation, peaked at 6 h, and then abated. This result is in agreement with a previous report (32) on ALA and glucose uptake in 3T3-L1 cells, where ALA stimulated glucose uptake before 6 h and then declined. The exact mechanism underlying ALA-stimulated AMPK activation remains to be defined. Here, for the first time, we investigated the mechanism of AMPK activation by ALA stimulation in C2C12 myotubes. The data showed that the tumor suppressor LKB1 may not be involved in the ALA-stimulation in C2C12 myotubes. The data showed that the upstream kinase that is mainly responsible for the ALA-stimulated AMPK activation in C2C12 myotubes. Our data showed that ALA modulates intracellular Ca\(^{2+}\) mobilization and increases the intracellular Ca\(^{2+}\) concentration. Ca\(^{2+}\), along with calmodulin, initiates a signaling pathway leading to AMPK activation (17, 19, 49). The AMPK activity in LKB1\(^{-/-}\) mouse embryo fibroblasts increased multiple folds upon stimulation by the Ca\(^{2+}\) ionophore ionomycin and/or A23187, clearly showing that CaMKK is an AMPKK along with LKB1 (17, 49).

CaMKK has two isoforms: CaMKK-\(\alpha\) and CaMKK-\(\beta\). While LKB1 is ubiquitously expressed, it is believed that CaMKKs, especially CaMKK-\(\beta\), are localized in neural tissues (1). However, skeletal muscle expresses only CaMKK-\(\alpha\) (24, 46, 47). Our results showed that ALA-stimulated AMPK activation was inhibited by chelation of intracellular free Ca\(^{2+}\), selective inhibition of CaMKK by STO-609, and silence of CaMKK-\(\alpha\) expression by siRNA. In addition, the association between AMPK and CaMKK was enhanced due to ALA treatment. These data indicate that the Ca\(^{2+}\)/CaMKK-mediated pathway was crucial for AMPK activation stimulated by ALA.

It has been demonstrated that hyperosmotic stress and the antidiabetic drug metformin activate AMPK independent of AMP within muscle cells (11). It has also been reported that metformin could modulate intracellular Ca\(^{2+}\) mobilization (3, 37). So, it is likely that metformin activates AMPK in muscle cells by the regulation of the intracellular Ca\(^{2+}\) concentration. More recently, two groups of researchers from different laboratories (9, 45) have reported that other fatty acids, palmitate and linoleate, activated AMPK, increased ACC phosphorylation, and fatty acid oxidation with no change in the AMP-to-ATP ratio in skeletal cells. To explain the AMPK activation, it has been proposed that AMPK might become a better substrate for LKB1 via a fatty acid-induced allosteric effect (45). However, in this study, we did not detect an alteration in the association between LKB1 and AMPK due to ALA treatment, indicating that LKB1 might not be involved in the activation of AMPK induced by ALA. The involvement of the CaMKK-dependent pathway in AMPK activation stimulated by fatty acids has not been previously examined. Data obtained in this study suggested that the CaMKK-dependent pathway has an important role in fatty acid-stimulated AMPK activation. This notion is fortified by a recent report (41) showing that thrombin activated AMPK in endothelial cells via a CaMKK-dependent pathway. And, very recently, CaMKK was demonstrated to
activate AMPK in skeletal muscle (24, 47). These results suggest that CaMKK-mediated AMPK activation may play an important role in cell responses to nutrients, hormones, and certain drugs in skeletal muscle.

In summary, our present study demonstrates that CaMKK mediates AMPK activation stimulated by ALA. ALA activates AMPK in C2C12 myotubes by the increasing intracellular Ca$^{2+}$ concentration, which then binds to and activates CaMKK, leading to AMPK activation without a change of the AMP-to-ATP ratio. As a function of AMPK activation, ALA stimulates fatty acid oxidation in C2C12 cells.

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