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Identification of Novel Approaches for Cardioprotection against Ischemia-reperfusion Injury

A dissertation submitted in partial satisfaction of the

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Xiang Yin

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ABSTRACT OF THE DISSERTATION

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Xiang Yin

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Professor Jing Huang, Chair

Heart attack is one of the major causes of death in elderly populations worldwide. Ischemia-reperfusion (I/R) injury is significantly involved in diseases such as heart attack and stroke, and it occurs as a result of the disruption and subsequent restoration of blood supply. The reperfusion process of the ischemic tissue is essential for the survival and recovery of the tissue, but at the same time initiates secondary damage. The I/R injury differs from the primary ischemic damage and is detrimental to patient survival. The I/R injury has been identified to be a result of several mitochondrial dysfunctions during reperfusion stage, including the production of reactive oxygen species (ROS), opening of the mitochondrial permeability transition pore (mPTP), loss of membrane potential, and

activation of downstream cell death signaling pathways. In clinical practice, there is a lack of effective treatment for I/R injury.

Here we report that tricarboxylic acid cycle intermediate α-ketoglutarate (α-KG), when administered at the onset of reperfusion, confers robust cardioprotection against I/R injury and reduces myocardial infarct size *in vivo* by ~70%. Previously, α-KG has been shown to bind to the mitochondrial F_1F_o-ATP synthase/ATPase using drug affinity responsive target stability (DARTS), a small-molecule target identification method. The structure of the mPTP has been studied for decades. It was suggested that ATP synthase plays a critical role in the mPTP opening. Here we provide evidence that α-KG regulates the interaction between ATP synthase to CypD. The opening of the mPTP has been suggested to be regulated by the interaction between ATP synthase to CypD. We further showed that cardioprotection by α-KG against I/R injury is abolished by CypD deficiency. Our findings link a basic metabolite to a critical mitochondrial component and provide novel approaches for cardioprotection against IR injury.

The dissertation of Xiang Yin is approved.

Heather R. Christofk

Michael E. Jung

Aldons J. Lusis

Jing Huang, Committee Chair

University of California, Los Angeles

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To my family for their love and support throughout my life.

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Chapter One

Chapter one is adapted from Yin. X et al. “The metabolite alpha-ketoglutarate protects against heart ischemia-reperfusion injury” (manuscript in preparation).

Contribution: Figure 1-1, 2 and 3: Xudong Fu, Jingyuan Li, Jing Huang, Xiang Yin designed the experiments, Xiang Yin, Xudong Fu prepared the materials. Jingyuan Li performed the experiments. Xiang Yin, Xudong Fu, Jingyuan Li, Zheqing Cai, and Jing Huang analyzed the data. Figure 1-4: Zheqing Cai, Hannah Jasick and Ian Mills designed, performed and analyzed the experiments. Xiang Yin analyzed the data. Figure 1-5 Xiang Yin and Laurent Vergnes performed the experiments and analyzed the data. Xiang Yin, Xudong Fu, Jingyuan Li, Zheqing Cai, and Jing Huang analyzed the data. Figure 1-6: Xudong Fu designed, performed, and analyzed the experiment, Xiang Yin analyzed the data. Daniel Braas performed the metabolic profiling. Xiang Yin wrote the manuscript. Mansoureh Eghbali provided guidance. Jing Huang designed the experiment and provided guidance.

Chapter Two

Chapter two is adapted from Yin. X et al. “The metabolite alpha-ketoglutarate protects against heart ischemia-reperfusion injury” (manuscript in preparation).

Contribution: Figure 2-2: Xudong Fu and Xiang Yin designed, performed, and analyzed the experiment, Xiang Yin analyzed the data. Daniel Braas performed the metabolic profiling. Figure 2-3 to 2-6: Xiang Yin designed, performed, and analyzed the data. Daniel Braas performed the metabolic profiling. Xiang Yin wrote the manuscript. Jing Huang provided guidance.

Chapter Three

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Contribution: Figure 3-2: Xiang Yin designed, performed, and analyzed the data. Laurent Vergnes helped with mitochondrial extraction. Heejun Hwang and Brett Lomenick helped with DARTS experiments. Figure 3-3: Yan Zhang, Xiang Yin, Jing Huang designed the experiments. Yan Zhang performed the experiments. Xiang Yin analyzed the data. Figure 3-4: Evan Taddeo provided *ppif-/-* mice. Xiang Yin, Jing Huang and Zheqing Cai designed the experiments. Zheqing Cai, Hannah Jasick and Ian Mills designed, performed and analyzed the experiments. Xiang Yin analyzed the data. Figure 3-5: Xiang Yin designed, performed, and analyzed the data. Figure 3-6: Xudong Fu, Jingyuan Li, Jing Huang, Xiang Yin designed the experiments, Xiang Yin, Xudong Fu prepared the materials. Jingyuan Li, Xiang Yin and Heejun Hwang performed the experiments. Xiang Yin analyzed the data. Xiang Yin wrote the manuscript. James N. Weiss, Guillaume Calmettes, Linsey Stiles, Evan Taddeo, Orian Shirihai, Ruiping Xiao, Yan Zhang, and Jing Huang provided guidance and helpful suggestions.

VITA

Education

2009-2013 B.S Biology
Peking University
Beijing, China

Experience

Graduate Student Researcher (June 2014 - Present)
Laboratory of Jing Huang, Ph.D., UCLA – Los Angeles, CA

- Led a research project focusing on the development of novel approaches for cardioprotection against heart ischemia-reperfusion injury
- Co-inventor of two patent applications of new approaches for anti-aging and cardioprotective strategies

Publications

Yin X *et al*. "The Metabolite α-ketoglutarate Protects Against Heart Ischemia-reperfusion Injury" (manuscript in preparation)

J Huang, X Fu, X Yin *et al.* "Glutarate Compounds for Treating Ischemia-Reperfusion Injuries". US Patent No.20170333373A1 (2017)

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X Yin, J Li, H Hwang, M Eghbali and J Huang. “Identification of Drugs that Protect Against Ischemia Reperfusion Injury” Oral presentation at Molecular and Medical Pharmacology Pharm Friday Seminar, UCLA, Los Angeles, Mar 2017

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Introduction

Cardiovascular disease is a leading cause of death worldwide[1]. In the United States, cardiovascular disease causes about 600 thousand deaths in the year 2017, accounting for a quarter of the total mortality[2,3]. Aging is a major factor contributing to cardiovascular disease, and the prevalence of cardiovascular disease in different age groups increase from about 11 percent at the age of 20 - 39 to over 80 percent at age over 65[3,4]. With the overall growth of elderly populations worldwide, the need of therapies to treat cardiovascular disease is also getting more important.

The major type of cardiovascular disease is coronary artery disease (CAD), which accounts for about 50% of the total deaths caused by cardiovascular disease[5]. CAD occurs as a result of blood plaque buildup inside the coronary arteries[6]. The blood flow can be narrowed or completely blocked by rupture of the plaque, and results in ischemia in the myocardium. Timely restoration of the blood supply is essential for patient survival and for reducing ischemic injury[7]. However, the process of reperfusion itself causes further injury to the myocardium, significantly affecting patient recovery and survival[8]. Although ischemia-reperfusion (IR) injury has been extensively studied for decades, there is still a lack of effective, clinically proven therapy for cardioprotection against I/R injury[9].

There are four major types of I/R injury: 1. Myocardial stunning. 2. Cardiac dysfunction ("no-reflow"). 3. Reperfusion arrhythmias. 4. Lethal reperfusion injury (increase in infarct size)[9]. Clinically, there have been various approaches toward treating IR injury, including antioxidants[10-13], anti-inflammatory agents[14], remote ischemic preconditioning[15], ischemic post-conditioning[16-18], and mPTP inhibitors[19,20]. Some

approaches showed effectiveness in improving the patient's recovery after myocardial infarction. However, these treatments either have their limitations in effectiveness and feasibility or failed in clinical studies at a larger scale[9,21]. For patients presenting ST-Elevation Myocardial Infarction (STEMI), in which case the patients have already had myocardial ischemia, timely reperfusion is required for patient survival and there is a limited therapeutic window for therapies that need to be applied before reperfusion[9,22,23]. Therefore, the development of new approaches to treat IR injury at the early reperfusion window is still of clinical significance.

In this dissertation, we investigated into a new approach for treatment against IR injury through the screening of a group of mitochondrial metabolites in the *in vivo* mouse myocardial infarction model. We discovered that the tricarboxylic acid cycle intermediate alpha-ketoglutarate (α-KG) confers cardioprotection when administered upon reperfusion. Treatment of α-KG reduced the infarct size by over 70% and improved heart hemodynamic parameters in the *ex vivo* Langendorff perfused heart model. The mechanism of α-KG to confer cardioprotection is not fully understood. Our result in CypD knockout mice showed that α-KG depends on CypD to confer cardioprotection. Our findings provided new insights into how exploiting endogenous mitochondrial metabolism could help with the clinical development of therapies for myocardial ischemia-reperfusion injury.

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Chapter One: Investigation of Novel Approaches that Confer Cardioprotection Against I/R Injury

Abstract

Mitochondrial metabolism is critically involved in ischemia-reperfusion (I/R) injury. Significant changes in the level of TCA cycle metabolites have been observed during ischemia-reperfusion. Previously several mitochondrial metabolites have been identified to extend lifespan in *C.elegans* through their regulation of mitochondrial functions. Here we describe approaches to screen metabolites in the mouse heart ischemia-reperfusion injury model, and the discovery that the TCA cycle metabolite alpha-ketoglutarate (α-KG) confers dramatic cardioprotection *in vivo* with over 70% infarct size reduction. The cardioprotection of α-KG was also further characterized in *ex vivo* Langendorff heart model and examined for gender differences.

Introduction

Pathways Involved in the Cause of I/R Injury:

Metabolism, Oxidative stress, Calcium Overload and mPTP Opening

Mitochondria serve as the center of energy production in cardiomyocytes. Metabolic changes, oxidative stress, calcium overload, and pH changes in the mitochondria play important roles in the initiation of I/R injury. Myocardium that undergoes ischemia-reperfusion displayed oxidative damage and elevated levels in ROS[1,2]. The mitochondrial metabolism plays a critical role in ROS production during ischemia-reperfusion[3,4].

The mitochondrial respiration chain is a group of five large complexes that act as the center of aerobic respiration and ATP synthesis in mitochondria. The TCA cycle is a major provider of NADH and $FADH_2$, the substrates for mitochondrial respiration chain. The major sources of NADH are pyruvate, isocitrate, alpha-ketoglutarate, and malate dehydrogenation. The Complex I (NADH:ubiquinone oxidoreductase) in the respiration chain further oxidized NADH into NAD^+ and H^+, and transport it to the downstream Complex III (ubiquinol:cytochrome c oxidoreductase) through coenzyme Q (CoQ). The Complex II (succinate-coenzyme Q reductase), generates $FADH_2$ through succinate dehydrogenation, and further transports the electrons from $FADH_2$ to Complex. Complex IV (cytochrome oxidase) receives the electrons transported from complex III and passes them to oxygen[5]. The normal function of the respiration chain requires sufficient supply of

oxygen, NADH, and $FADH_2$, and a lack of oxygen and nutrients supply during ischemia could lead to a stop of the electron flow into Complex IV[3,4].

Chouchani *et al.* in 2014 identified a universal pattern of succinate accumulation in ischemic tissue and provided evidence showing the rapid succinate oxidization upon early reperfusion is an important contributor to ROS production[3]. Reverse electron transport (RET) theory was used to explain the changes in succinate during ischemia and reperfusion: During ischemia, the lack of oxygen and stop of electron flow into Complex IV does not immediately stop the function of Complex I and Complex II. The electrons generated from Complex I drive the reverse chemical reaction at Complex II, and malate is further reduced into succinate, resulting in the accumulation of succinate by 5-20 folds. Upon reperfusion, the abundance of Complex II substrate succinate and the lack of Complex I substrate NADH together lead to rapid electron production at Complex II. The CoQ pool becomes over reduced and results in reverse electron transport from Complex II to Complex I, initiating ROS production and oxidative stress[3,4,6,7].

Calcium plays an important and complicated role during ischemia-reperfusion. The homeostasis of intracellular Ca^{2+} is essential for cardiac muscle contraction in normal conditions. It was observed that the level of intracellular Ca^{2+} increases during the early ischemia stage as well as during the reperfusion stage[8-14]. But a detailed mechanistic understanding of the abrupt increase of intracellular Ca^{2+} under ischemia-reperfusion is still lacking[15-18]. The sarcoplasmic reticulum (SR) is a major source of Ca^{2+} and it was suggested that the dysfunction of Calcium Release Channel (CRC) on the SR under I/R plays a critical role in the increase of intracellular Ca^{2+} [17,18]. Santulli *et al.* in 2015 also

showed that diastolic SR Ca^{2+} leak causes Ca^{2+} overload during reperfusion and is mainly responsible for the cardiac dysfunctions following heart failure[21].

The "pH paradox" was also involved in mitochondrial dysfunctions during ischemia-reperfusion[15]. It was observed that during ischemia both intra- and extracellular pH significantly decreased[22]. The pH decrease is most likely a result of anerobic respiration and accumulation of lactate. During reperfusion, the intra- and extracellular pH returning to normal levels accompanies with I/R injury, and there is evidence that acidic pH inhibits the opening of the mitochondrial permeability transition pore (mPTP) and protects against I/R injury[23-26].

Both oxidative stress and calcium overload contribute to mPTP opening, disruption of the mitochondrial membrane potential, and activation of cell death signaling[3,4,27-30]. Mitochondria have the ability to take up and release Ca^{2+} through its Ca^{2+} uniporter, and the mitochondrial permeability transition (mPT) is closely linked with mitochondrial Ca^{2+} overload[31,32]: In isolated mitochondria, if the matrix Ca^{2+} concentration rises out of the physiological range, the mitochondrial membrane will transform into a high-permeability stage, allowing the influx of molecules with sizes up to 1.5 kDa. This phenomenon was termed as the mitochondrial permeability transition[33,34]. The hypothesis of the mPTP was proposed to explain the mPT[29,33,34]. Helstrap *et al.* demonstrated in 1999 that mPT occurs in the reperfusion stage but does not occur during ischemia[6]. The reason why mPT does not occur during ischemia was still not clear. It was suggested that factors such as the acidic intra- and extracellular environment and the lack of oxidative stress during ischemia inhibited the mPTP opening[15,27]. Although there is not a thorough understanding of the mechanisms of mPT, the oxidative stress, Ca^{2+} overload and pH were thought to be

interconnected with each other and contribute to the mPTP opening and activation of cell death signaling[15,27,35]. The involvement of oxidative stress, Ca^{2+} overload and pH in I/R injury were also supported by the results of approaches targeting these pathways[23,26,36-40].

Cell Death Signaling and Inflammation

In cardiomyocytes, cell death signaling is activated under various stimuli, such as oxidative stress[41], hypoxia-reoxygenation[42,43], and the mPTP opening[44,45]. Cell death signaling is involved in I/R injury possibly in two ways: (1) Activation of death signaling result in cardiomyocyte death and tissue damage and impairs heart functions. Activation of apoptosis signaling has been observed in 5% to 30% of myocytes in the area at risk within 16 hours of ischemia-reperfusion[46-49]. In the long-term range, abnormal apoptosis in cardiac myocyte persists for months in patients with heart failure[50-52]. (2) The inflammatory response accompanying the cardiomyocyte death also contribute to the reperfusion injury. The exact cause and effect relationship between inflammatory responses and activation of cell death signaling has not been fully understood[53-55]. There is emerging evidence suggesting that myocardial innate immune response is activated during ischemia-reperfusion through the interaction between damage-associated molecular patterns (DAMPs) released from damaged cardiac myocytes and pattern recognition receptors (PRRs)[56-58].

Necrosis, apoptosis, and autophagy are three major types of cell death pathways, and they all have been shown to be involved in cardiac myocyte death under I/R[27,59,60],

and post-ischemic cardiac functional recovery[61-66]. Bcl-2 (B-cell lymphoma 2) protein family[67-70], tumor suppressor p53[47,69], calcium[71], oxidative stress[72-74], and the mPTP opening[45,59,70,74] are shown to be involved in the activation of necrosis and apoptosis during I/R.

Activation of apoptosis during I/R has been shown in various species[47,62,75,76]. Apoptosis was shown to occur in ischemic tissue and surrounding tissue after I/R, accompanied with a decrease in Bcl-2 protein[77] and an increase in Bax (BCL2 Associated X)[76] and Fas receptor expression[78]. Infarct size by I/R injury could be reduced by removal of the proapoptotic protein Bax[70], removal of Fas[79], overexpression of the antiapoptotic protein Bcl-2[27,68,80], and anti-apoptotic therapies[81]. But whether apoptosis is the major form of cell death responsible for infarct size is still under debate[27]. Although approaches inhibiting apoptosis have been shown to confer cardioprotection[82-84], studies also showed that necrotic and autophagic death can be reduced by antiapoptotic proteins as well[70,80,85,86], and only a small portion of the infarct tissue displayed apoptosis as assessed by TUNEL assay[27].

Necrosis during I/R was suggested to be a result of endoplasmic reticulum (ER) stress, calcium overload and the mPTP opening[87-90]. Previously necrosis was considered an uncontrolled process, but recent studies suggested a process of regulated necrosis (necroptosis) mediated by RIP1, RIP3 signaling and the mPTP opening[91-95]. Studies also showed that RIP3 deficiency[95] or necrosis inhibitor[96] to reduce infarct size.

