Lithium downregulates phosphorylated acetyl-CoA carboxylase 2 and attenuates mitochondrial fatty acid utilization and oxidative stress in cardiomyocytes

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Abstract. Acetyl-CoA carboxylase 2 plays a crucial role in regulating mitochondrial fatty acid oxidation in cardiomyocytes. Lithium, a monovalent cation known for its cardioprotective potential, has been investigated for its influence on mitochondrial bioenergetics. The present study explored whether lithium modulated acetyl-CoA carboxylase 2 and mitochondrial fatty acid metabolism in cardiomyocytes and the potential therapeutic applications of lithium in alleviating metabolic stress. Mitochondrial bioenergetic function, fatty acid oxidation, reactive oxygen species production, membrane potential and the expression of proteins involved in fatty acid metabolism in H9c2 cardiomyocytes treated with LiCl for 48 h was measured by using a Seahorse extracellular flux analyzer, fluorescence microscopy and western blotting. Small interfering RNA against glucose transporter type 4 was transfected into H9c2 cardiomyocytes for 48 h to induce metabolic stress mimicking insulin resistance. The results revealed that LiCl at a concentration of 0.3 mM (but not at a concentration of 0.1 or 1.0 mM) upregulated the expression of phosphorylated (p-)glycogen synthase kinase-3 beta and downregulated the expression of p-acetyl-CoA carboxylase 2 but did not affect the expression of adenosine monophosphate-activated protein kinase or calcineurin. Cotreatment with TWS119 (8 μM) and LiCl (0.3 mM) downregulated p-acetyl-CoA carboxylase 2 expression to a similar extent as did treatment with TWS119 (8 μM) alone. Moreover, LiCl (0.3 mM) inhibited mitochondrial fatty acid oxidation, improved coupling efficiency and the cellular respiratory control ratio, hindered reactive oxygen species production and proton leakage and restored mitochondrial membrane potential in glucose transporter type 4 knockdown-H9c2 cardiomyocytes. These findings suggested that low therapeutic levels of lithium can downregulate p-acetyl-CoA carboxylase 2, thus reducing mitochondrial fatty acid oxidation and oxidative stress in cardiomyocytes.

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Introduction

Growing evidence suggests that disruptions in mitochondrial energy metabolism can contribute to the development of various cardiac diseases (1-5). Among the fuel sources utilized by the myocardium, increased fatty acid oxidation plays a pivotal role in driving the pathogenesis of cardiomyopathy (1-7). The inhibition of fatty acid oxidation has been demonstrated to

alleviate cardiac dysfunction (2,4,6,8). Acetyl-CoA carboxylases (ACCs) are key enzymes responsible for catalyzing the carboxylation of acetyl-CoA into malonyl-CoA (9). Mammalian cells contain two isoforms of ACC, namely ACC1 and ACC2. ACC1 serves as the rate-limiting enzyme responsible for fatty acid biosynthesis in the cytoplasm of adipocytes and hepatocytes. ACC2 is located in the outer mitochondrial membrane of cardiomyocytes and governs mitochondrial fatty acid oxidation (9,10). Therapeutics targeting ACC2 have the potential to regulate mitochondrial fatty acid utilization, reduce oxidative stress and confer cardioprotective benefits.

For the preceding five decades, lithium has served as the first-line medication for treating bipolar disorder (11). Human and preclinical studies have both demonstrated that in addition to its mood-stabilizing effects, lithium can enhance the activity of mitochondrial complex I while hindering the formation of free radicals and reducing lipid peroxidation and DNA damage (12-14). Furthermore, low-dose lithium has been shown to promote longevity (15,16) and reduce the risk of heart failure (17,18). These findings suggest that low-dose lithium may possess cytoprotective properties and thus may play an essential role in regulating metabolic stress. However, whether lithium exerts cardioprotective effects through the modulation of cardiac metabolism remains unclear. Laboratory evidence has indicated that lithium directly targets glycogen synthase kinase-3 beta (GSK-3β) (19), a crucial protein kinase that inhibits the activity of ACC (20,21). The present study investigated whether lithium can regulate the activity of ACC2 and modulate mitochondrial fatty acid oxidation in cardiomyocytes, with a focus on the potential of lithium for mitigating metabolic stress. The results revealed that at a low physiological concentration (0.3 mM), LiCl upregulated the expression of phosphorylated (p-)GSK-3β and downregulated the level of p-ACC2 in H9c2 cardiomyocytes. Additionally, when used in combination with the GSK-3β inhibitor TWS119, LiCl (0.3 mM) downregulated the expression of p-ACC2, an effect comparable to treatment with TWS119 alone. Furthermore, LiCl (0.3 mM) inhibited mitochondrial fatty acid oxidation, enhanced coupling efficiency and the cellular respiratory control ratio, suppressed reactive oxygen species (ROS) production and proton leakage and restored mitochondrial membrane potential in glucose transporter type 4 (GLUT4)-knockdown H9c2 cardiomyocytes. Taken together, these findings suggest that at a low physiological concentration, lithium can inhibit mitochondrial fatty acid utilization and mitigate oxidative stress in cardiomyocytes, potentially through its upregulation of p-GSK-3β and downregulation of p-ACC2.

Materials and methods

Cell culture and treatment. The H9c2 cell line (cat. no. 60096) was purchased from the Bioresource Collection and Research Center and cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich; Merck KGaA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich; Merck KGaA) at 37˚C in a humidified atmosphere with 5% CO_2. To retain the differentiation capacity and mitochondrial respiratory activity, a subculture was performed when the cells reached 80% confluence. The culture medium was changed every 2-3 days. H9c2 cells were treated with LiCl (Sigma-Aldrich; Merck KGaA) at concentrations of 0.1 mM (i.e., subtherapeutic), 0.3 mM (i.e., low therapeutic), or 1.0 mM (i.e., high therapeutic) for 48 h. Additionally, these cells were cotreated with LiCl and a GSK-3β inhibitor, namely TWS119 (Sigma-Aldrich; Merck KGaA), at a concentration of 8 μM for 48 h to evaluate whether lithium downregulated p-ACC2 by modulating GSK-3β activity. Each experiment was performed at least three times.

GLUT4-knockdown cellular model. To simulate insulin resistance and induce metabolic stress in H9c2 cardiomyocytes, a GLUT4-knockdown cellular model was established. H9c2 cardiomyocytes were seeded at a density of $2x10^5$ cells per well on a 6-well plate and transfected when reaching ~80% confluence. Transfection was performed with either 50 nM GLUT4 small interfering (si)RNA (sense 5'-GCUGUUUUC UACUAUUCAAtt-3', antisense 5'-UUGAAUAGUAGA AAACAGCat-3'; cat. no. s73928; Thermo Fisher Scientific, Inc.) or 50 nM negative control siRNA (sense 5'-UAACGA CGCGACGACGUAAtt-3', antisense 5'-UUACGUCGUCGC GUCGUUAtt-3'; cat. no. 4390843; Thermo Fisher Scientific, Inc.) using Lipofectamine® RNAiMax Transfection Reagent (Thermo Fisher Scientific, Inc.) for 48 h at 37˚C. The protein knockdown efficiency of GLUT4 and protein expression levels of carnitine palmitoyl transferase 1, the mitochondrial enzyme responsible for the translocation of fatty acids from the cytosol to the mitochondrial matrix, were assessed 48 h after siRNA transfection (Fig. S1). LiCl was administered 24 h post-initiation of transfection. For experiments in which GLUT4-knockdown was combined with LiCl administration, cells were incubated for an additional 48 h. In groups without LiCl treatment, cells were incubated for an equivalent 48 h period, matching the incubation time of the LiCl-treated groups.

Measurement of mitochondrial bioenergetic function. Mitochondrial bioenergetic function was assessed using the XFe24 Extracellular Flux Analyzer (Seahorse Bioscience) and Seahorse XF Cell Mito Stress Test kit (Seahorse Bioscience). H9c2 cells were initially seeded at a density of 7,000 cells per well on a Seahorse XFe24 culture plate and cultured with DMEM supplemented with 10% FBS for 48 h. On the day of the assay, the culture medium was substituted with Seahorse assay medium supplemented with 25 mM glucose, 1 mM pyruvate and 2 mM glutamine. Subsequently, a series of injections was administered, including 1.5 μM oligomycin, 3 μM carbonyl cyanide p-trifluoromethoxy phenylhydrazone (FCCP) and 0.5 μM rotenone/antimycin A. Basal respiration (last rate measurement before oligomycin injection-minimum rate measurement after rotenone/antimycin A injection), adenosine triphosphate (ATP) production (last rate measurement before oligomycin injection-minimum rate measurement after oligomycin injection), proton leakage (minimum rate measurement after oligomycin injection-minimum rate measurement after rotenone/antimycin A injection) and maximal respiration (maximum rate measurement after FCCP injection-minimum rate measurement after rotenone/antimycin A injection) were determined using our previously described methods (22). In addition, coupling efficiency and the cell respiratory control

ratio were analyzed as these measures are internally normalized bioenergetic parameters used to assess the proportion of mitochondrial respiratory activity contributing to ATP generation (i.e., coupling efficiency) and the degree of change in mitochondrial respiratory activity attributable to proton leakage (i.e., the cell respiratory control ratio) (23,24). Coupling efficiency was calculated by dividing ATP production by basal respiration. The cell respiratory control ratio was calculated by dividing maximal respiration by proton leakage.

Measurement of mitochondrial fatty acid oxidation. Mitochondrial fatty acid oxidation was assessed using the XFe24 Extracellular Flux Analyzer (Seahorse Bioscience) and Seahorse XF substrate oxidation stress test kit (Seahorse Bioscience). H9c2 cells were initially seeded at a density of 7,000 cells per well on a Seahorse XFe24 culture plate and cultured with DMEM supplemented with 10% FBS for 48 h. On the day of the assay, the culture medium was substituted with Seahorse assay medium containing 25 mM glucose, 1 mM pyruvate and 2 mM glutamine. During the substrate oxidation stress test, etomoxir was injected at a concentration of 40 μM. Mitochondrial fatty acid oxidation was estimated by monitoring the change in the oxygen consumption rate following etomoxir injection.

Measurement of mitochondrial ROS. Mitochondrial ROS levels were assessed using MitoSox Red dye (Invitrogen; Thermo Fisher Scientific, Inc.) and a fluorescence microscopy system (EVOS M5000 Imaging System; Thermo Fisher Scientific, Inc.) in accordance with our previously described methods (22). In brief, H9c2 cells were seeded at a density of 5,000 cells per well on a 96-well plate and cultured with DMEM supplemented with 10% FBS for 48 h. Prior to fluorescence microscopy, the H9c2 cells were loaded with MitoSox Red at a concentration of 5 μM and Hoechst 33423 (Sigma-Aldrich; Merck KGaA) at a concentration of 1 μg/ml for a 30-min incubation period at 37˚C. Fluorescence intensity was then measured in four randomly selected fields in each well and quantified using ImageJ 1.52a software (National Institutes of Health).

Measurement of mitochondrial membrane potential. Mitochondrial membrane potential was assessed using the TMRE Mitochondrial Membrane Potential Assay Kit (Cayman Chemical Company) and fluorescence microscopy (EVOS M5000 Imaging System; Thermo Fisher Scientific, Inc.). In brief, H9c2 cells were seeded at a density of 5,000 cells per well on a 96-well plate and cultured with DMEM supplemented with 10% FBS for 48 h. Prior to fluorescence microscopy, the H9c2 cells were loaded with tetramethylrhodamine ethyl ester at a concentration of 125 nM and HOECHST 33423 at a concentration of 1 μg/ml for a 30-min incubation period at 37˚C in accordance with the manufacturer's instructions. Fluorescence intensity was measured in four randomly selected fields within each well and quantified using ImageJ 1.52a software (National Institutes of Health).