Autophagy was shown to play a bilateral role in the survival of cardiac myocytes under I/R[97,98]. Autophagy was shown to be activated by a low level of ATP induced adenosine monophosphate-activated protein kinase (AMPK) activation[99,100] and hypoxia

induced hypoxia-inducible factor 1 alpha (HIF-1α) signaling[101] in cardiac myocytes during ischemia. During the reperfusion stage, some studies suggested that Beclin-1 mediated the activation of autophagy[99], but some studies also suggested impaired autophagy during this stage[102]. The effect of autophagy on I/R injury is rather complex. It was suggested that autophagy during ischemia helps with ATP production[99], and mitophagy during reperfusion compensates for mitochondrial injury[103]. While uncontrolled autophagy was suggested to contribute to cardiac myocyte death[97,104].

Inflammatory response is also observed in I/R heart, but the exact cause and effect relationship is poorly understood. Necrosis is a critical initiator of inflammation: intracellular components that could serve as danger signals released from necrotic cells can activate inflammatory responses[55]. Studies showed that neutrophil infiltration was increased upon reperfusion[105,106], and that they are a major source of ROS and proteases in the inflammatory responses[15]. However, there was no clear evidence showing whether neutrophil accumulation is a part of the cause of IR injury or is just a response towards cardiac myocyte death[106]. The importance of inflammation in I/R injury was more emphasized by experimental[107-111] and clinical studies[112,113], in which the use of anti-inflammatory methods conferred cardioprotection against I/R injury.

RISK and SAFE Pathways

The Reperfusion Injury Salvage Kinase (RISK) pathway and the Survivor Activating Factor Enhancement (SAFE) pathway are two major signaling pathways involved in cardioprotection mechanisms under I/R. Various approaches have been investigated to reduce infarct sizes by activating of these two pathways.

The concept of the RISK pathway was first proposed by Hausenloy and Yellon in 2004. Anti-apoptotic signaling pathways such as phosphatidylinositol-3-OH kinase (PI3K)–Akt and extracellular signal regulated kinases (Erk 1/2) are critically involved in I/R injury, and up-regulation of these pathways by pharmacological manipulations confers cardioprotection. Therefore, they are termed as the Reperfusion Injury Salvage Kinase (RISK) pathway[114]. Approaches activating RISK pathways such as administrations of insulin[115], insulin-like growth factor-1 (IGF-1)[116], transforming growth factor-β1 (TGF-β1)[117], urocortin[118,119] and bradykinin[120] have been shown to confer cardioprotection. The mechanisms of the RISK pathway to confer cardioprotection were not fully understood. It was suggested that inactivation of pro-apoptotic proteins[121,122], caspases[123], inhibition of cytochrome c release[124] and activation of protein kinase C[125] may be involved[114]

The SAFE pathway primarily involves the TNFα signaling pathway. This concept was proposed by Lacerda and Lecour *et al.* in 2009 based on a series of their previous findings that manipulations of TNFα signaling could confer cardioprotection independent of the RISK pathway[126-128]. However, the role of TNFα signaling in cardioprotection is complex. Some studies showed that increased TNFα levels contribute to dysfunction and heart failure[129], and knock out of TNFα, or use of TNFα antibodies conferred cardioprotection[130]. However, other studies also showed that deficiency of TNFα receptors increases infarct size[131], and increased hypoxic injury[132]. It was later suggested that optimization in dosage and timing of TNFα is key to promote cell survival[127]. Just like the RISK pathway, the mechanism of the SAFE pathway is also not completely clear. Protein kinase C, sphingolipid pathway and mitochondrial K+ ATP dependent channel was found to be activated by TNFα preconditioning[126]. The STAT-3 signaling pathway was

also shown to mediate the SAFE pathway signaling and is also involved in the cardioprotection by ischemic post-conditioning[133].

Approaches to Confer Cardioprotection:

Preconditioning and Postconditioning

Preconditioning and postconditioning are endogenous protective procedures that showed promising potency in conferring cardioprotection. Ischemic preconditioning (IPC) was first described in a study by Murry *et al.* in 1986, where a session of four cycles of 5 minutes of ischemia with reperfusion before 40 minutes ischemia followed with reperfusion significantly reduced the infarct size versus the control group[134]. The beneficial effect of IPC was further confirmed in multiple experimental studies[135-137] and clinical studies[138,139]. Apart from IPC, it was later discovered that pharmaceutical agents, such as adenosine, bradykinin, nitric oxide donors, adrenergic and muscarinic receptor could generate cardioprotection in a similar manner as IPC[140,141]. IPC was shown to induce two distinct forms of cardioprotection. The first one occurs immediately and only lasts for 2-3 hours; the second occurs 12-24 hours later, with a duration of 48-72 hours[141,142]. The actual mechanism of IPC was suggested to involve ROS production, ROS-activation of Protein Kinase C, tyrosine kinase, and MAPK signaling pathways [143-145]. Studies also suggested transient mPTP opening and ROS during PC are required for PC to confer cardioprotection[146,147], and cyclophilin D (CypD, a component of the mPTP) knockout mice are resistant to IPC[148]. The RISK pathway was also recruited by IPC upon

reperfusion and could also be a contributor to cardioprotection[142,149]. Ischemic postconditioning (IPost) was first demonstrated to confer cardioprotection by Zhao *et al*.[150] in dog and by Staat *et al.* in human[151]. Both RISK and SAFE pathways were suggested to be part of the mechanism of cardioprotection by IPost[133,152,153]. Although IPost showed effectiveness in some studies[154-156], a considerable number of clinical studies reported no benefits by IPost[142,157-160].

IPC and IPost have limited applications since they require interventions direct on the heart, and the patient's response to the treatment may vary depending on the timing and the situation[161]. Studies further showed that remote ischemic conditioning (RIC), i.e. applying ischemia at noncardiac tissue, confers cardioprotection against I/R injury as well[139,162-165]. There was not a clear mechanism of the cardioprotection by RIC. Results from previous studies suggested that neural pathway[166], humoral pathway[167], and systemic responses could be mediating pathways to activate protective signaling in the myocardium[168]. Pharmacological approaches that mimic the effects of preconditioning and postconditioning were also found to be effective in conferring cardioprotection[169,170].

Other Mechanical Approaches

Apart from ischemic preconditioning (IPC), ischemic postconditioning (IPost), remote ischemic conditioning (RIC), the hypothermia method was also shown to be promising in a small-scale clinical study, and similarly, the method was applied before PCI, therefore there is a potential limit of therapeutic window for this method as well[171].

Pharmacological Approaches

A large amount of effort has been devoted to the development of pharmacological interventions to help reduce I/R injury. Despite a large number of pharmacological approaches that were shown to confer cardioprotection in the animal models, most of them failed to prove effectiveness in clinical trials[53]. Cyclosporine A (CsA), an mPTP inhibitor, showed mixed effectiveness in reducing infarct sizes in patients with acute myocardial infarction[172-175]. The effectiveness of CsA was further challenged by the results of a multicenter, double-blinded, randomized Phase III study by Cung *et al.*, which showed no benefit of CsA when administered before percutaneous coronary intervention (PCI)[175]. Another mPTP inhibitor TRO40303 failed to show effectiveness in the clinical phase as well[176,177]. Other approaches including atrial natriuretic peptide[178,179], metoprolol[180], exenatide[181] also showed mixed outcomes in clinical trials. Until now, there is still a lack of effective treatment for I/R injury, and the success rate of the translation from pre-clinical research into clinical phase was low due to a number of factors: (1) Differences in the actual physiological conditions between animal models and human patients; (2) Differences in the duration of ischemia between preclinical study and clinical practice (< 1 hour vs usually several hours); (3) MI in animal models were performed through external compression on the artery of healthy animals, while human patients already suffered atherosclerosis, buildup of plaque and are subject to inflammatory responses[161]. (4) Off-target effects and toxicity also limited the therapeutic window for pharmacological approaches[53,172].

Currently, there is still a lack of effective treatment for I/R injury in clinical practice. Mitochondrial metabolites play critical roles in targeting the mitochondrial components and regulate critical events in the cell, and in this chapter, we used the in vivo mouse myocardial infarction model to screen endogenous metabolites in their ability to confer cardioprotection against I/R injury.

Materials and Methods

In vivo mouse myocardial I/R injury model[182]

Animal studies were performed using animal research protocols approved by UCLA. Male mice (12 - 14 weeks; Charles River Laboratories) were anesthetized with ketamine (80 mg kg^{-1} intraperitoneally) and xylazine (8 mg kg^{-1} intraperitoneally), intubated endotracheally and ventilated with a ventilator (CWE SAR-830/P). The hearts were then exposed through a left thoracotomy procedure in the fourth intercostal space. The pericardium was opened, and a 7-0 Prolene suture was tightened around the proximal left anterior descending coronary artery to induce myocardial infarction. The ligature was removed after 45 min of ischemia and the mice were given a bolus of either PBS (vehicle control), α-KG (800 μM final in the blood), octyl-α-KG (800 μM final in the blood), succinate (800 μM final in blood), succinyl-CoA (800 μM final in the blood), or oligomycin (10 nM final in blood) through tail vein injection upon reperfusion.

The mice were then allowed to recover from anesthesia. 24 hours later the mice were anesthetized, and the ligature was tightened to inject 1% Evans Blue dye through the right ventricle. Area at risk (AAR) is represented as the region not stained by blue dye. The ventricles of the hearts were then sliced transversely into 2 mm thick slices. The slices were then incubated in 1% triphenyl tetrazolium chloride (TTC) at 37 °C for 15 min to distinguish the non-infarct and infarct areas. The infarcted area was displayed as the area not stained by TTC. Infarct size was expressed as Infarct Area / AAR (%).

In vivo mouse myocardial I/R injury model for gender difference studies

Animal studies were performed under animal research protocols approved by the research facility**.** C57BL6 male and female mice at the age of 12-15 weeks old were used. Mice were subject to ischemia for 40 minutes. At start the reperfusion and the mice received a bolus of either PBS (vehicle control), α-KG (800 μM final in the blood).

After 3 hours of reperfusion, 50 μl of blood was collected for assaying troponin I. At 24 hours of reperfusion, mice received echocardiography for measurement of cardiac function. Then mice were euthanized, and the heart was harvested. Myocardial infarct size was measured in the heart by triphenyl tetrazolium chloride (TTC) staining described above.

Measurement of plasma troponin I by ELISA

Troponin I ELISA was performed using Ultra-sensitive mouse cardiac Troponin-I ELISA kit according to the manufacturer's instructions (Life Diagnostics Inc., Cat # CTNI-1-US).

Ex vivo Langendorff heart model[182]

Male mice were heparinized (200 U intraperitoneally) to prevent blood coagulation and anesthetized with sodium pentobarbital (50 mg kg−1 intraperitoneally). The heart was isolated and put in ice-cold Krebs–Henseleit (KH) buffer (11.1 mM glucose, 118 mM NaCl,

4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, and 2 mM $CaCl_2$) at pH 7.4, and bubbled with 95% O_2 / 5% CO_2. The heart was cannulated through aorta in a Langendorff apparatus and perfused with KH buffer at 37 °C. Upon equilibration, the aorta was then clamped for 30 min to induce normothermic ischemia (37 °C) globally, and then subject to 60 min reperfusion with KH buffer with the addition of vehicle (PBS) or α-KG (800 μM). A catheter (1.4F Millar SPR-671) connected to a pressure transducer (Power Lab, AD Instruments) was inserted into the left ventricle (LV) to measure heart functional parameters: left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP) and heart rate (HR). The left ventricular developed pressure (LVDP) was calculated as LVDP = LVSP – LVEDP and the rate pressure product (RPP) as RPP = HR × LVDP. The maximum rate of rise of LV pressure (dP/dt_{max}) and the maximum isovolumetric rate of relaxation ($-dP/dt_{min}$) were obtained from the recordings[182].

U87 cell culture

U87 MG (ATCC® HTB14™) Homo sapiens brain likely glioblastoma cells were obtained from ATCC and cultured according to the instructions. Cells were seeded to 6-well plates in complete growth medium (DMEM (Dulbecco's Modified Eagle Medium, high glucose with 140 mM sodium pyruvate, Thermo Fisher) + 10% FBS (Fetal bovine serum, Thermo Fisher #16000044)). After the cells have attached to the bottom of the well, the cells are incubated for 24 hours with vehicle or α-KG treatment. Metabolite extraction was then performed for quantitative analysis of intracellular metabolites levels.

Metabolic profile analysis[183]

To extract metabolites from cultured cells in the 6-well plates, the cells are first rinsed with ice-cold 150 mM NH4AcO (pH 7.3). 800 µL 50% ice-cold methanol was added to each well. Cells were scraped off using a cell scraper. The solution was then transferred to 1.5 mL Eppendorf tubes. For each sample, 10 nmol norvaline was added as internal standard, and 400 µL chloroform was added to extract the metabolites. Samples were then vortexed for 5 min on ice, centrifuged at 4 degrees, 12 000 g. The aqueous phase was transferred into a glass vial for vacuum drying. 70% ACN was used to resuspend the metabolites, and a volume of 5 µL per each sample was loaded onto a Phenomenex Luna 3u NH2 100A (150 × 2.0 mm) column. UltiMate 3000RSLC (Thermo Scientific) with mobile phases A (5 mM NH4AcO, pH 9.9) and phase B (ACN) with a flow rate of 300 µL/ min was used for chromatographic separation. The gradient was set to run from 15% A to 95% A over 18 minutes, 9 minutes isocratic at 95% A, and re-equilibration for 7 minutes.

Metabolites analysis was performed using a Thermo Scientific Q Exactive mass spectrometer under the polarity switching mode (+3.0 kV / -2.25 kV). To quantify metabolites, TraceFinder 3.1 (Thermo Scientific) was used to calculate the area under the curve using retention time and accurate mass measurements (≤ 3 ppm). Relative amounts of metabolites were further calculated by summing up all isotope forms of a metabolite and the values were then normalized according to the cell number and the internal standard.[183]

Isolation of mitochondria from mouse heart[184]

Animal studies were performed under approved animal research protocols by UCLA. Mitochondria from 3-month-old C57BL/6 mice were isolated as previously described[184]. Mouse hearts or livers were extracted, minced at 4 °C in MSHE + BSA (70 mM sucrose, 210 mM mannitol, 5 mM HEPES, 1 mM EGTA, and 0.5% fatty-acid free BSA, pH 7.2), and rinsed. All subsequent steps were performed on ice or at 4 °C. The tissue was disrupted in 10 volumes of MSHE + BSA with a glass Dounce homogenizer (5–6 strokes) and the homogenate was centrifuged at 800 × g for 10 min to remove tissue debris and nuclei. The supernatant was decanted through a cell strainer and centrifuged at 8,000 × g for 10 min. The dark mitochondrial pellet was resuspended in 800 µL MSHE + BSA and re-centrifuged at 8,000 × g for 10 min. The supernatant was further removed, and the pellet was resuspended in 30 µL MAS buffer (Sucrose 70 mM, Mannitol 220 mM, KH_2PO_4 5 mM, $MgCl_2$ 5 mM, HEPES 2 mM, EGTA 1 mM, BSA fatty acid-free 0.2 %, pH 7.4). The protein concentration in the resuspended mitochondria was further determined by BCA assay using Pierce™ BCA Protein Assay (Thermo Fisher Scientific, 23225).

Measurement of oxygen consumption rate (OCR) in isolated mouse heart mitochondria[183,184]

Oxygen consumption rate of isolated mouse heart mitochondria was measured by running coupling assay as described[184,185] using XF24 Seahorse extracellular flux analyzer (Agilent). Basically, 20 µg of mitochondria suspended complete MAS buffer (MAS + 10 mM succinate + 2 µM rotenone) were seeded into a 24-well XF24 Seahorse

plate by centrifugation at 2,000*g* for 20 min at 4 °C. Before the assay, 500 µl complete MAS buffer with vehicle (water + 1% DMSO) or α-KG or oligomycin was added to the wells with quadruplicates and incubated at 37 °C for 30 min before the OCR measurement. Mitochondrial respiration begins in a coupled state 2; state 3 is initiated by the addition of 2 mM ADP as Complex V substrate; state is induced by addition of 2.5 µM oligomycin; and state 3u is induced by 4 µM FCCP (uncoupling reagent to induce maximal respiratory capacity). At the end stage, 1.5 µg/ml antimycin A was added at the end of the assay.

Results

Identification of Small Molecules That Confer Cardioprotection Against I/R Injury

Through our initial screening of TCA cycle metabolites in the *in vivo* mouse myocardial infarction model, we discovered that α-KG, octyl-α-KG, and succinyl-CoA conferred cardioprotection *in vivo* at a final blood concentration of 800 μM (Blue: non-ischemia area, Red and White: area at risk (AAR). White: infarct area. Infarct size = infarct area / AAR, Figure 1-1). Among the molecules screened, α-KG conferred the most significant cardioprotection (> 70% reduction in infarct size) (Figure 1-1 and Figure 1-2). In the TCA cycle, α-KG is a product of isocitrate by oxidative carboxylation through isocitrate dehydrogenase (IDH). α-KG further gets dehydrogenated by α-KG dehydrogenase (α-KGDH), generates NADH and succinyl-CoA. α-KG can also be produced from glutamate through anaplerotic reactions catalyzed by glutamate dehydrogenase.