Western blot analysis. Western blotting was performed as previously described (25). Briefly, H9c2 cells were lysed using protein extraction reagent (cat. no. 78501; Thermo Fisher Scientific, Inc.). Protein concentrations were determined using Qubit™ Protein Assay Kits (Thermo Fisher Scientific, Inc.). Subsequently, 30 μg protein/lane was separated using 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. This process was followed by the electrophoretic transfer of the separated proteins onto equilibrated polyvinylidene difluoride membranes. These membranes were then blocked with 5% skimmed milk for 1 h at room temperature. Following this blocking procedure, the membranes were incubated overnight at 4˚C with specific antibodies against total ACC2 (1:2,000; monoclonal; cat. no. ab45174; Abcam), p-(p-) ACC2 (1:500; polyclonal; cat. no. 07303; Millipore), total AMP-activated protein kinase (AMPK; 1:500; monoclonal; cat. no. 5831; Cell Signaling), p-AMPK (1:1,000; polyclonal; cat. no. 07681; Millipore), calcineurin (1:10,000; monoclonal; cat. no. ab109412; Abcam), total GSK-3β (1:1,000; monoclonal; cat. no. 9315; Cell Signaling), p-GSK-3β (1:1,000; polyclonal; cat. no. 9336; Cell Signaling) and GLUT4 (1:500; monoclonal; cat. no. sc-53566; Santa Cruz). After washing with PBS containing Tween 20 (0.1%) for 15 min at room temperature, a peroxidase-conjugated secondary antibody (anti-rabbit IgG; 1:1,000; cat. no. HAF008; or anti-mouse IgG; 1:500; cat. no. HAF007; R&D Systems, Inc.) was added for incubation for 1 h at room temperature. Bound antibodies were detected using an enhanced chemiluminescence detection system (MilliporeSigma) and the results were analyzed using AlphaEaseFC 4.0.0.34 software (ProteinSimple). Targeted bands were normalized to glyceraldehyde 3-phosphate dehydrogenase (1:50,000; monoclonal; cat. no. M171-1; MBL) or β-actin (1:10,000; polyclonal; cat. no. ab6274; Abcam) to confirm equal protein loading.

Statistical analysis. Quantitative data are presented as mean ± standard error of the mean. Statistical significance in H9c2 cells exposed to various conditions was determined using a one-way analysis of variance followed by Tukey's post hoc test. Statistical analysis was performed using SigmaPlot 12.3 software (Systat Software, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of lithium on ACC2 in H9c2 cardiomyocytes. In H9c2 cardiomyocytes treated with 0.3 mM LiCl for 48 h, the expression of p-ACC2 was significantly downregulated compared with the control (Fig. 1A). However, LiCl at concentrations of 0.1 and 1.0 mM had only a minimal effect on the expression of p-ACC2. Notably, no significant differences were observed in the expression of total ACC2 between the control group and H9c2 cardiomyocytes treated with LiCl at concentrations of 0.1, 0.3 and 1 mM.

Effects of lithium on GSK-3β in H9c2 cardiomyocytes. As GSK-3β is a potential regulator of ACC2, it was investigated whether lithium regulated GSK-3β activity in H9c2 cardiomyocytes. Compared with the control, LiCl at a concentration of 0.3 mM upregulated the expression of p-GSK-3β by 55.4% (Fig. 1B). By contrast, LiCl at concentrations of 0.1 and 1.0 mM did not significantly affect the expression

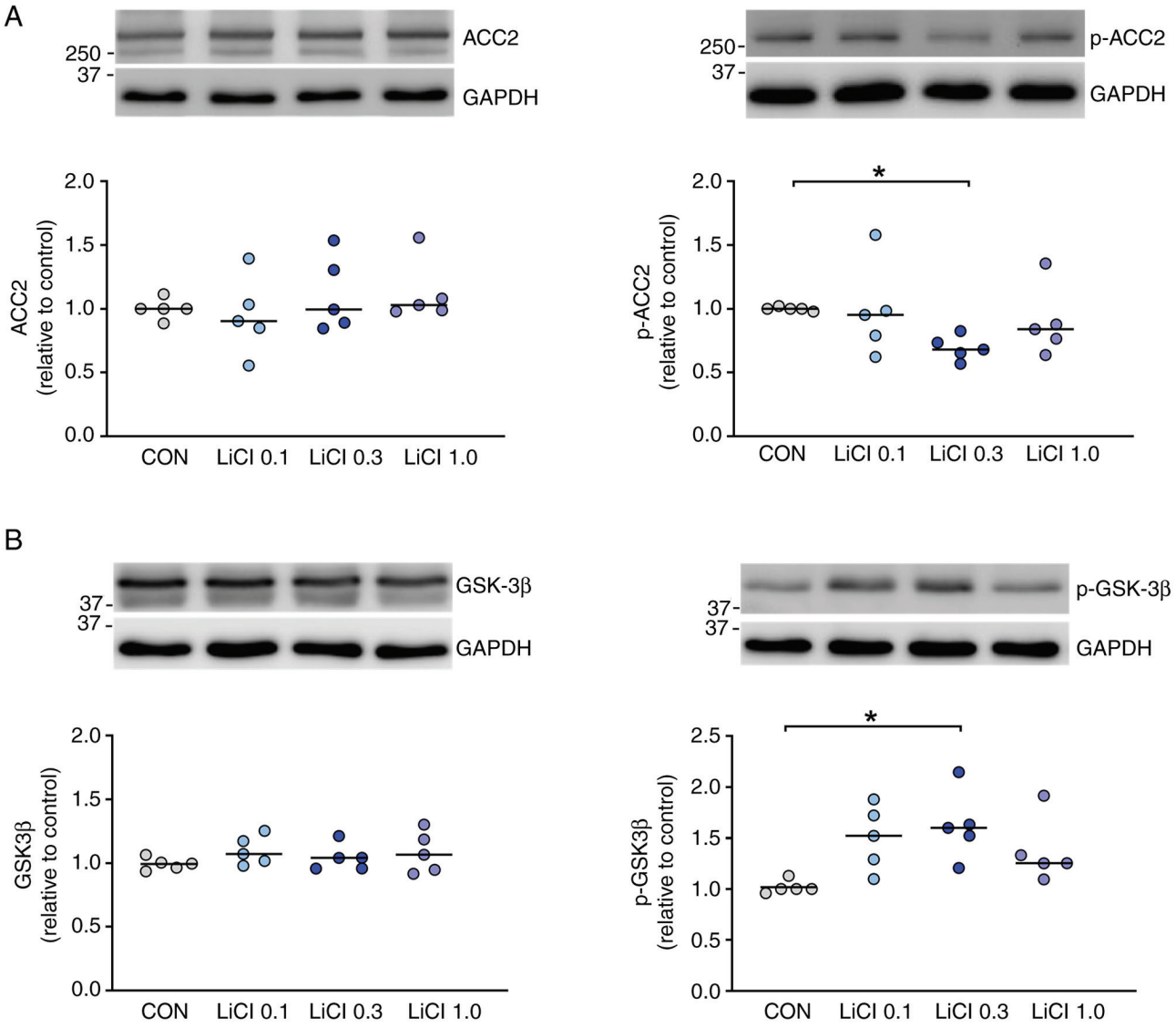

Figure 1. Expression ACC2 and GSK-3β in H9c2 cardiomyocytes treated with LiCl. (A) Compared with control cells (n=5), H9c2 cells treated with LiCl at 0.3 mM (n=5) for 48 h exhibited downregulated expression of p-ACC2. However, LiCl at 0.1 (n=5) or 1.0 mM (n=5) had no significant effect on the expression of p-ACC2. Additionally, the expression of total ACC2 did not significantly differ between the control cells and those treated with LiCl at concentrations of 0.1, 0.3 and 1 mM. (B) H9c2 cells treated with LiCl at 0.3 mM (n=5) for 48 h exhibited upregulated expression of p-GSK-3β relative to the control cells (n=5). However, LiCl at 0.1 (n=5) and 1.0 mM (n=5) did not significantly affect the expression of p-GSK-3β. The expression of total GSK-3β did not significantly differ between the control cells and those treated with LiCl at concentrations of 0.1, 0.3 and 1 mM. *P<0.05. ACC2, acetyl-CoA carboxylase 2; GSK-3β, glycogen synthase kinase-3 beta; p-, phosphorylated; CON, control.

of p-GSK-3β in H9c2 cardiomyocytes. No significant differences in the expression of total GSK-3β were observed between the control group and H9c2 cardiomyocytes treated with LiCl at concentrations of 0.1, 0.3 and 1 mM. To confirm whether lithium downregulated the expression of p-ACC2 by modulating GSK-3β activity, H9c2 cardiomyocytes were treated with TWS119, a GSK-3β inhibitor. The expression of p-ACC2 in H9c2 cardiomyocytes was downregulated to a similar extent in cells subjected to combined treatment with TWS119 (8 μM) and LiCl (0.3 mM) and in those treated with TWS119 (8 μM) alone (Fig. 2). These findings suggest that lithium downregulated the expression of p-ACC2 through the modulation of GSK-3β activity. As the activities of ACC1 and ACC2 are regulated by numerous protein kinases and phosphatases, the present study also examined the effects of lithium on AMPK and calcineurin, the two principal protein kinases involved in the regulation of ACC2 activity in H9c2 cardiomyocytes. It was observed that LiCl did not significantly affect the expression level of total or p-AMPK or calcineurin in H9c2 cardiomyocytes (Fig. S2).

Effects of lithium on mitochondrial bioenergetic function in GLUT4-knockdown H9c2 cardiomyocytes. To explore the therapeutic potential of lithium for mitigating metabolic stress, mitochondrial bioenergetic function was evaluated in GLUT4-knockdown H9c2 cardiomyocytes treated with lithium. Compared with the control cells, the GLUT4-knockdown H9c2 cardiomyocytes exhibited greater proton leakage (Fig. 3). Additionally, the GLUT4-knockdown H9c2 cardiomyocytes exhibited greater reductions in coupling efficiency and the cell respiratory control ratio compared with the control cells. Furthermore,

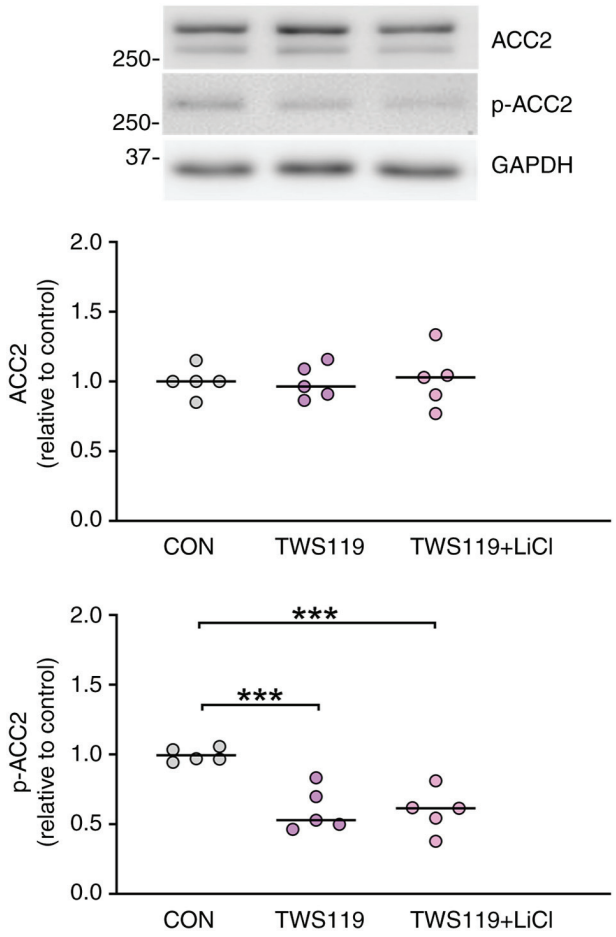

Figure 2. Expression of ACC2 in H9c2 cardiomyocytes treated with a glycogen synthase kinase-3 beta GSK-3β inhibitor. Cotreatment with TWS119 (8 μM) and LiCl (0.3 mM) downregulated the expression of p-ACC2 in H9c2 cells to a similar extent as did treatment with TWS119 (8 μM) alone. The expression of total ACC2 did not significantly differ between the control cells and those treated with TWS119 (8 μM alone or cotreatment with TWS119 [8 μM] and LiCl [0.3 mM]). ***P<0.001. ACC2, acetyl-CoA carboxylase 2; GSK-3β, glycogen synthase kinase-3 beta; p-, phosphorylated; CON, control.