Previously α-KG has been shown to bind to ATP synthase and regulates its activity.[184] Consistently, another ATP synthase inhibitor oligomycin, when post-ischemic administered at 10 nM, also conferred cardioprotection *in vivo* and reduces the infarct size by ~ 55% (Figure 1-1 and Figure 1-2). The product of α-KG through α-KGDH, succinyl-CoA, also conferred cardioprotection. α-KG has low membrane permeability across U87 cell membranes (Figure 1-6). Previous studies used a membrane permeable α-KG derivative, octyl-α-KG, which hydrolyzes upon entering into the cell and releases α-KG, to deliver α-KG across the cell membrane[184]. However, in the *in vivo* mouse

myocardial infarction model, the cardioprotection conferred by octyl-α-KG was not as good as α-KG.

α-KG Protects Against I/R Injury in the *ex vivo* Langendorff Perfused Heart Model and Improves Cardiac Functional Recovery

The cardioprotection by α-KG was also observed using an isolated Langendorff perfused heart I/R injury model[182]. The isolated Langendorff perfused heart is an *ex vivo* model used for research in cardiovascular protection. It allows for assessments of the treatment's direct effects on the heart without the effect of global factors such as hormonal or neuronal influences.[186,187]

Isolated Langendorff perfused heart was subjected to 30 min ischemia with 60 min reperfusion and treated with control or 800 μM α-KG upon reperfusion (Fig 1-3 A). Heart hemodynamic parameters were examined to evaluate the cardioprotection by control or α-KG on heart functional recoveries.

The α-KG treated group showed significant improvements in left ventricle developed pressure (LVDP) (α-KG: ~ 70% recovery vs Control: ~ 20% recovery). Rate pressure product (RPP) was also significantly improved (α-KG: ~ 70% recovery vs Control: ~ 20% recovery). α-KG also significantly improved dP/dt_{max} recovery compared with control groups (α-KG: ~ 50% recovery vs Control: ~ 20% recovery) (Fig 1-3 B and C). These results suggest that post-ischemic administration of α-KG confers significant cardioprotection against I/R injury, resulting in improvements in heart hemodynamic parameters (functional recovery).

Cardioprotection by α-KG Differ in Male and Female Mice

The gender difference in cardioprotection by α-KG was also investigated in the *in vivo* mouse myocardial infarction model (Fig 1-4). Male or female mice were subject to 40 minutes ischemia followed by 24 hours reperfusion, vehicle (water) or 800 μM α-KG was administered through tail vein injection upon reperfusion. All four groups showed similar AAR/LV (area at risk / left ventricle) ratios (Control male: 50% ± 3%, n = 8, Control female: 48% ± 5%, n = 11, α-KG male: 51% ± 10%, n = 11, α-KG female: 50% ± 8%, n = 10, no significance, mean ± S.D, Fig 1-4 B). For male groups, the control male group have an average infarct size (IS/AAR) of 51% ± 7% (n = 8), and the α-KG male group has an average infarct size of 22% ± 6% (n = 11), indicating 57% infarct size reduction ($P < 0.0001$). For female groups, the control female has an average infarct size of 42% ± 12% (n = 11), and the α-KG female has an average infarct size of 30% ± 8% (n = 11), indicating 29% infarct size reduction ($P = 0.019$). The male control group showed larger mean infarct sizes compared with female control group (Control: Male: 51% vs Female: 42%, $P = 0.063$). The male α-KG group showed smaller mean infarct sizes compared with female α-KG group (α-KG: Male: 22% vs Female: 30%, $P = 0.02$).

The Troponin I level was significantly lower in α-KG male group vs control male group, but no significant difference between α-KG female group vs control female group (Control male: 20 ± 8, n = 9, Control female: 14 ± 10, n = 10, α-KG male: 12 ± 6, n = 10, α-KG female: 14 ± 6, n = 11, ng/mL, mean ± S.D, Fig 1-4 C) . No differences in the percentage of left ventricular fractional shortening were observed (Control male: 49 ± 4,

n = 9, Control female: 47 ± 7, n = 10, α-KG male: 49 ± 5, n = 10, α-KG female: 50 ± 8, n = 11, ng/mL, mean ± S.D, Fig 1-4 D)

Discussion

Discovery of a new approach to confer cardioprotection against I/R injury through post-ischemic administration of α-KG

We discovered a new approach that reduces the infarct size by >70% through post-ischemic administration of α-KG. This approach is advantageous in three aspects: (1) α-KG showed dramatic potency in conferring cardioprotection and reducing infarct size. The over 70% infarct size reduction by α-KG surpasses most approaches tested previously[15,137,188-191]. (2) α-KG is safe for clinical use. As an endogenous metabolite, α-KG has been shown as safe for patients at dosages higher than what was used to confer cardioprotection[192-194]. In addition, in a previous clinical study by Kjellman *et al.*, a much higher dosage of α-KG (28 g in human, equivalent to ~38 mM final in the blood) was used for myocardial protection in heart surgery[195]. Therefore, there is abundant evidence supporting the safety of α-KG. (3) Many approaches require the administration of agents or operations before PCI[15]. Therefore, patients need both timely reperfusion to reduce ischemic time and additional procedures before PCI at the same time to reduce myocardium injury[15,53]. Post-ischemic approaches allow patients to receive PCI in a timely manner, therefore may further reduce the ischemia time and improve the patient's overall

survival and recovery. Our finding allows for investigations of combination therapies with other pre-ischemic approaches to get additive or synergistic effects.

α-KG Confers A Direct Protective Effect on the Myocardium

Cardioprotection can be conferred either directly through cardiac myocytes, or through remote approaches such as remote ischemic conditioning[53]. In our study, α-KG improved the heart hemodynamic parameters LVDP, DPP and dP/dt_{max} in the *ex vivo* Langendorff heart model isolated from peripheral tissue and other organs, indicating the cardioprotection by α-KG confers a direct protective effect on the myocardium.

Correlation between Complex V Inhibition and Cardioprotection

Oligomycin, a commonly used Complex V inhibitor with a binding site to Complex V subunit OSCP (oligomycin sensitivity conferring protein), also confers cardioprotection (~55% infarct size reduction). α-KG was previously shown to bind to Complex V and inhibit ATP production[184]. However, the dosages of oligomycin and α-KG used in the in vivo myocardial infarction model did not inhibit Complex V activity in isolated mouse heart mitochondria (Figure 1-5). Complex V has been suggested to be a key component of the mPTP in the model proposed by Giorgio *et al.*[196]. It is possible that both oligomycin and α-KG share a similar mechanism to confer cardioprotection through Complex V, and this topic will be discussed in detail in chapter three.

α-KG Confers Better Cardioprotection Than Octyl-α-KG

Octyl-α-KG, which has better permeability across U87 cell membrane[184], was not advantageous than α-KG. Metabolic profiling of mouse myoblast H9c2 with α-KG treatment in either normoxia or reoxygenation showed that α-KG has much higher membranes permeability across H9c2 (Figure 2-2).

In addition, timely cardioprotection is needed to reduce lethal I/R injury[53]. To release α-KG. octyl-α-KG requires additional hydrolysis reactions upon entering into the cells. Therefore, the effectiveness of octyl-α-KG could be mitigated by the possibility that not enough α-KG was released during the early stage of reperfusion to confer full-extent cardioprotection.

Female Mice are More Resistant to I/R Injury but Less Responsive to α-KG Treatment

The infarct size in the female control group was significantly smaller than male control groups, consistent with the observations in the previous studies[197-200]. Hormone levels may contribute to the female mice resistance to I/R injury, as estrogen administration was shown to reduce the infarct size[199].

The infarct size reduction by treatment of α-KG is larger in male versus female mice (57% vs 29%), showing the female mice are less responsive to α-KG treatment. This gender difference may partially result from the different genders' initial responses to ischemia-reperfusion and may also be a result of different genders' responses to α-KG. Lagranha 2011 *et al.* discovered that α-KGDH phosphorylation level is different in male

and female mice, and this phosphorylation level difference might affect the metabolism and effectiveness of α-KG[200]. However, there is a lack of in-depth study and mechanism in related areas.

Figures

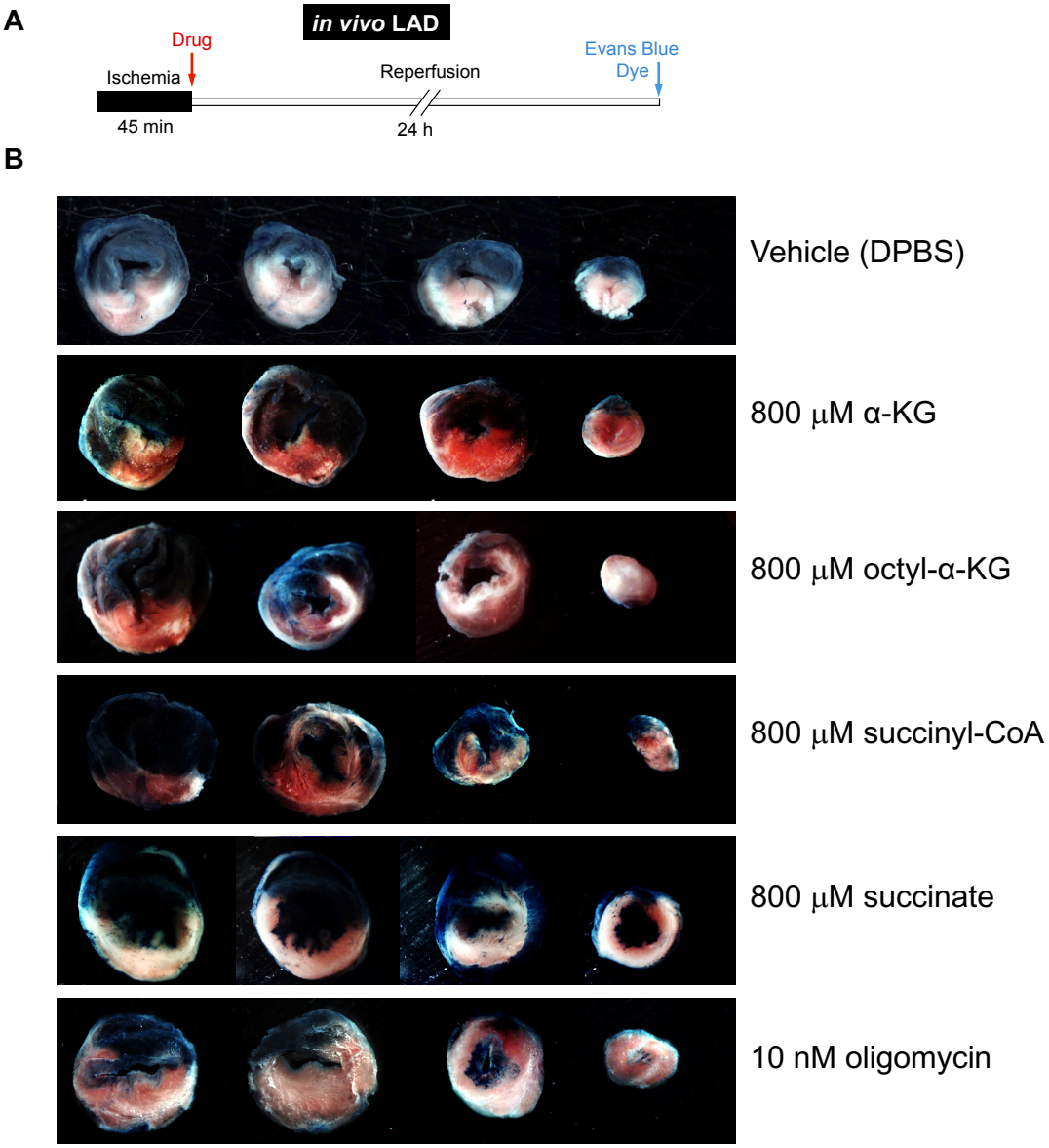

Figure 1-1: Screening of mitochondrial metabolites in the mouse myocardial infarction model. (A) Experiment design of in vivo mouse myocardial infarction model to assess I/R injury. (B) Representative results showing the extents of infarct size reduction conferred by treatments with small molecules. Blue: Non-ischemic tissue, Red: Ischemic tissue, recovered from myocardial infarction, White: Infarct tissue with lethal I/R injury.

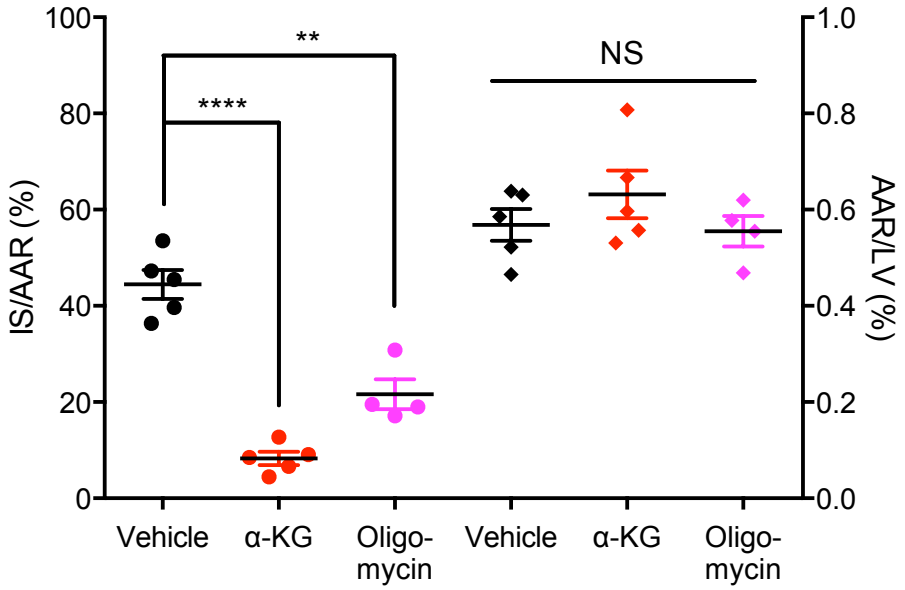

Figure 1-2: α-KG and oligomycin confer significant cardioprotection *in vivo*. Quantification of myocardial infarct size. Data show the results of 4-5 biological replicates. N = 5. Bars indicate mean ± SEM. ****$P < 0.0001$, **$P = 0.0012$, NS (not significant), by unpaired *t*-test, two-tailed, two-sample unequal variance.

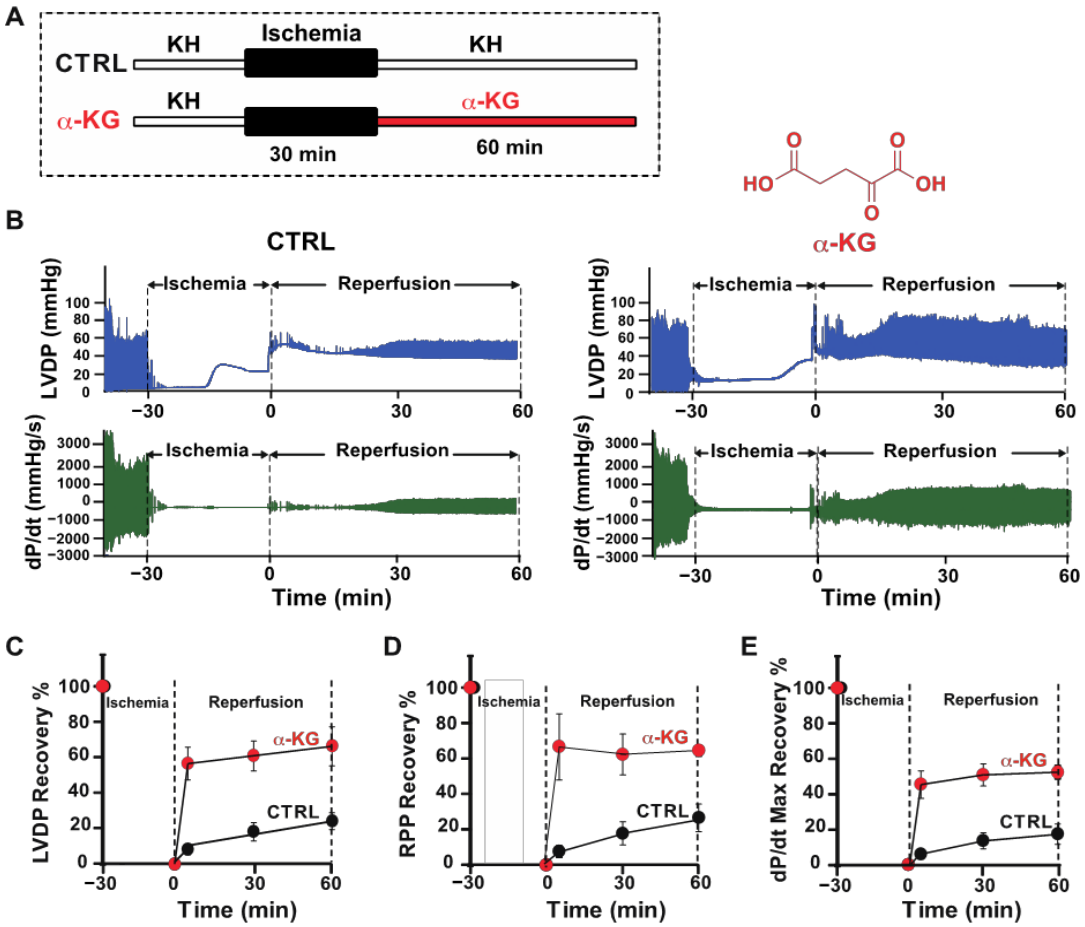

Figure 1-3: The effect of α-KG treatment in *ex vivo* Langendorff mouse heart ischemia-reperfusion model. (A) Experimental protocol for *ex vivo* Langendorff mouse heart mouse model. (B) Representatives of the left ventricular developed pressure (LVDP) and dP/dt_{max} and dP/dt_{min} as a function of time. (C) Left ventricular developed pressure (LVDP) as a function of time. (D) Rate pressure product (RPP) as a function of time. (E) The maximum rate of left ventricle (LV) pressure rise (dP/dt_{max}) as a function of time.