GLUT4-knockdown H9c2 cardiomyocytes treated with LiCl (0.3 mM) exhibited a reduction in proton leakage and improvement in coupling efficiency and cell respiratory control ratio compared with cells not treated with LiCl. These findings indicated that LiCl at a concentration of 0.3 mM enhanced mitochondrial bioenergetic function in GLUT4-knockdown H9c2 cardiomyocytes.

Effects of lithium on mitochondrial ROS and membrane potential in GLUT4-knockdown H9c2 cardiomyocytes. The effects of lithium on mitochondrial ROS and membrane potential in GLUT4-knockdown H9c2 cardiomyocytes are illustrated in Fig. 4. Compared with the control cells, the GLUT4-knockdown H9c2 cardiomyocytes exhibited higher levels of mitochondrial ROS. By contrast, the GLUT4-knockdown cells treated with LiCl (0.3 mM) had mitochondrial ROS levels similar to those in the control cells. Furthermore, the GLUT4-knockdown H9c2 cardiomyocytes exhibited reduced mitochondrial membrane potential in contrast to both the control cells and the LiCl (0.3 mM)-treated GLUT4-knockdown cells. These findings suggested that LiCl at a concentration of 0.3 mM attenuated mitochondrial ROS and restored the mitochondrial membrane potential in GLUT4-knockdown H9c2 cardiomyocytes.

Effects of lithium on mitochondrial fatty acid oxidation in GLUT4-knockdown H9c2 cardiomyocytes. The effects of lithium on mitochondrial fatty acid oxidation in GLUT4-knockdown H9c2 cardiomyocytes are depicted in Fig. 5. Compared with the control cells, the GLUT4-knockdown H9c2 cardiomyocytes exhibited a greater elevation in mitochondrial fatty acid oxidation. Additionally, when GLUT4-knockdown H9c2 cardiomyocytes were treated with LiCl (0.3 mM), they exhibited a greater reduction in mitochondrial fatty acid oxidation compared with GLUT4-knockdown H9c2 cardiomyocytes not treated with LiCl. These findings suggested that LiCl at a concentration of 0.3 mM attenuated the increase in mitochondrial fatty acid oxidation in GLUT4-knockdown H9c2 cardiomyocytes.

Discussion

Studies have suggested that ACC2 can be inactivated through phosphorylation (9,10). In the present study, for the first time to the best of the authors' knowledge, LiCl at a concentration of 0.3 mM, representing a low therapeutic level but not a supraphysiological level, was observed to downregulate the expression of p-ACC2 in H9c2 cardiomyocytes but not to affect the expression of total ACC2. The downregulation of p-ACC2 induced by lithium may activate ACC2 and subsequently elevate the level of malonyl-CoA (9). Such an increase in malonyl-CoA levels can inhibit carnitine palmitoyltransferase 1, which is crucial for the transport of long-chain fatty acyl-CoAs into the mitochondria for β-oxidation (26,27). Additionally, the present study also revealed that LiCl at a low therapeutic level attenuated mitochondrial fatty acid oxidation in H9c2 cardiomyocytes.

The inhibition of fatty acid oxidation has been demonstrated to have beneficial effects related to heart failure associated with diabetes mellitus (2,4,8,28,29). In patients with diabetes, heart failure often presents a shift in cardiac fuel substrate utilization toward an increased reliance on more mitochondrial fatty acid oxidation, a change driven by insulin resistance (1,6). Such an increase in fatty acid oxidation can overwhelm mitochondria, resulting in oxidative stress (6). The subsequent generation of ROS from lipid-overburdened mitochondria may worsen insulin resistance and accelerate the progression of heart failure (6). Thus, inhibiting fatty acid oxidation in patients with diabetic cardiomyopathy may offer cardioprotection by reducing oxidative stress, promoting glucose utilization and enhancing cardiac efficiency (6,30-32). Supporting this hypothesis, a previous study found that a low dose of lithium (0.36±0.03 mM) mitigated cardiac dysfunction in an experimental animal model to investigate sleep deprivation (33). The current study corroborated the cardioprotective potential of lithium in a GLUT4-knockdown cellular model designed to simulate insulin resistance (34-36). This finding agrees with previous findings on the benefits of lithium on