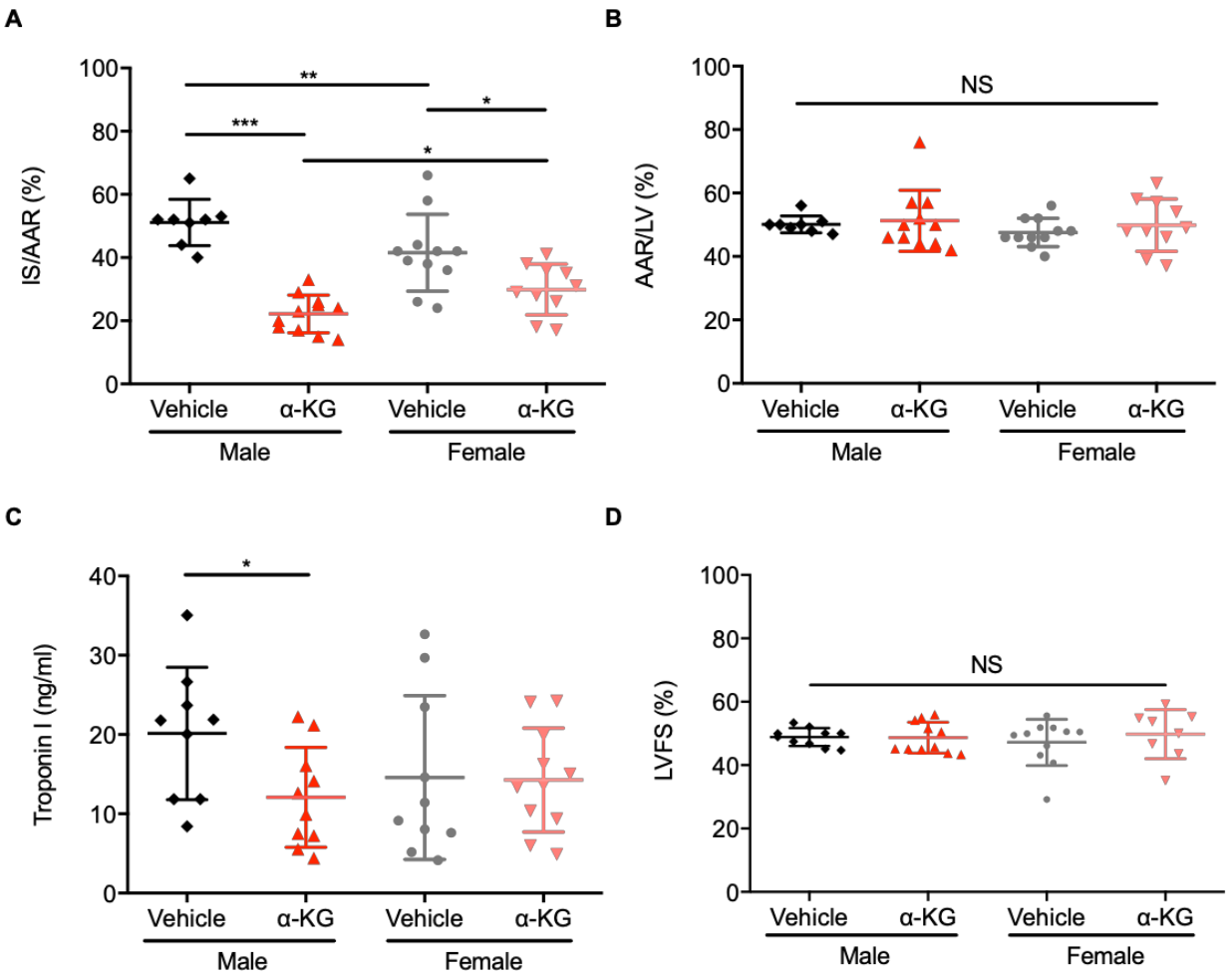

Figure 1-4: Cardioprotection by α-KG in male and female mice. Data shown results of 9-12 biological replicates. Bars indicate mean ± SD. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, NS (not significant), by unpaired *t*-test, two-tailed, two-sample unequal variance.

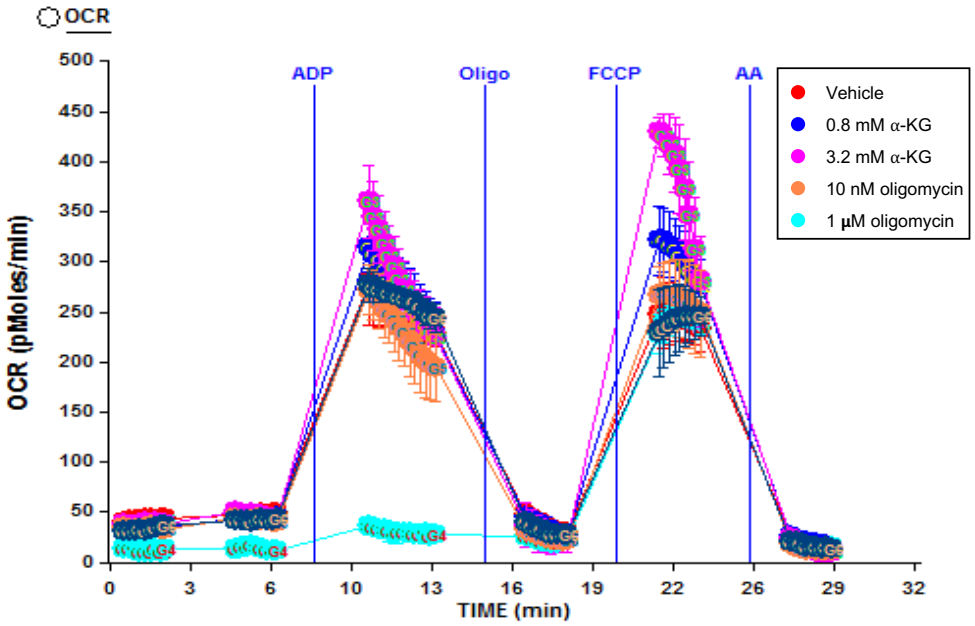

Figure 1-5: Representative seahorse Oxygen Consumption Rate results showing the effects of different concentrations of α-KG and oligomycin on Complex I driven respiration in isolated mouse heart mitochondria. At the dosages used in the *in vivo* mouse myocardial infarction model, α-KG (800 µM and 3.2 mM) and oligomycin (10 nM) did not inhibit the Complex V activity in isolated mouse heart mitochondria.

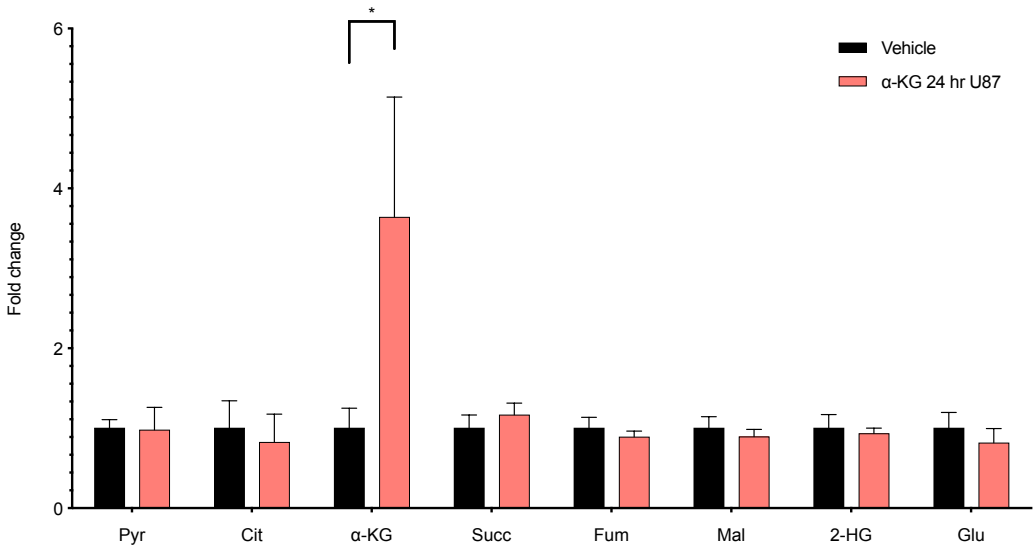

Figure 1-6. Metabolic profiling of U87 cells showing intracellular levels of TCA cycle metabolites, 2-hydroxyglutarate (2-HG) and glutamate (Glu). U87 cells are treated with vehicle (water) or 800 μM α-KG treatment for 24 hours in complete growth medium (DMEM, high glucose with sodium pyruvate and glutamate + 10% FBS). Bars indicate mean ± SD, n = 5, *P < 0.05, **P < 0.01, ***P < 0.001. By unpaired two-tailed, two-sample unequal variance t-test.

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Chapter Two: Characterization of α-KG Metabolism in H9c2 Rat Myoblast under Hypoxia-Reoxygenation

Abstract

Mitochondrial metabolism undergoes significant changes during ischemia-reperfusion. Metabolism a key initiator of IR injury, and metabolic approaches confer cardioprotection against I/R injury. α-KG is an intermediate of the TCA cycle and treatment of α-KG will likely change the metabolism in cardiomyocytes during IR. In this chapter, we investigated the metabolic changes of H9c2 rat myoblast under hypoxia-reoxygenation with α-KG treatment.

Introduction

Alpha-ketoglutarate (α-KG) is an important biological compound. It plays several roles in metabolism: (1) An intermediate in the tricarboxylic acid (TCA) cycle, serves as the co-substrate of α-KG dehydrogenase (α-KGDH) to produce NADH and succinyl-CoA; (2) Co-substrate of alanine transaminase (ALT) and aspartate transaminase (AST) for production of glutamate, and a product of glutamate dehydrogenase; (3) Co-substrate of α-ketoglutarate-dependent hydroxylase. Especially as a co-substrate of HIF (Hypoxia-inducible factor) prolyl-hydroxylase (PHD), to facilitate the oxygen-sensor mechanism in the HIF signaling pathway[1].

The most prominent role of α-KG is as a TCA cycle intermediate. The TCA cycle plays a key role in providing NADH and $FADH_2$ for the mitochondrial respiration chain. The production of NADH is accomplished by isocitrate dehydrogenase, α-KGDH, malate dehydrogenase, and pyruvate dehydrogenase. α-KGDH is sensitive to hydrogen peroxide and its activity is inhibited under oxidative stress[2]. However, α-KGDH is also a source of reactive oxygen species (ROS)[3]. Intracellular calcium concentration also regulates the α-KGDH activity. A low calcium concentration (< 20 μM) activates α-KGDH, and a higher concentration (> 100 μM) inhibits α-KGDH[4,5]. Studies in brain mitochondria also suggested that α-KGDH is a rate-limiting step in the TCA cycle[4-6]. The α-KGDH is closely associated with age-related diseases. In the brain of Alzheimer's disease patients, both decreases in α-KGDH activity and a lack of α-KGDH-enriched neurons were observed[7-9]. Chin *et al.* showed in 2014 that α-KG increases lifespan in *C.elegans* through inhibiting

the ATP synthase and mTOR signaling[10]. The study by Lucas and Szweda also observed age-dependent inactivation of α-KGDH during cardiac reperfusion[11].

Glutamate can be produced from α-KG. During the myocardial arrest and early reperfusion, a decrease in intracellular glutamate has been reported[12]. Multiple studies also observed a low availability of α-KG after trauma or heart surgery, and the addition of α-KG improved the patients' recovery[13,14]. The cause of low availability of α-KG was suggested to be a result of rapid consumption of α-KG during the ischemia stage[15]. Newsholme *et al.* also proposed a possibility that α-KG could be recruited to the immune system and the kidney in the form of glutamine[16]. Svedjeholm *et al.* also reported that glutamine being exported from the heart at a large-scale during heart surgery[14,17,18]. Multiple studies also suggested the trend of low availability of glutamate and α-KG in reperfused myocardium, and supplementation of α-KG or glutamate improves the heart functional recovery[13,14,19-22].

Chouchani *et al.* in 2014 suggested the TCA cycle metabolism plays a critical role in the cause of I/R injury. They suggested that the accumulation of succinate during ischemia and its rapid oxidative upon reperfusion was the probable cause of excessive ROS production. It is worth noting that their experimental results also showed a decrease of α-KG in liver and kidney during ischemia.

α-KG also plays a role in the hypoxia-inducible factor (HIF)-1α transcription factor signaling pathway. In normoxia, HIF-1α keeps getting hydroxylated by HIF prolyl-hydroxylases (PHD) and is rapidly degraded by proteasome. PHD is an α-KG-dependent dioxygenase and the hydroxylation process uses oxygen and α-KG. During hypoxia, PHD is inhibited and the accumulation of HIF-1α activates downstream gene expressions as

hypoxia responses[23]. Experimental evidence showed that HIF-1α activation confers cardioprotective effects and attenuates I/R injury[24]. In renal ischemia, the protective effects of preconditioning may partially attribute to HIF-1α–dependent Bcl-2 signaling[25]. In intestinal ischemia, HIF-1α was shown to mediate pathogenic inflammatory responses[26]. Mice deficient in PHD showed resistance against I/R injury through HIF-1α targeted genes[27].

In this chapter, we investigated the metabolism profile of H9c2 cells during a simulated I/R using the hypoxia chamber. We also examined the metabolic changes in H9c2 cells treated with α-KG upon reoxygenation and examined the contribution of α-KG treatment to cell metabolism.

Materials and Methods

Cell culture and hypoxia chamber incubation

H9c2(2-1) (ATCC® CRL-1446™) rat myoblasts were obtained and cultured according to instructions provided by ATCC. Cells were seeded to 12-well plates in complete growth medium (DMEM (Dulbecco's Modified Eagle Medium, ATCC® 30-2002™) + 10% FBS (Fetal bovine serum, Theromo Fisher #16000044)). After overnight incubation, the medium is replaced with DMEM without glucose (Thermo Fisher #A1443001), and the cell culture plates are placed in the hypoxia chamber (Billups-Rothenberg Inc. MIC-101). 95% N_2 + 5% CO_2 gas was injected into the hypoxia chamber at 25 L / minute for 5 minutes to reduce the oxygen level to < 0.5%. The chamber was then sealed and incubated at 37 centigrade for 2 hours. After the incubation, the chamber is opened, exposed to air and the medium is replaced with complete growth medium. Metabolite extraction is performed at different time points.

13C5 labeled α-KG

Alpha-ketoglutaric acid (13C5, 99%, > 90% purity, CLM-2411-PK, Cambridge Isotope Laboratory) was obtained from Cambridge Isotope Laboratory.

Metabolic profile analysis

To extract metabolites from cultured cells in the 12-well plates, the cells are first rinsed with ice-cold 150 mM NH4AcO (pH 7.3). 400 μL 70% ice-cold methanol was added to each well. Cells were scraped off using a cell scraper. The solution was then transferred to 1.5 mL Eppendorf tubes. For each sample, 5 nmol norvaline was added as internal standard, and 200 μL chloroform was added to extract the metabolites. Samples were then vortexed for 5 min on ice, spun down at 4 centigrade, 12 000 g, and the aqueous layer transferred into a glass vial and vacuum dried. 70% ACN was used to resuspend the metabolites, and a volume of 5 μL per each sample was loaded onto a Phenomenex Luna 3u NH2 100A (150 × 2.0 mm) column. The metabolic profiling steps were as described in the previous chapter.[28]

Results

α-KG Has High Permeability across H9c2 Myoblast Cell Membrane

We analyzed the metabolic profiles of H9c2 rat myoblasts treated with vehicle (water) or 800 μM 13C5 α-KG. In normoxia, the intracellular α-KG level in H9c2 cells significantly increased by 15-fold within 30 minutes, and the increased intracellular α-KG level persisted over 24 hours (Figure 2-2 A and B).

We also examined the permeability of α-KG across H9c2 cells in a simulated low-oxygen - reoxygenation treatment using hypoxia chamber. H9c2 cells were subject to 95% N_2 + 5% CO_2 for 2 hours. Upon reoxygenation, the cells were treated with vehicle (water) or 800 μM 13C5 α-KG. The intracellular level of α-KG increased by ~ 80 folds within 10 minutes, and > 90% of total α-KG was labeled (Figure 2-4, Figure 2-5 B and C).

The addition of α-KG did not significantly change the intracellular levels of 2-hydroxyglutarate or glutamate (Figure 2-3 A). Isotope labeled 2-hydroxyglutarate was less than 1% (Figure 2-5 B and C), and isotope labeled glutamate was less than 5% (Figure 2-5 B and C), indicating that α-KG largely remained unmetabolized during the early reoxygenation stage.

The ATP level was significantly decreased during reoxygenation, and supplementation with α-KG increased the ATP level at 30 minutes (Figure 2-5 A). Succinate level is increased by α-KG treatment during reoxygenation, and >70% of succinate comes from labeled 13C5 α-KG, indicating that labeled α-KG contributed to the increased activity of α-KGDH. The succinyl-CoA level was not measurable in the

metabolic profiling method. And the downstream metabolites of succinate, fumarate and malate, mostly remain unlabeled. Changes in the levels of these metabolites during reoxygenation were not as significant as α-KG and succinate (Figure 2-4, Figure 2-5 B and C).

Discussion

α-KG did not show high membrane permeability across U87 cells (Figure 1-6)[10]. However, the membrane permeability of α-KG across H9c2 cells is significantly higher compared with U87 (Figure 2-1). And it appears that during reoxygenation, the addition of α-KG still significantly increased the intracellular level of α-KG in H9c2 cells within 10 minutes. The conversion of α-KG into other metabolites did not appear to be the major routes for added α-KG. The majority of α-KG remained unmetabolized, and the absolute changes in the levels and labeling of succinate appear to come from a small portion of labeled α-KG.

The conversion between 2-HG and α-KG was not significant, since there is not a detectable amount of labeled 2-HG in H9c2 cells treated with 13C5 labeled α-KG either in normoxia or during the reoxygenation stage.

The ATP level was significantly increased by α-KG treatment at 30 minutes after reoxygenation. This increase might be a result of increased α-KG metabolism through α-KGDH, which further increased the NADH levels and oxidative phosphorylation. However, this change was only significant at 30 minutes after reoxygenation, indicating a short-term effect of added α-KG on ATP production.

The membrane permeability of α-KG across the cardiac myocytes is an important factor determining the potential mechanisms of the cardioprotection by α-KG against I/R injury. Here we showed that α-KG has a high membrane permeability across H9c2 myoblasts, and the intracellular level of α-KG was significantly increased by α-KG treatment, suggesting that α-KG may play a non-metabolic role as well.

Figures

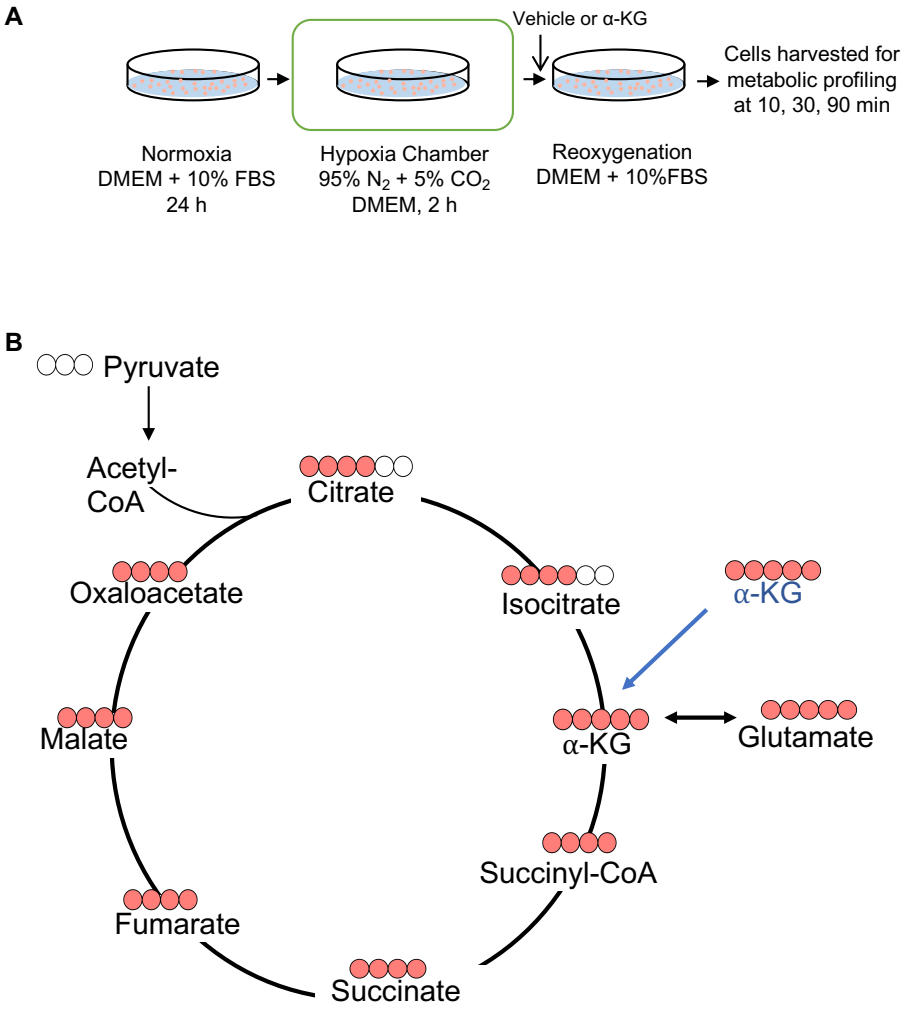


Figure 2-1. (A) Experiment design for metabolic profiling of H9c2 cells using the hypoxia chamber. (B) 13C isotope flow diagram illustrating the labeling of carbon atoms in metabolites in the TCA cycle and in glutamate, under the treatment with 13C5 α-KG (all five carbon atoms labeled).

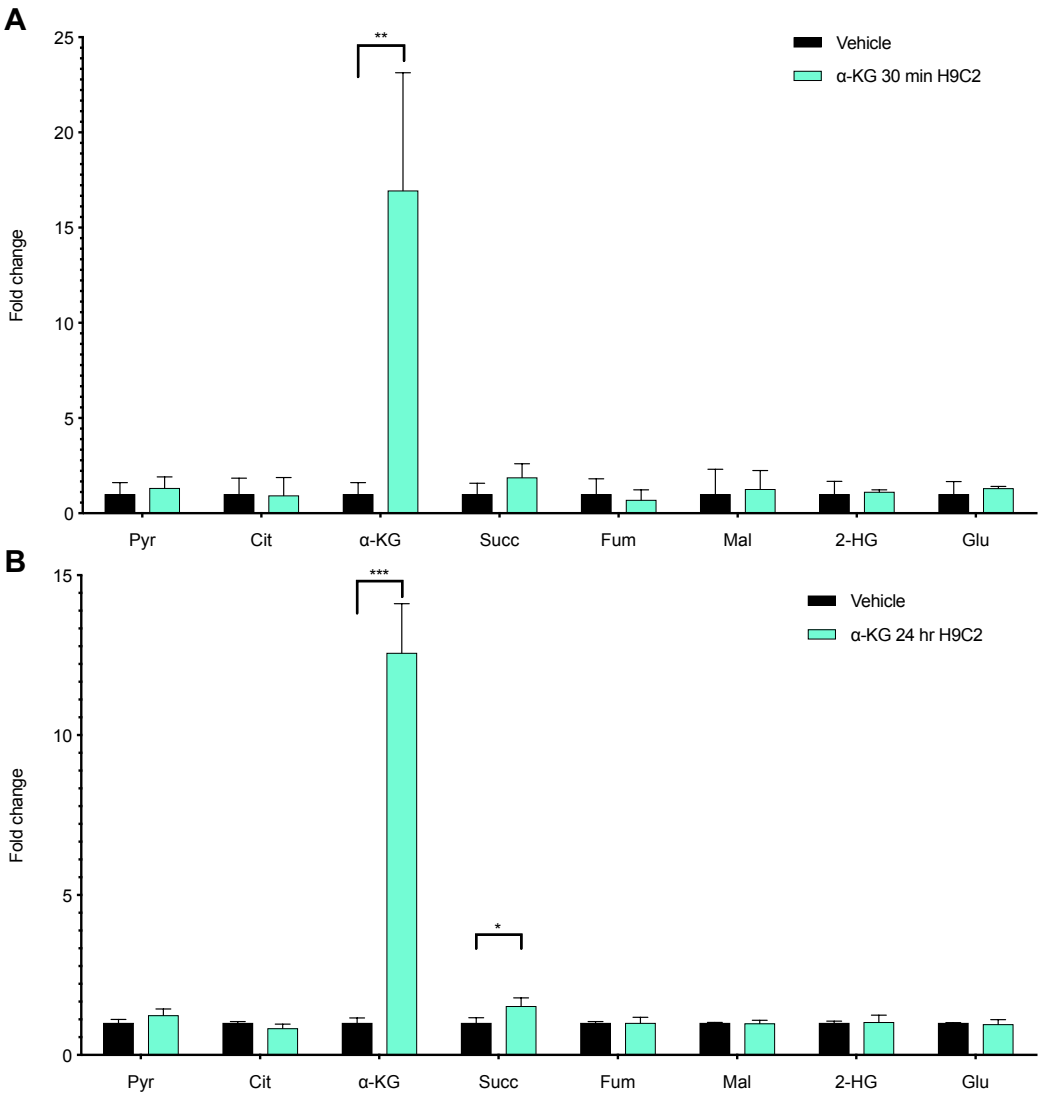


Figure 2-2. Metabolic profiling of H9c2 cells in normoxia. H9c2 cells are treated with vehicle (water) or 800 μM α-KG treatment for 30 minutes or 24 hours in complete growth medium (DMEM, high glucose with sodium pyruvate and glutamate + 10% FBS), the metabolites are extracted at the end of the treatment and further analyzed. (A) Intracellular levels of TCA cycle metabolites, 2-hydroxyglutarate (2-HG) and glutamate (Glu) in H9c2 cells under treatment for 30 minutes. (B) Intracellular levels of metabolites in H9c2 cells under treatment for 24 hours. Bars indicate mean ± SD, n = 5, *P < 0.05, **P < 0.01, ***P < 0.001. By unpaired two-tailed, two-sample unequal variance t-test.

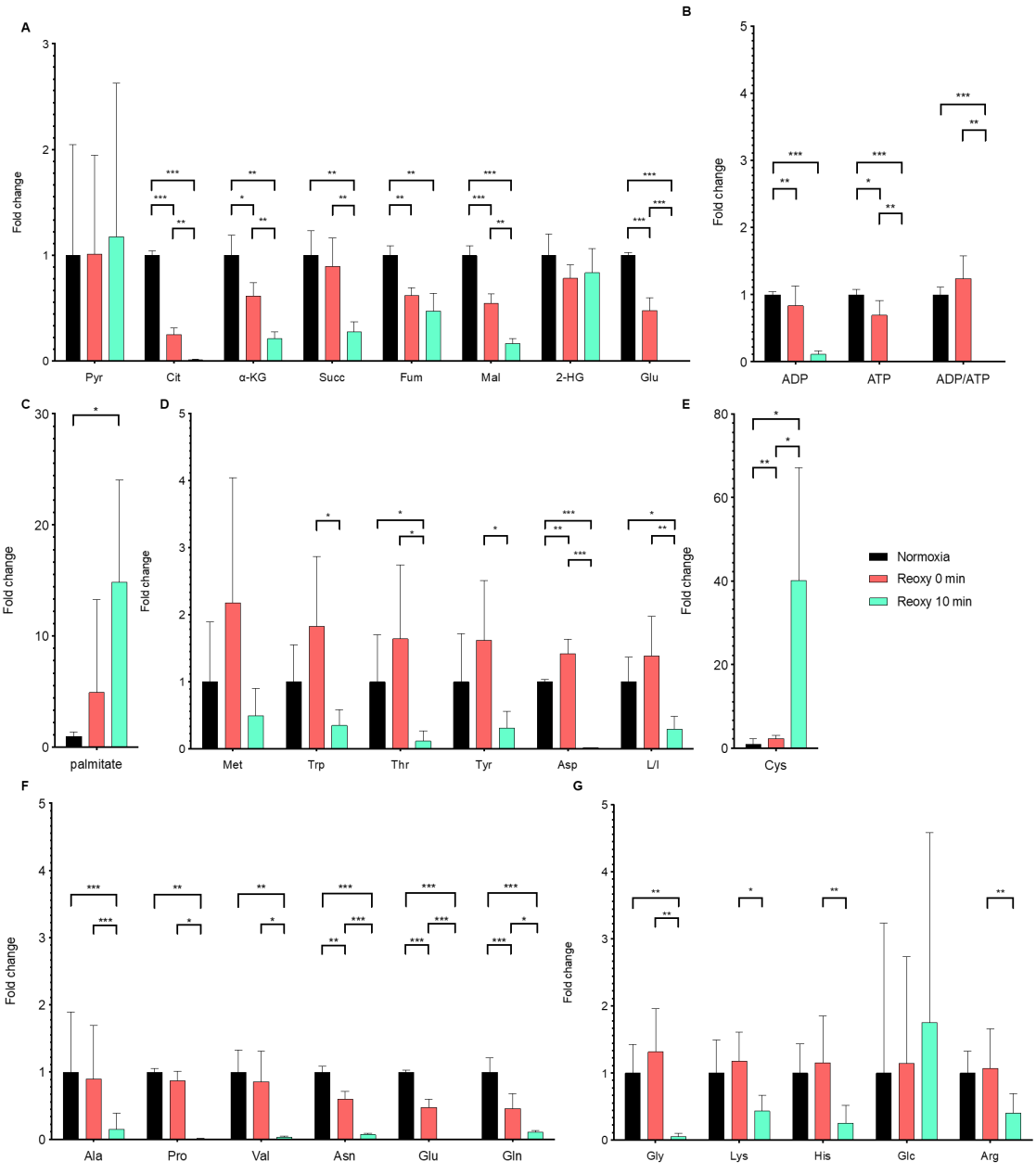
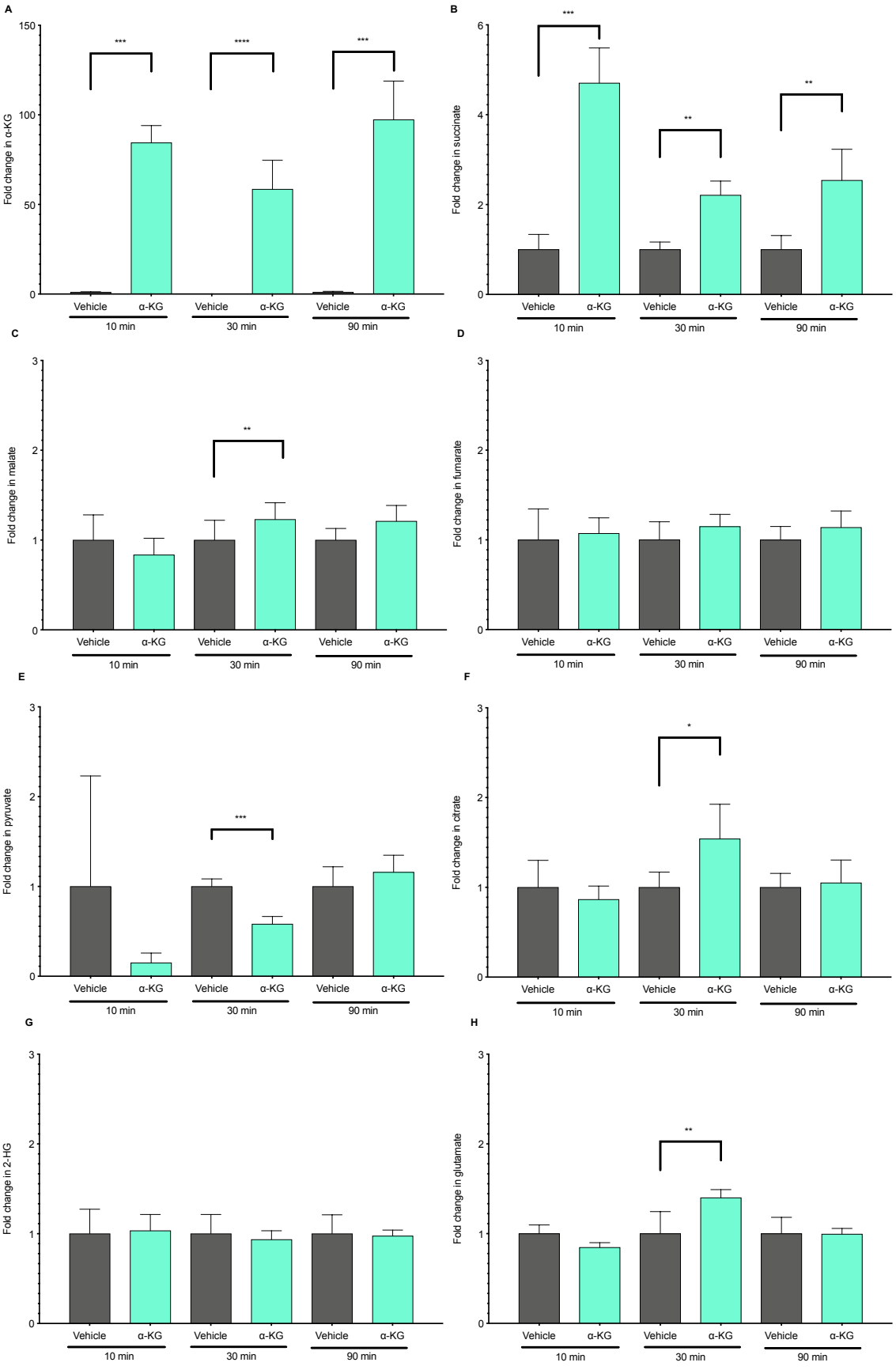


Figure 2-3. Metabolic profiling H9c2 cells during oxygen deprivation-reoxygenation. H9c2 are placed in hypoxia chamber with 95% N_2 + 5% CO_2 in DMEM (no glucose, no sodium pyruvate, no glutamate) for 2 hours to mimic the effect of ischemia. Upon reoxygenation, the medium is replaced with complete growth medium with either vehicle (water) or 800 μM α-KG. Metabolites were extracted at 0 or 10 minutes upon reoxygenation. Intracellular levels of (A) TCA cycle metabolites, 2-hydroxyglutarate (2-HG) and glutamate (Glu), (B) ADP, ATP, and ADP/ATP ratio, (C) Palmitate, (D-G) Amino acids. Reoxy: reoxygenation. Bars indicate mean ± SD, n = 5, *P < 0.05, **P < 0.01, ***P < 0.001. By unpaired two-tailed, two-sample unequal variance t-test.