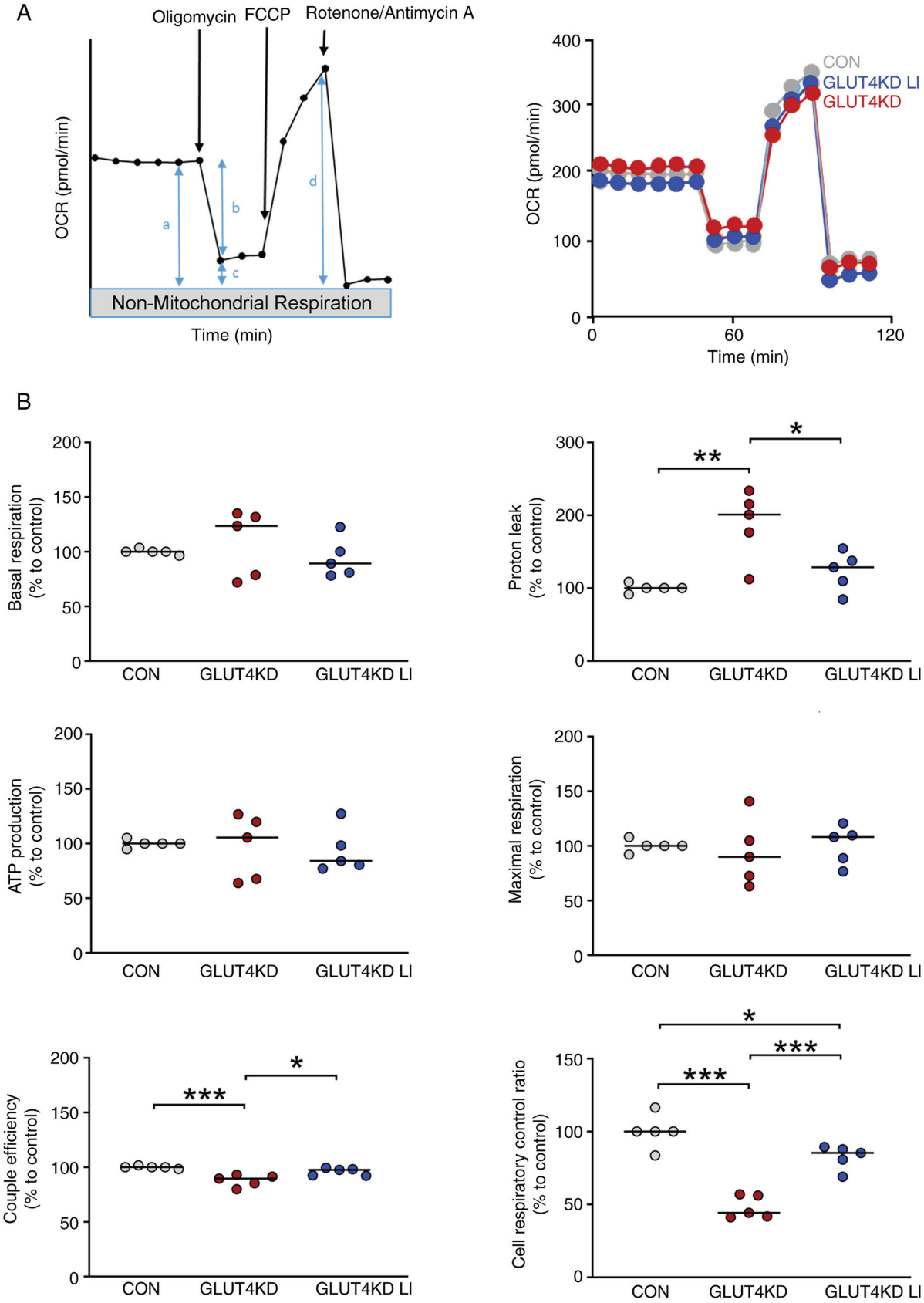

Figure 3. Mitochondrial bioenergetic function in GLUT4-knockdown H9c2 cardiomyocytes treated with LiCl. (A) Schematic and representative tracing of oxygen consumption rates, following the sequential injection of oligomycin (1.5 μM), carbonyl cyanide p-trifluoromethoxy phenylhydrazone (3 μM) and rotenone/antimycin A (0.5 μM). The derived bioenergetic parameters are indicated as a, basal respiration; b, ATP production; c, proton leakage; d, maximal respiration; b/a: coupling efficiency; d/c: cell respiratory control ratio. (B) The knockdown of GLUT4 with small interfering RNA at 50 nM in H9c2 cardiomyocytes for 48 h (n=5) resulted in elevated proton leakage, impaired mitochondrial coupling efficiency and a reduced cell respiratory control ratio compared with the control cells (n=5). Treatment with LiCl at a concentration of 0.3 mM (n=5) attenuated proton leakage and enhanced mitochondrial coupling efficiency and the cell respiratory control ratio in GLUT4-knockdown H9c2 cardiomyocytes. *P<0.05, **P<0.01, ***P<0.001. GLUT4, glucose transporter type 4; KD, knockdown; CON, control.