A
Fold change in α-KG
150
100
50
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***
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***
Vehicle
α-KG
10 min
30 min
90 min
B
Fold change in succinate
6
4
2
0
***
**
**
C
Fold change in malate
3
2
1
0
**
D
Fold change in fumarate
E
Fold change in pyruvate
***
F
Fold change in citrate
*
G
Fold change in 2-HG
H
Fold change in glutamate
**

Figure 2-4. Metabolic profiling H9c2 cells after oxygen deprivation-reoxygenation using the same protocol described above, with treatment of vehicle (water) or 800 μM 13C labeled α-KG (13C5). Metabolites were extracted at 10, 30, 90 upon reoxygenation. Intracellular levels of (A) α-KG, (B) succinate, (C) malate, (D) fumarate, (E) pyruvate, (F) citrate, (G) 2-HG, (H) glutamate. Reoxy: reoxygenation. Bars indicate mean ± SD, n = 5, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. By unpaired two-tailed, two-sample unequal variance t-test.

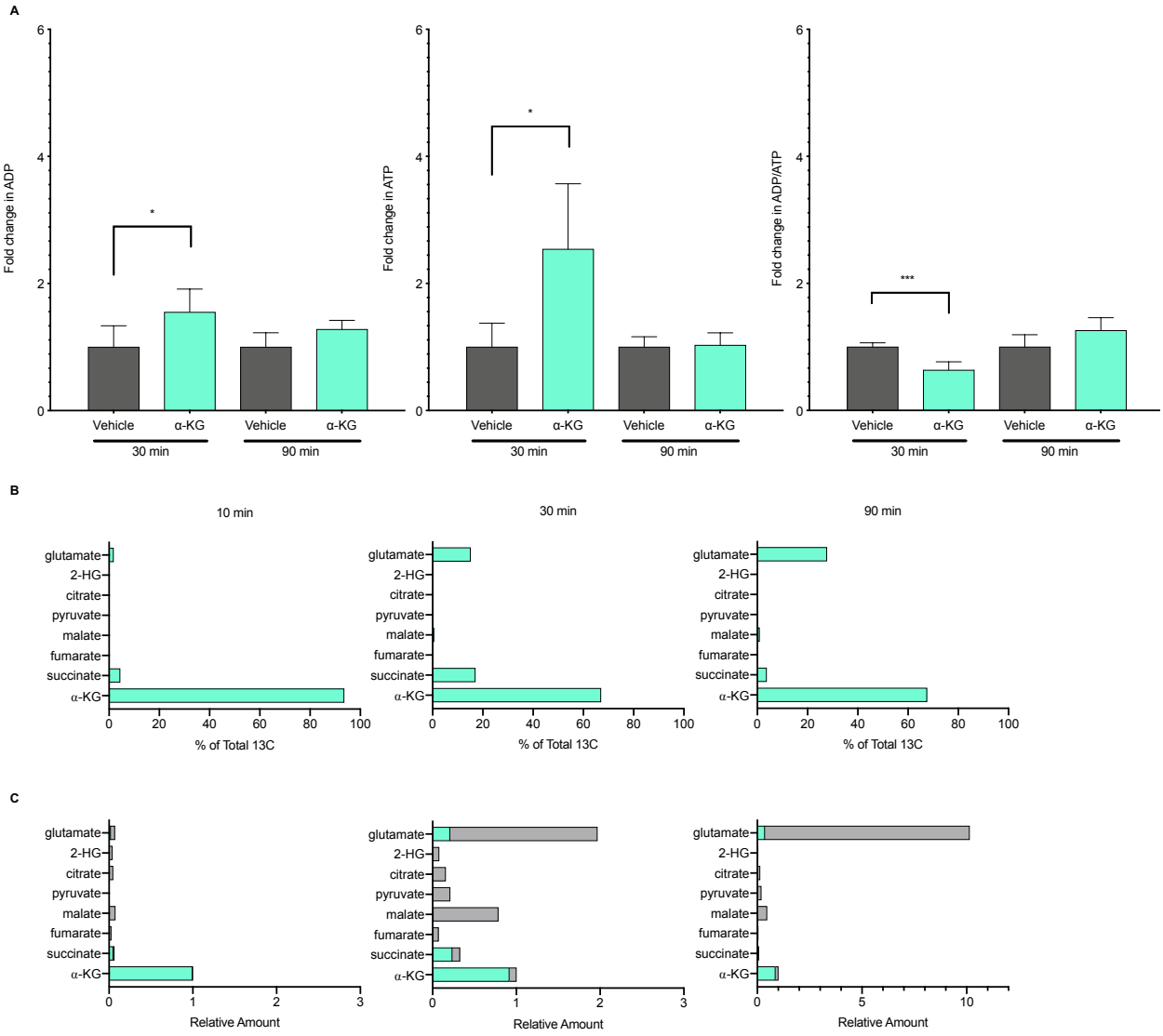

Figure 2-5. Metabolic profiling H9c2 cells after oxygen deprivation-reoxygenation using the same protocol described above, with the treatment of vehicle (water) or 800 μM 13C5 α-KG. Metabolites were extracted at 10, 30, 90 upon reoxygenation. Intracellular levels of (A) ATP, ADP and ADP/ATP ratio, (B) Distribution of 13C among intracellular metabolites in H9c2 cells treated with 800 μM 13C5 α-KG at 10, 30 and 90 minutes after hypoxia-reoxygenation, as shown in percentage of total 13C added. (C) Comparison of the absolute amount of metabolites in labeled and unlabeled forms. In Figure 2-5 C, only the primary conversion of metabolites from 13C5 α-KG was taken account into, since labeled metabolite generated from secondary conversion was less than 1% of the total amount of metabolites. Reoxy: reoxygenation. Bars indicate mean ± S.D., n = 5, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. By unpaired two-tailed, two-sample unequal variance t-test.

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Chapter Three: Investigation of the Mechanism of Cardioprotection by α-KG: The Connection with CypD-ATP Synthase Interaction

Abstract

Our finding showed that α-KG confers dramatic cardioprotection against IR injury *in vivo*. Mitochondria act as a key player in initiating oxidative stress and is one of the major targets to reduce I/R injury. Previously it has been shown that α-KG binds to ATP synthase and inhibits its activity. It was discovered that ATP synthase plays a critical role in the mitochondrial permeability transition pore (mPTP). In this chapter, we investigated the possible mechanism of which α-KG confers cardioprotection through both *in vitro* and *in vivo* methods.

Introduction

The Composition and Structure of mPTP: An Unsolved Problem

The mitochondrial permeability transition (mPT) is an event that is characterized as a rapid increase in the inner mitochondrial membrane permeability.[1] Raaflaub *et al.* in 1953 first discovered and described this event[2,3]. Haworth and Hunter in 1979 introduced the term "permeability transition", and proposed the concept that mPT is caused by the opening of mitochondrial permeability transition pore (mPTP) - a hypothetical channel that once opens, allows the flux of molecules under 1.5 kDa.[4-6] The opening of the mPTP is also extensively associated with other changes in the mitochondria, including mitochondrial swelling, loss of membrane potential (loss of proton electrochemical gradient), inhibition of respiration, ATP depletion, the release of cytochrome c and activation of cell death signaling[7,8].

The composition and structure of the mPTP have been extensively investigated for decades yet remain unsolved. Several models have been proposed to explain the mPTP formation. The original model of mPTP suggests that the adenine nucleotide translocator (ANT) on the inner membrane, the voltage-dependent anion channel (VDAC) on the outer membrane and cyclophilin D (CypD) in the mitochondrial matrix together form the mPTP (Figure 3-1 A)[9,10].

The findings that cyclosporine A (CsA) inhibits the inner membrane permeability transition and cyclophilin D is a target of CsA lead to the hypothesis that Cyclophilin D is a potential component of the mPTP[11-15]. Other CypD inhibitors were also capable to inhibit

opening of the mPTP [16,17]. This hypothesis was further supported by genetic evidence that mitochondria isolated from CypD knockout (*ppif*-/-) mice are resistant to swelling or permeability transition, and overexpression of CypD lead to mPT and cell death[18-20]. However, mice with deficiency in CypD still showed limited mPTP activities[18,21]. Since CypD itself cannot form a channel-like structure across the membrane, these findings suggest that although CypD appears to be a critical component of the mPTP, it does not constitute the pore structure of the mPTP.

The adenine nucleotide translocator (ANT) is a protein family of transporters to transport ATP and ADP across the inner membrane[22]. The hypothesis of ANT to be a component of the mPTP was supported by both pharmacological and biochemical evidence. Bongkrekic acid, an inhibitor of ANT, inhibits calcium-induced mPTP opening[14]. ADP and ATP also inhibit mPTP[10]. Vyssokikh *et al*. further showed that ANT and CypD are structurally connected[23], and Crompton and Halestrap *et al.* showed that the reconstituted ANT-CypD complex displays mPTP-like channel activity[9,24]. This model was further challenged by the observations from genetic studies by Kokoszka *et al*., in which the mPTP activity persisted in ANT1/2 double-null mice. But the ANT1/2 double-null mice in the study displayed reduced the mPTP sensitivity to calcium, and the inhibitory effect of bongkrekic acid was also abolished, suggesting a regulatory role of ANT in the opening of mPTP[25].

The hypothesis of voltage-dependent anion channel (VDAC) being a component of mPTP was also proposed[26,27]. The hypothesis was supported by the finding that the mPT was inhibited by when the VDAC channel activity is blocked[28,29]. The structural link between the VDAC and other hypothesized mPTP components and their ability to form

channel-like structures were also demonstrated by Crompton *et al.*[9]. However, genetic studies provided evidence against this hypothesis. Baines *et al.* showed that fibroblasts from VDAC1/3 double-null mice with VDAC2 knockdown were still sensitive to calcium-induced the mPT and oxidative stress[30]. These findings put the essential role of VDAC in question and raise the question whether the outer membrane plays a role in the mPTP at all.

A revised model of mPTP was later proposed to reflect the findings from genetic studies, and several new candidates of the mPTP components were proposed (Figure 3-1 B)[10]. The SLC25 family of inner mitochondrial membrane carriers included a large amount of cross-membrane proteins, including ANT. The mitochondrial phosphate carrier (PiC) in the SLC25 family was also proposed as a component of the mPTP[31]. The PiC was found to be closely associated with ANT and CypD structurally[32,33], and it provides a phosphate binding site-which could explain the Pi senility of the mPTP. The PiC was also capable of forming channel-like structures[34]. And genetic deletion of PiC desensitizes the mPTP[35]. The involvement of Bax and Bak proteins in the formation of the outer membrane structure of mPTP was suggested by Karch *et al.*, who discovered that loss of Bax/Bak desensitizes the mPTP and increases mitochondrial calcium retention capacity[36] (Figure 3-1 B).

ATP synthase is a large cross-membrane structure on the mitochondrial inner membrane. In 2013, two research groups proposed two new hypotheses suggesting the involvement of ATP synthase in mPTP. Bonora *et al.* and Alavian *et al.* proposed that the c-subunit ring of the F_oF_1 ATP synthase forms the channel structure of the mPTP[37,38]. Giorgio *et al*. proposed that dimers of ATP synthase form the mPTP[39]. This model was

supported by the evidence that Bz-423, an ATP synthase inhibitor, modulates the interaction between ATP synthase and CypD, and induces the mPTP opening[39,40].

The two F-ATP synthase mPTP models were not perfect in explaining all experimental observations. In 2017 He *et al*. showed that the mPT persists in the absence of ATP synthase subunit *c*, OSCP, and ATP6 and ATP8 membrane subunits[41,42]. However, their genetic mutations or perturbations did not completely remove ATP synthase from the mitochondria and the core structure of ATP synthase was still maintained. Given the complexity in the composition and structure of the ATP synthase, it is possible that the ATP synthase lacking certain subunits could still form mPTP-like channels under certain circumstances to display properties like mPTP. Antoniel *et al.* used genetic mutations instead of knockout to study the involvement of ATP synthase, and demonstrated the involvement of ATP synthase in mPTP acvitiy[43]. In 2019, Neginskaya *et al*. reported evidence showing that mitochondria deficient of ATP synthase c-subunit could form smaller channels displaying CsA sensitivity[44].

The composition and structure of the mPTP remain an unsolved problem. Although current ATP synthase models explained the experimental observations, they are far from enough to explain the mechanism of the mPTP opening. It is possible that there is not a uniform mechanism of the mPTP, and the mPTP forms through different structures under various physiological and biochemical conditions.

The mPTP as a Target for Cardioprotection Against I/R Injury

The mPTP plays a critical role in ischemia-reperfusion (I/R) injury. Oxidative stress, Ca^{2+} overload, and pH paradox trigger the mPTP opening. Prolonged mPTP opening lead to mitochondrial dysfunctions such as swelling, loss of membrane potential and ATP depletion. These dysfunctions further lead to cytochrome c release and activation of cell death signaling[8]. The mPTP opening has been well observed during the early reperfusion stage, and the administration of mPTP inhibitor cyclosporine A (CsA), either before ischemia or upon reperfusion, conferred cardioprotection against I/R injury and reduced the infarct size[45,46]. The involvement of mPTP in I/R injury was also supported by observations in genetic studies and cardiomyocytes devoid of CypD were resistant to oxidative damage. CypD knockout mice showed resistance against I/R injury and reduced infarct size compared with wildtype[18].

A lot of effective cardioprotective approaches against I/R injury have been shown to involve inhibition of mPTP opening. For example, both ischemic preconditioning (IPC) and remote ischemic conditioning were shown to inhibit mPTP opening, and IPC does not further reduce the infarct size in CypD knockout mice[47-49]. Targeting the mPTP pathway has been the focus of development of new cardioprotective strategies over the past decade, and several mPTP inhibitors have been tested in clinical trials. However, the results were not promising[50,51]. The use of cyclosporine A as a pharmacological intervention is limited by its off-target and immunosuppressive effects. Until now, there is still a lack of potent mPTP inhibitor suitable for clinical practice to confer cardioprotection against I/R injury[50,52,53].

Previously, Chin *et al.* discovered that the metabolite alpha-ketoglutarate (α-KG) binds to ATP synthase and inhibits its activity[54]. Given the possible role of ATP synthase

in the mPTP and I/R injury, we investigated into the structural interplay between α-KG and ATP synthase-CypD complex. We also examined whether α-KG depends on CypD to confer cardioprotection.

Materials and Methods

Drug Affinity Responsive Target Stability (DARTS)[55]

H9c2 or HEK293 cells were lysed in M-PER buffer (Thermo Scientific, 78501) with the addition of protease inhibitors (Roche, 11836153001) and phosphatase inhibitors (50 mM NaF, 10 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 2 mM Na_3VO_4). Chilled TNC buffer (50 mM Tris-HCl pH 8.0, 50 mM NaCl, 10 mM CaCl2) was added to the protein lysate, and protein concentration of the lysate was measured by the BCA Protein Assay kit (Pierce, 23227). The protein lysate was then incubated with vehicle control (H2O) or varying concentrations of α-KG for 3 hrs at room temperature with continuous rotation in an Eppendorf tube. Pronase (Roche, 10165921001) digestions were performed for 5 min at room temperature and stopped by protease inhibitors.

Western blotting

H9c2 or HEK293 cells were lysed in M-PER buffer (Thermo Scientific, 78501) with the addition of protease inhibitors (Roche, 11836153001) and phosphatase inhibitors. The cell lysate was then centrifuged at 4 centigrade for 5 minutes. The supernatant was collected and added with 5x loading buffer. For mitochondrial lysate samples in the immunoprecipitation assay, the samples were obtained according to manufacturer's instruction. The samples were subjected to SDS-PAGE on 4–12% Bis-Tris gradient gel (Invitrogen, NP0322BOX) and western blotted for ATP synthase subunit ATP5B (Sigma,

AV48185), ATP5O (ArigoBio, ARG57362), CypD (Abcam, ab64935), GAPDH (Ambion, AM4300), NDUFV1(Santa Cruz Biotechnology, sc-138808).

Immunoprecipitation

Immunoprecipitation of ATP synthase was performed with isolated bovine or mouse heart mitochondrial lysate using anti-ATP synthase monoclonal antibody covalently linked to protein G-Agarose beads according to the manufacturer's protocol (MS501 immunocapture kit; Abcam). Basically, isolated bovine or mouse heart mitochondrial lysate were incubated with non-ionic detergent (0.5% n-Dodecyl β-D-maltoside, Sigma Aldrich, D4641) on ice for 30 minutes. The lysate was then centrifuged at 21000 x g for 30 minutes at 4 centigrade. The supernatant was collected, and added with protease inhibitors (Roche, 11836153001). 10 µl of ATP synthase immunocapture beads were added to 100 µg mitochondrial lysate, and incubated with vehicle (water or DMSO), or 800 µM α-KG overnight at 4 centigrade on a rotator. The mixture was then washed 5 times using the washing buffer (0.05% n-Dodecyl β-D-maltoside), and the beads were collected. The beads were then eluted using SDS elution buffer (1% SDS), and the protein samples were identified using western blot.

In vivo mouse myocardial injury model in CypD knockout mice

Animal studies were performed under approved animal research protocols. *Ppif (+/-)* mice were obtained from Evan Taddeo from Orian Shirihai lab at UCLA. *Ppif (-/-)* and

wildtype mice were then generated through breeding *ppif (+/-)* mice. The *in vivo* mouse myocardial I/R injury model was the same as described in Chapter one.