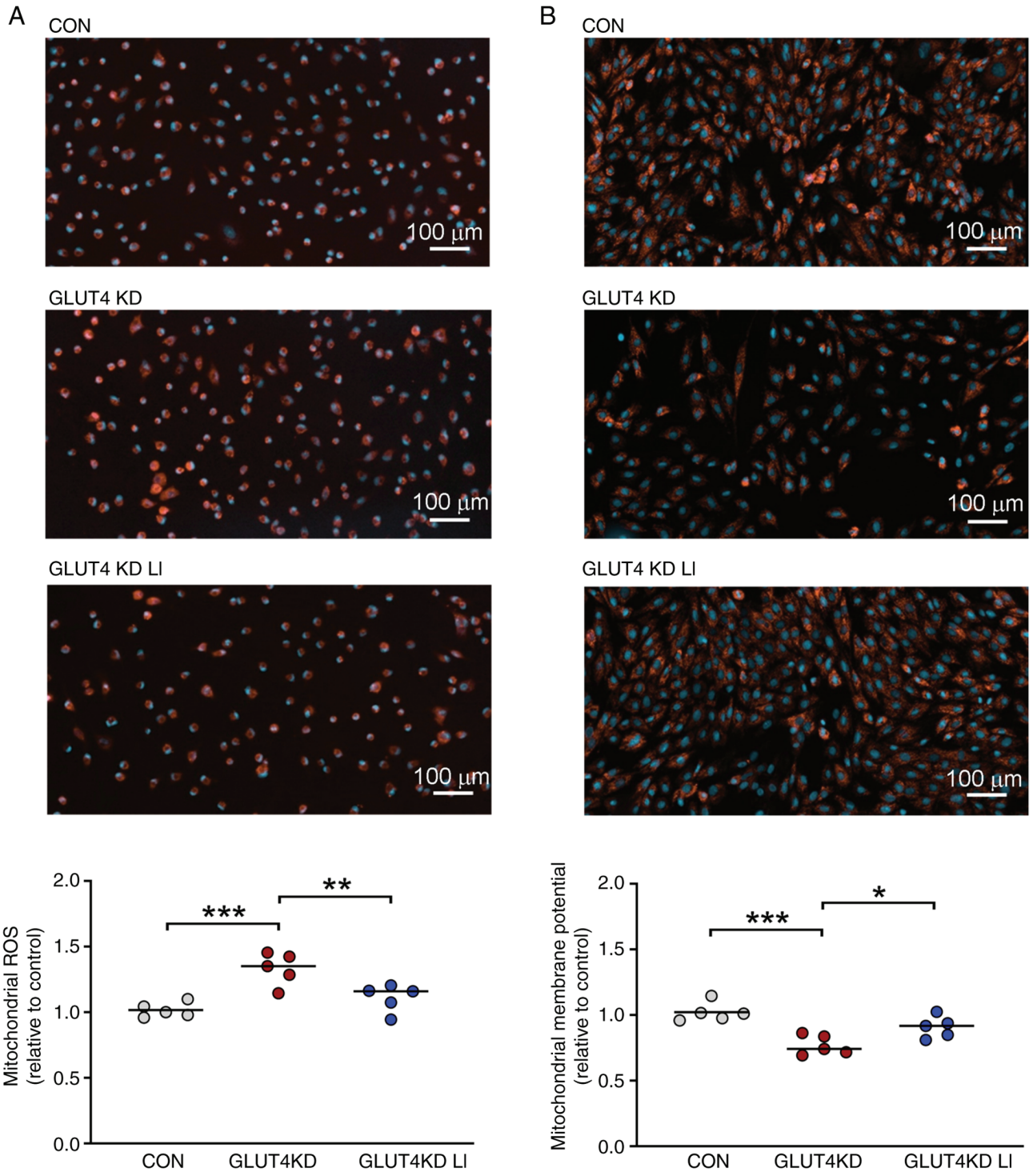

Figure 4. Mitochondrial ROS and membrane potential in GLUT4-knockdown H9c2 cardiomyocytes treated with LiCl. (A) Compared with control cells (n=5), knockdown of GLUT4 with small interfering RNA at 50 nM in H9c2 cardiomyocytes for 48 h (n=5) resulted in an elevation of mitochondrial ROS. Treatment with LiCl at 0.3 mM (n=5) reduced the levels of mitochondrial ROS in GLUT4-knockdown H9c2 cells. (B) Compared with the control cells (n=5), knockdown of GLUT4 with siRNA at 50 nM in H9c2 cardiomyocytes for 48 h (n=5) led to the suppression of mitochondrial membrane potential. LiCl at 0.3 mM (n=5) restored the mitochondrial membrane potential in GLUT4-knockdown H9c2 cells. *P<0.05, **P<0.01, ***P<0.001. ROS, reactive oxygen species; GLUT4, glucose transporter type 4; CON, control.

cardiac metabolism (12-14,37). Nonetheless, failing hearts show varying mitochondrial fatty acid oxidation patterns, which could increase or decrease depending on the heart failure type (4). For example, in heart failure associated with conditions such as hypertension and ischemia, myocardial fatty acid oxidation tends to decline. Hence, the implications of the present findings might not extend to all types of heart failure given the variability in underlying disease processes.

ROS-induced proton leakage is mediated by adenine nucleotide translocase (38,39), a protein that is a central component of the mitochondrial permeability transition pore (40). The opening of the mitochondrial permeability transition pore,

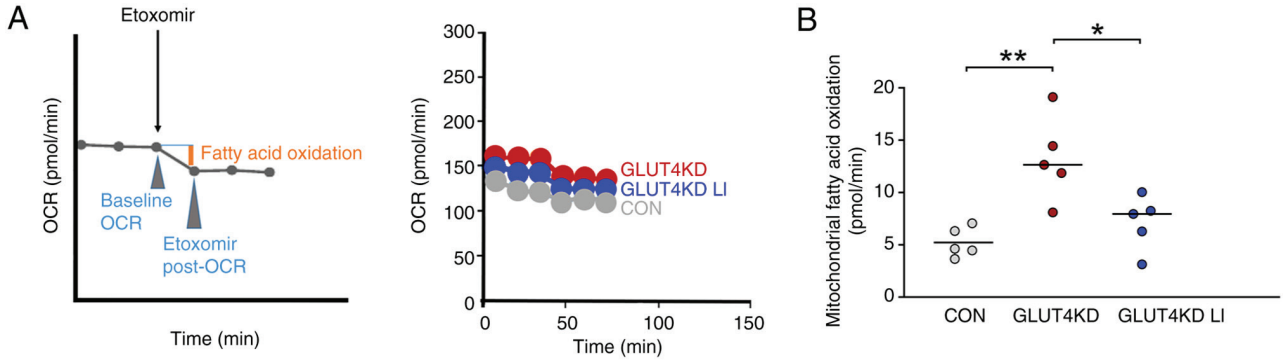

Figure 5. Mitochondrial fatty acid oxidation in GLUT4-knockdown H9c2 cardiomyocytes treated with LiCl. (A) Schematic and representative tracing of OCRs following the acute injection of etomoxir at 40 μM. Mitochondrial fatty acid oxidation was estimated by measuring the change in the OCR after etomoxir injection. (B) Knockdown of GLUT4 with small interfering RNA at 50 nM for 48 h induced mitochondrial fatty acid oxidation to a greater extent in H9c2 cardiomyocytes (n=5) than in control cells (n=5). LiCl at 0.3 mM (n=5) inhibited mitochondrial fatty acid oxidation in GLUT4-knockdown H9c2 cardiomyocytes. *P<0.05, **P<0.01. GLUT4, glucose transporter type 4; OCR, oxygen consumption rate; KD, knockdown; CON, control.

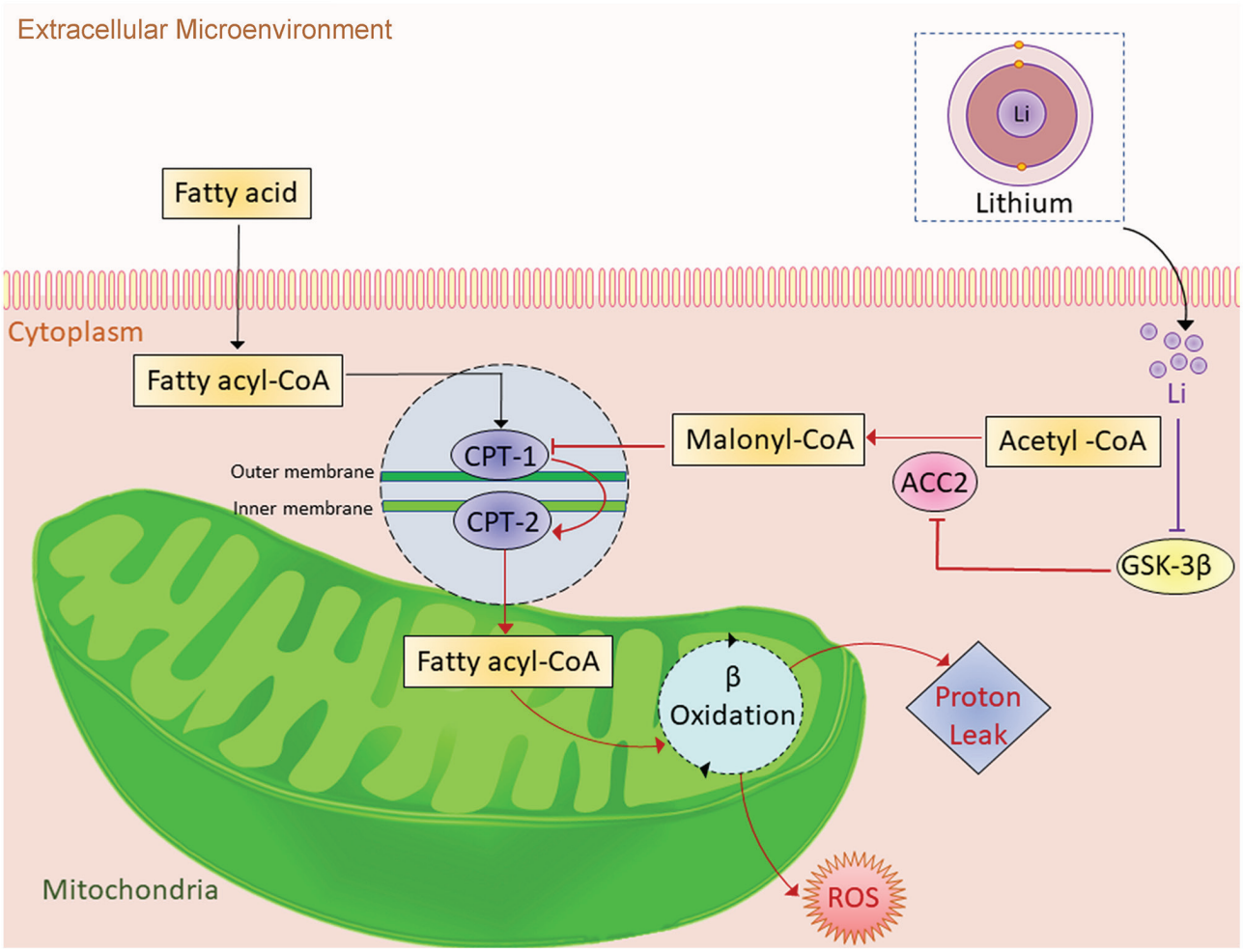

Figure 6. Illustration of the proposed mechanisms underlying lithium's effects on mitochondrial fatty acid oxidation and oxidative stress in cardiomyocytes. Low-dose lithium downregulates GSK-3β activity in cardiomyocytes and activates ACC2, thereby upregulating malonyl-CoA expression, which in turn leads to the inhibition on CPT1 activity, transportation of fatty acyl-CoAs into mitochondria for β-oxidation and generation of ROS and proton leakage. GLUT4, glucose transporter type 4; ACC2, acetyl-CoA carboxylase 2; CPT1, carnitine palmitoyltransferase 1; ROS, reactive oxygen species.

particularly under pathological conditions, leads to the dissipation of mitochondrial membrane potential, which in turn triggers the mitochondrial pathway of apoptosis (41). Studies have demonstrated that lithium exerts a cardioprotective effect by enhancing the threshold at which the mitochondrial permeability transition pore is activated by ROS (42,43). The present study demonstrated that low-dose lithium hinders ROS generation and proton leakage while restoring

mitochondrial membrane potential in GLUT4-knockdown H9c2 cardiomyocytes.

GSK-3β signaling plays a pivotal role in the regulation of multiple mitochondrial functions, including energy bioenergetics, biogenesis and apoptosis (44). The inhibition of GSK-3β has been shown to reduce the apoptosis of cardiomyocytes and alleviate cardiac dysfunction (45-48). In the present study, LiCl at a concentration of 0.3 mM inhibited GSK-3β and activated ACC2 in H9c2 cardiomyocytes. Although studies have reported that lithium may regulate the activity of AMPK and calcineurin (49-52), the present study revealed that lithium at a concentration of 0.3 mM had no significant effect on the expression of p-AMPK or that of calcineurin in H9c2 cardiomyocytes. These findings suggested that the biological effects of lithium may be concentration dependent. As summarized in Fig. 6, the results of the present study suggested that at a low therapeutic concentration, lithium inhibits mitochondrial fatty acid utilization and mitigates oxidative stress in cardiomyocytes. These effects are probably achieved through the inhibition of GSK-3β and the activation of ACC2.

In the present study, lithium had a biphasic dose-response effect on ACC2 and GSK-3β activities. This phenomenon may stem from interactions among multiple signaling pathways targeted by lithium (53), resulting in dose-response curves that significantly differ from the monotonic curves typically seen in pharmaceuticals acting on specific receptors (54). Numerous studies have identified the biphasic dose-response effects of lithium on a wide range of signaling pathways in multiple cell types (55-58). To gain a comprehensive understanding of the mechanisms underlying the effects of lithium on mitochondrial energy metabolism in cardiomyocytes at the system level, future research involving large-scale multiomics data is warranted.

In conclusion, the findings of the present study indicate that lithium directly regulated mitochondrial fatty acid utilization and mitigated oxidative stress in cardiomyocytes at low therapeutic concentrations. These findings suggest that lithium possessed cardioprotective properties and may attenuate metabolic stress in the myocardium. Considering the inherent limitations of *in vitro* research, future *in vivo* studies are necessary to confirm the cardioprotective effects of lithium in metabolic stress.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

PHC, YHY, YHK and YJC conceived and designed the study. PHC, YHK and YJC conducted the experiments and analyzed the data. PHC, YHK and YJC confirm the authenticity of all the raw data. PHC and CCC acquired funding. PHC, TWL, SHL, TVH and CCC contributed to the data interpretation and prepared the manuscript. TVH assisted with data visualization and created graphs. PHC composed the first draft of the manuscript. YHK, YHY and YJC reviewed and edited the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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