Isolation, culture and adenoviral infection of rat ventricular myocytes

Neonatal rat ventricular cardiomyocytes (NRVMs) were isolated from 1-day old Sprague-Dawley rats as described[56].

Complex I enzymatic activity assay

The Complex I diaphorase enzymatic activity assay was measured using the Complex I Enzyme Activity Microplate Assay Kit (Abcam, ab109721) according to the manufacturer's instructions.

LDH and ATP cell viability assays

Cell viability was assessed by measuring the ATP content described previously[57]. The LDH concentration in the culture medium was spectrophotometrically assayed using a kit (Sigma MAK066) according to the manufacturer's protocols[58].

RNA interference–mediated gene silencing

The siRNAs used for gene silencing was designed in 19 nucleotides length, with a dTdT overhang at the 3′ terminus, using the tool provided by Invitrogen. Lipofectamine

RNAiMAX (Invitrogen) was used for transfection of the siRNA into cardiomyocytes according to the manufacturer's instructions. 72 h after siRNA transfection, western blotting was used to assess the efficiency of gene knockdown[58].

Mitochondrial Membrane Potential Assay

H9c2 cells were incubated and treated under hypoxia-reoxygenation as described in Chapter 2. Upon reoxygenation, the membrane potential was measured using Tetramethylrhodamine, Methyl Ester, Perchlorate (TMRM) (Thermo Fisher T668) according to the manufacturer’s instructions.

Statistical analyses

Data represent biological replicates. Appropriate statistical tests are used for every figure. Data meet the assumptions of the statistical tests described for each figure. Mean ± S.D. is plotted in all figures unless stated otherwise.

Results

α-KG Affects ATP synthase - CypD interaction

Previously α-KG was shown to bind to ATP synthase by Chin *et al.*[54]. Bernadi *et al*. provided evidence showing that ATP synthase and cyclophilin D (CypD) interact with each other and form mPTP[39,40]. Therefore, it is possible when α-KG binds to the ATP synthase, the CypD-ATP synthase interaction is affected as well. To test this hypothesis, we used Drug Affinity Responsive Target Stability (DARTS)[59] to examine whether the presence of α-KG affects CypD stability against protease digestion by binding to ATP synthase. DARTS is a drug-target identification technology developed by Lomenick *et al.*[59] for investigation of molecule-protein interactions. The principle of DARTS is shown in Figure 3-2 A. Basically, when a protein is bound by a molecule, its conformation changes and its stability against protease digestion changes, and this change can be detected semi-quantitatively by western blotting or quantitatively by mass spectrometry. DARTS with H9c2 cell lysates revealed that α-KG increases the stability of ATP5B, ATPTO, and CypD against protease digestion(Figure 3-2 B), and results from DARTS with recombinant CypD solution (no ATP synthase is present) showed α-KG does not directly affect the stability of CypD protein against protease digestion without the presence of ATP synthase (Figure 3-2 C). These findings also confirmed the previous discovery by Bernadi *et al.* that ATP synthase interacts with CypD[40] and suggested that α-KG may affect the interaction between CypD protein and ATP synthase complex.

Bernadi *et al.* showed that ATP synthase inhibitors such as oligomycin and Bz-423, affect the ATP synthase - CypD binding in different ways using the co-immunoprecipitation method with anti-ATP synthase beads[40]. When Bz-423 is present, the amount of CypD bound to ATP synthase was less compared to control group[40]. Bz-423 was shown to induce the mPTP opening and activates apoptotic signaling. And oligomycin increases the amount of CypD bound to the ATP synthase[39,40]. In our previous investigation, both oligomycin and α-KG confer cardioprotection against I/R injury (Chapter one, Figure 1-2). We used the same co-immunoprecipitation method to test the effect of α-KG on CypD – ATP Synthase interaction. Similar to oligomycin, the presence of α-KG increased the amount of CypD bound to the ATP synthase(Figure 3-2 D).

α-KG Depends on CypD to Confer Protection Against Oxidative Damage

We examined whether CypD is required for α-KG to protect against oxidative damage. Neonatal rat ventricular myocytes (NRVMs) were treated with either vehicle (water) group or α-KG and incubated in complete growth medium with vehicle (water) or 400 μM H_2O_2 for 24 hours. ATP content and the LDH release were measured to indicate cell viability. Treatment with α-KG significantly improved cell viability compared with vehicle groups (Figure 3-3 A and B). We further examined whether α-KG depends on the expression of CypD to confer protection against oxidative damage using CypD siRNA. In NRVMS treated with CypD siRNA, α-KG did not further confer increase ATP content or reduce LDH release (Figure 3-3 C and D).

α-KG Depends on CypD to Reduce Infarct Sizes *In Vivo*

Next, we examined whether α-KG depends on CypD to confer cardioprotection against I/R injury using the *in vivo* myocardial infarction model in CypD knockout (*ppif-/-*) mice. α-KG still reduced the infarct size (IS/AAR) in wildtype by 39% compared with vehicle treatment (wildtype: vehicle: 69% ± 9%, α-KG: 42% ± 12%, $p < 0.0001$, n = 11 ~ 12, Figure 3-4 A). But α-KG does not reduce the infarct size in *ppif-/-* mice (*ppif-/-*: vehicle: 57% ± 13%, α-KG: 56% ± 10%, $p > 0.05$, n = 11 ~ 12, Figure 3-4 A). In addition, although *ppif-/-* mice displayed smaller infarct size versus wildtype ($p < 0.05$, 17% reduction), their infarct sizes are still larger than α-KG treated wildtype mice (*ppif-/-*: vehicle: 57% ± 13%, wildtype: α-KG: 42% ± 12%, $p < 0.05$). And infarct sizes in α-KG treated wildtype mice are smaller than α-KG treated *ppif-/-* mice ($p < 0.05$). The AAR/LV ratio is similar among all groups (wildtype: vehicle: 45% ± 12%, α-KG: 48% ± 8%, *ppif-/-*: vehicle: 45% ± 8%, α-KG: 46% ± 8%, n = 11 ~ 12, Figure 3-5 B). LVDP was also significantly improved by α-KG in wildtype, but not in CypD knockout mice(Figure 3-5 C).

The Effect of α-KG on Membrane Potential in H9c2 Cells upon Reoxygenation

The effect of α-KG on membrane potential in H9c2 cells undergone oxygen deprivation-reoxygenation was examined. The membrane potential is needed for ATP synthase to produce ATP and the membrane potential is decreased in mitochondria having mPTP opening. Tetramethyl rhodamine, methyl ester (TMRM) was used as the membrane potential indicator in H9c2 cells and the fluorescence level was measured

during normoxia and reoxygenation (Figure 3-5). Treatment with α-KG only slightly increased the membrane potential during reoxygenation, and the difference is only significant at 2 hours after reoxygenation.

Succinyl-CoA May be Involved in the Cardioprotection by α-KG

Succinyl-CoA is a downstream metabolite of α-KG and confers cardioprotection *in vivo* as well (Figure 1-1). Through a screening of protein targets using DARTS, we discovered that succinyl-CoA binds to the mitochondrial Complex I subunit NDUFV1 (Figure 3-6 A), and succinyl-CoA inhibits Complex I diaphorase activity in the Complex I diaphorase activity assay (Figure 3-6 B).

α-KG metabolizes through α-KGDH to generate NADH and succinyl-CoA. To examine whether the metabolism of α-KG into succinyl-CoA play a role in the cardioprotection by α-KG. We tested the cardioprotection by α-KG with co-treatment of α-KGDH inhibitor CPI-613, or CPI-613 + succinyl-CoA. α-KG + CPI-613 did not reduce the infarct size compared with vehicle. And the cotreatment of α-KG + CPI-613 + succinyl-CoA further reduced the infarct size (Figure 3-6 D).

Discussion

α-KG May Confer Cardioprotection By Regulating ATP Synthase - CypD Interaction

We discovered that α-KG affects the structural interaction between ATP Synthase and CypD and α-KG does not confer cardioprotection in CypD knockout mice. These results suggest α-KG may confer cardioprotection by regulating ATP Synthase - CypD interaction. However, this mechanism needs further validation in the following aspects: (1). The mPTP structural model is still under debate, and the mechanisms of the mPTP were not fully understood. We need a complete understanding of the mPTP structure to better clarify the mechanism of α-KG. (2) The mPTP opening is a mitochondrial response to stimuli like ROS, calcium, and pH plays a role in mediating the cell death signaling. It is possible that α-KG acts through the upper stream pathway (i.e. antioxidative effects) and indirectly affect mPTP opening. (3) Although the *ex vivo* Langendorff model showed the cardioprotection by α-KG is at least partially from a direct effect on the myocardium, it is possible that α-KG works through other organs or tissue to confer remote cardioprotection that is dependent on CypD. (4) Direct evidence showing α-KG inhibits the mPTP opening in cardiac myocytes under I/R is also needed to support the hypothesis. It is possible that α-KG is relying on other functions of CypD instead of its role in mPTP opening. (5) A few studies discovered changes in the metabolism pattern of CypD knockout mice. Tavecchio *et al*. showed that in the mouse embryonic fibroblasts (MEFs) from CypD knockout mice embryos, there is an increase in the α-KG level compared to wildtype[60]. Also, CypD knockout mouse displayed a global proteomic pattern difference compared with wildtype, especially with upregulation of peroxiredoxin-6, a H_2O_2 scavenger[61]. Therefore, an in-depth analysis of CypD knockout mice metabolism is also needed to further investigate the mechanism.

In addition, an experimental design with littermate is important for knockout mice experiments to reduce the impact of variations in genetic backgrounds[62]. Our CypD knockout mice experiment was not performed with the littermate experiment design. Therefore, a highly controlled littermate experiment might better clarify the involvement of CypD in the cardioprotection by α-KG.

α-KG May Work Through Additional Mechanisms

The infarct size in the wildtype α-KG group is smaller than CypD knockout vehicle group, suggesting the possibility of multiple mechanisms. The metabolism role of α-KG may be involved in the cardioprotection by α-KG as well. In the TCA cycle, α-KGDH converts α-KG into succinyl-CoA and generates NADH. Our results showed that succinyl-CoA confers cardioprotection, and inhibition of α-KGDH using an α-KGDH inhibitor CPI-613 abolished the cardioprotection by α-KG, suggesting that α-KGDH might be critical in the cardioprotection by α-KG. This potential mechanism needs further investigation to be better understood.

Figures

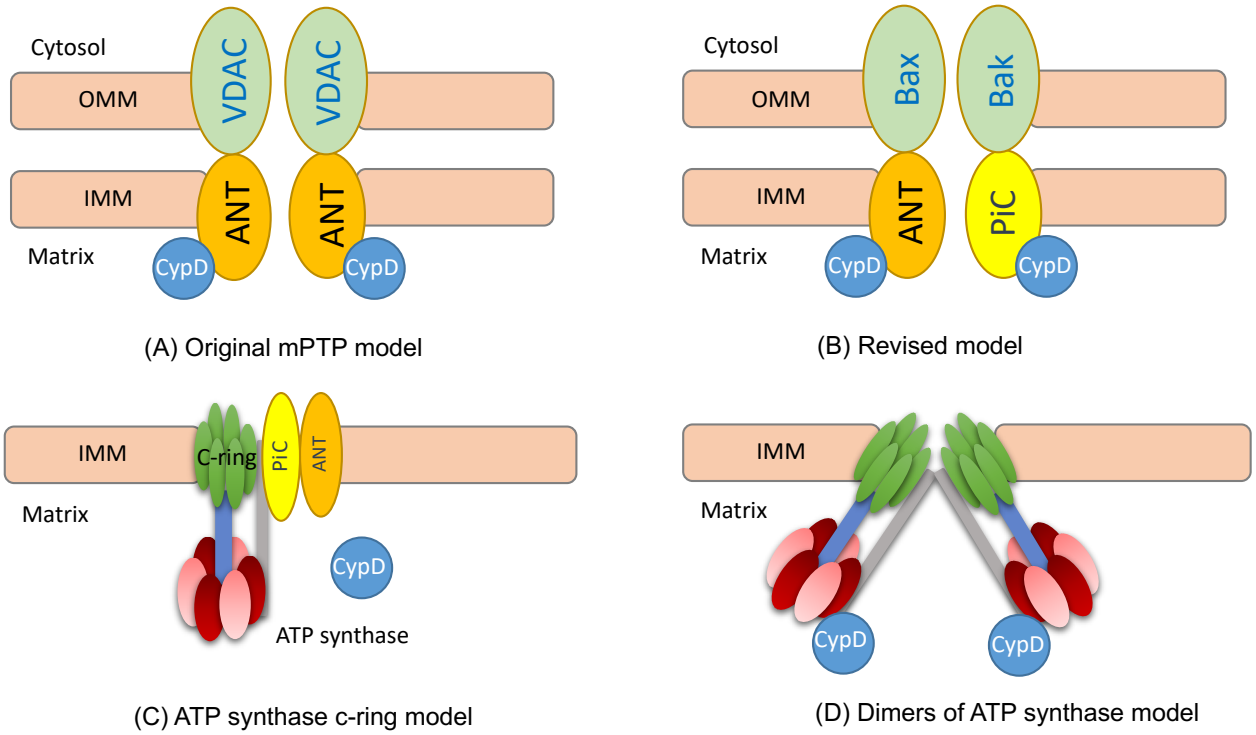

Figure 3-1: Models of mPTP. (A) The original model suggesting the involvement of VDAC, ANT, and CypD. (B) A revised model including the possible involvement of PiC and Bax/Bak. (C) The model of mPTP formed by ATP-synthase c-ring. (D) The model of mPTP formed by dimers of ATP-synthase.

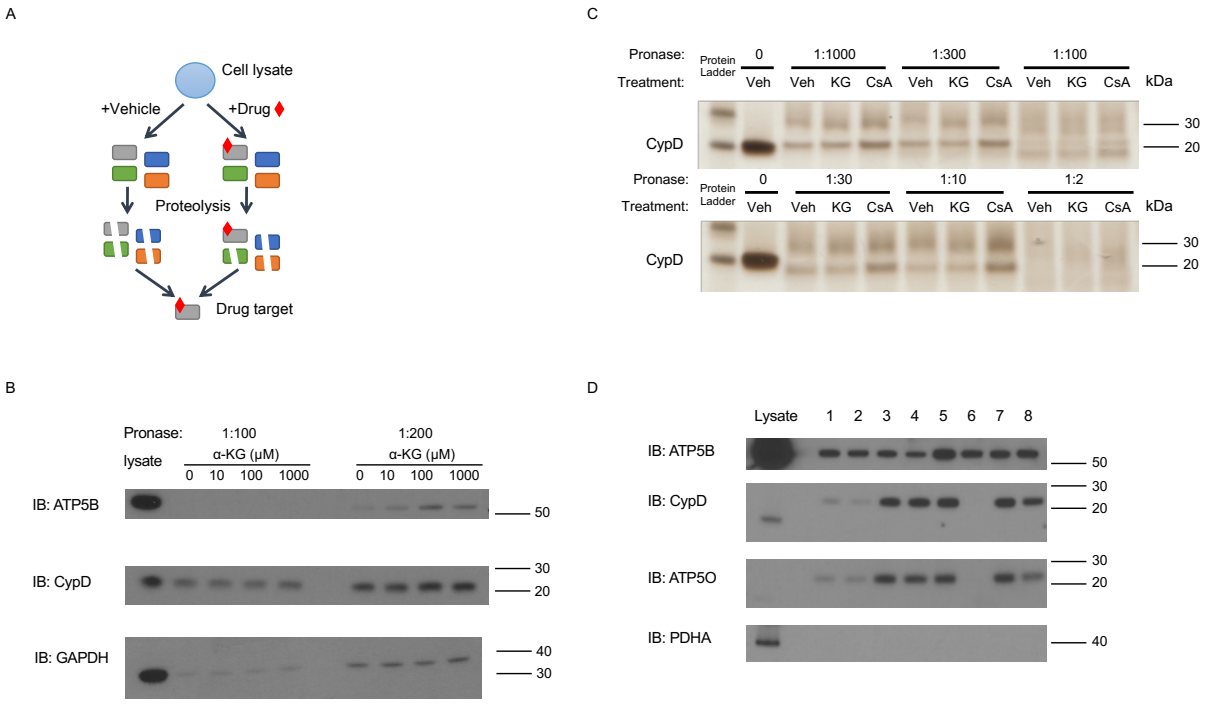

Figure 3-2: (A) Scheme of DARTS. (B) DARTS experiment using H9c2 cell lysate showing the effect of α-KG on the stabilities of ATP5B, CypD, ATP5O and GAPDH against protease digestion. (C) DARTS experiment using recombinant CypD protein showing the effect of α-KG on the stabilities of CypD against protease digestion. (D) Representative results of immunoprecipitation assay using mouse heart mitochondrial lysate with anti-ATP synthase immunoprecipitation beads. 1,2. Vehicle, 3. 0.8 mM α-KG, 4. 1.6 mM α-KG, 5. 3 μM oligomycin, 6. 1 μM CsA, 7. 1% DMSO, 8. 50 μM Bz-423 (1% DMSO).

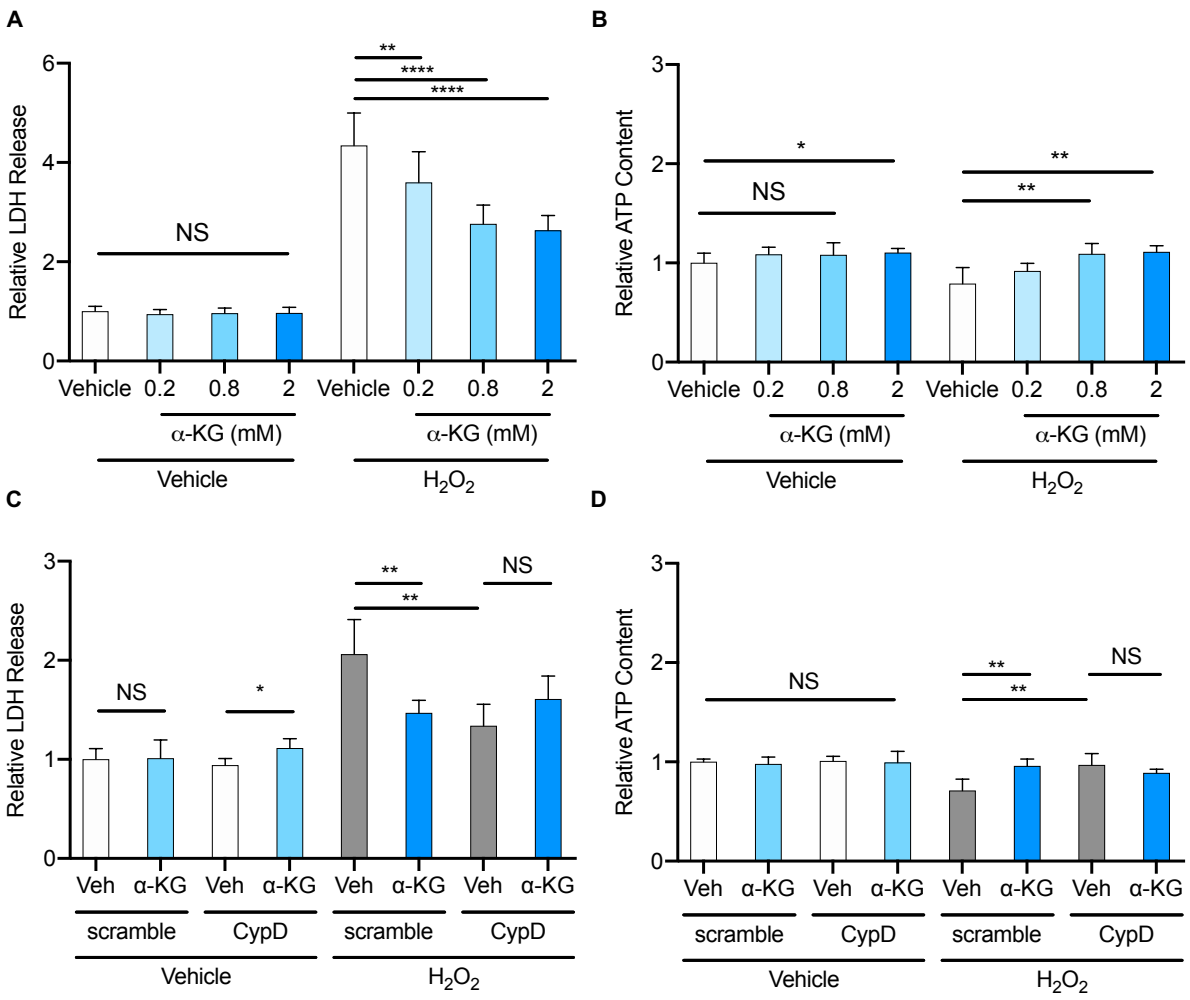

Figure 3-3: α-KG increases ATP production (A) and decreases LDH release (B), but does not affect apoptosis pathways (C) in neonatal rat ventricular cardiomyocytes under H2O2 treatment. Bars indicate mean ± SD, n=12. ****P < 0.0001, **P <0.01, by unpaired t-test, two-tailed, two-sample unequal variance. (D and E) α-KG depends on CypD to confer protection against oxidative damage. Bars indicate mean ± SD, n=6. ****P < 0.0001, **P <0.01, by unpaired t-test, two-tailed, two-sample unequal variance.

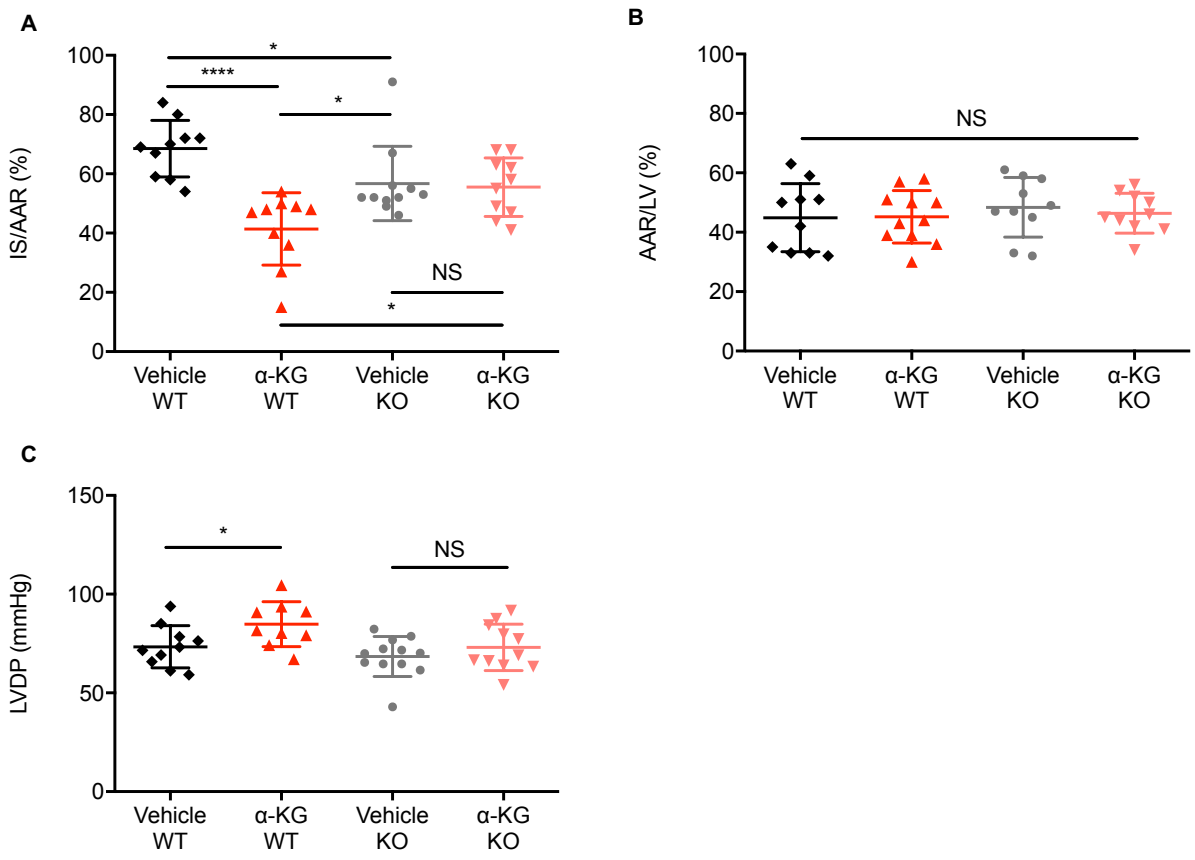

Figure 3-4: The effect of α-KG treatment on (A) Infarct size shown as IS/AAR (B) AAR/LV (C) LVDP in the *in vivo* myocardial infarction model in CypD knockout mice. WT, wildtype. KO, CypD knockout. Only male mice were used. IS/AAR, infarct area as a percentage of area at risk, AAR/LV, area at risk as a percentage of the left ventricle. LVDP, left ventricular diastolic pressure. Data shown results of 9-12 biological replicates, *P < 0.05, **P < 0.01, ****P < 0.0001, by unpaired two-tailed, two-sample unequal variance t-test. Mean ± S.D. plotted.

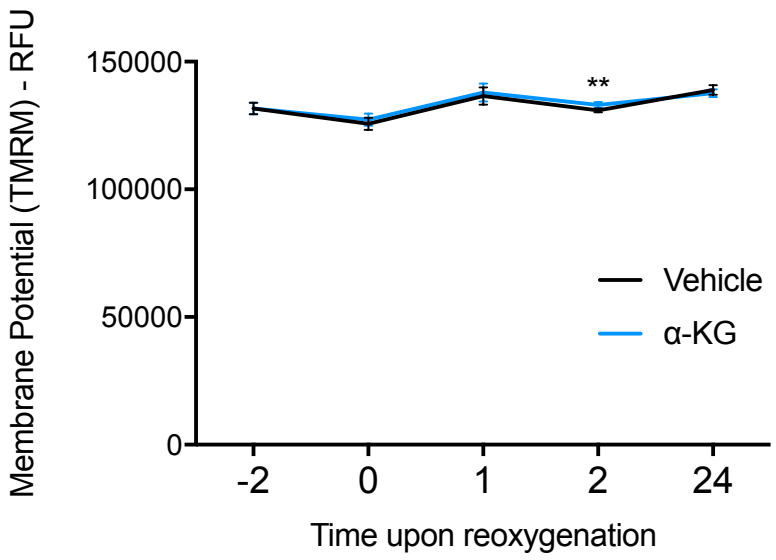

Figure 3-5: The effect of α-KG treatment on membrane potential in H9c2 cell under hypoxia-reoxygenation. Data showing results of 5 biological replicates, **P < 0.01, by unpaired two-tailed, two-sample unequal variance t-test. Mean ± S.D. plotted.

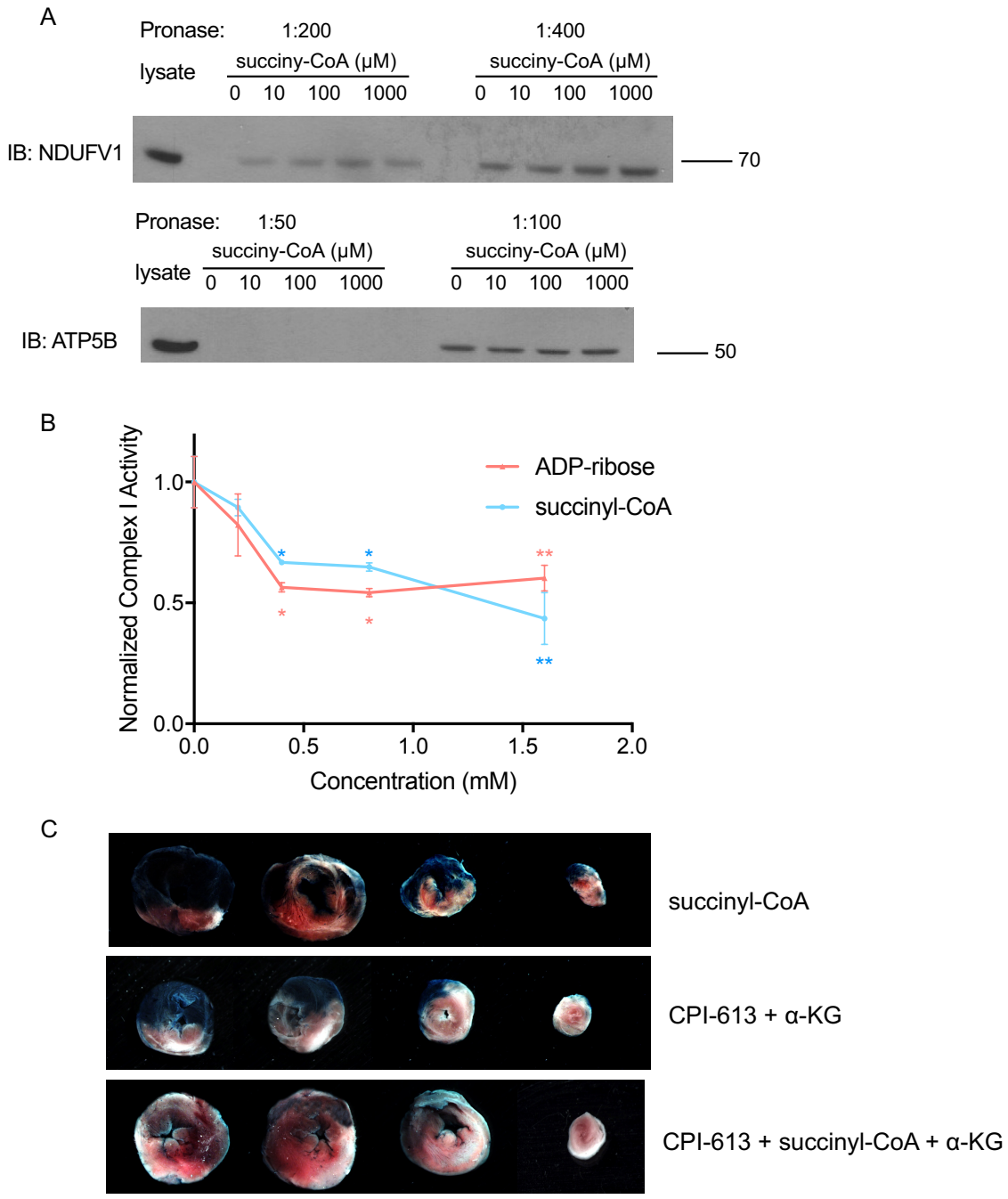

Figure 3-6: (A) DARTS method showing the effect of succinyl-CoA on the stability of NDUFV1. (B) Mitochondrial complex I activity assay showing the effect of succinyl-CoA on Complex I diaphorase activity. *P < 0.05, **P < 0.01, by unpaired two-tailed, two-sample unequal variance t-test. Mean ± S.D. plotted. (C) Representative results from the *in vivo* mouse myocardial infarction model showing the effect of succinyl-CoA and CPI-613 on infarct sizes.

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Conclusion and Future directions

In this dissertation, we discovered a novel approach that confers cardioprotection against ischemia-reperfusion (I/R). Post-ischemic administration of the tricarboxylic acid (TCA) cycle intermediate alpha-ketoglutarate (α-KG) reduces the infarct size by ~70% and significantly improves heart hemodynamic parameters. The cardioprotection by α-KG also showed higher potency in male mice versus female mice. The mechanism of α-KG to confer cardioprotection is still unclear. Our results in cyclophilin D (CypD) knockout mice showed that α-KG cannot confer cardioprotection in mice deficient in CypD, suggesting that α-KG may depend on CypD to confer cardioprotection. Our immunoprecipitation analysis showed that α-KG affects the interaction between CypD and ATP synthase, and this interaction has been shown to be potentially connected with the mPTP opening[1,2]. Therefore, it is possible that α-KG functions through regulating the ATP synthase - CypD interaction and further affect the mitochondrial permeability transition pore (mPTP) opening to confer cardioprotection.

The cardioprotection by α-KG also appears to be even more potent than CypD deficiency, and this suggests that there might be additional mechanisms of the cardioprotection by α-KG. It has long been proposed and supported by clinical observations that the cellular energetics and TCA cycle metabolism are impaired during the reperfusion stage[3-7]. The metabolic effect of α-KG through α-KGDH may also contribute to its cardioprotective effects. α-KGDH is a major source of NADH in the TCA cycle, and the product of α-KGDH, succinyl-CoA also appeared to be cardioprotective. Therefore, it is possible that the treatment of α-KG improves the overall cellular energy

production and increased the intracellular succinyl-CoA to result in cardioprotection. This possibility is partially supported by our finding that succinyl-CoA confers cardioprotection, and co-treatment with CPI-613 abolishes the cardioprotection by α-KG.

However, to further understand the mechanism of cardioprotection by α-KG, there are several issues remaining to be elucidated: (1) The hypothesis of α-KG regulating ATP synthase - CypD interaction to inhibit the mPTP opening requires more support from additional experimental results. Testing α-KG in the calcium retention capacity assay using mitochondria isolated from the reperfused heart is an important experiment that will provide insight of whether α-KG directly affect the mPTP opening in response to calcium overload. (2) The CypD knockout mice experiment was not performed with littermate design. The accuracy and reliability of the experiment result could be further improved with a littermate design. However, a littermate design might not be very practical in this case, since it will require a much larger number of breeding pairs and a longer time to operate the experiment. (3) The metabolic effects of α-KG on the myocardium under I/R was not characterized. Our metabolic profiling of H9c2 cells using a simulated oxygen deprivation - reoxygenation procedure does not reflect the real changes in myocardium under I/R. Therefore, it is important to examine the metabolism of myocardium under I/R with α-KG treatment in the *in vivo* mouse myocardial infarction model. (4) The composition and structure of mPTP remain unclear, and the ATP synthase - CypD complex model is only supported partially based on the experimental observations. Therefore, if there is a more thorough understanding of the mPTP structure and mechanism, our conclusion might need to be revised accordingly.

Use of α-KG as a post-ischemic treatment to protect against I/R injury has four advantages: (1) Post-ischemic treatment allows a less-stringent therapeutic window in clinical practice compared with other approaches, therefore this approach has a potentially wider range of application in patients with myocardial infarction; (2) α-KG is an endogenous metabolite and its safety in clinical practice has been well established: A lot of pharmaceutical approaches in this field failed in clinical phases due to toxicity and off-target adverse effects. The approach of α-KG might be advantageous since the dosage range of α-KG that is effective in our study has been tested as safe in previous clinical practice[8,9]. (3) The extent of infarct size reduction by α-KG is the most dramatic among all major approaches compared with other approaches[10-15]. These advantages make α-KG a very strong candidate as a pharmacological approach to reduce I/R injury. (4) A lot of approaches to reduce I/R injury are used before ischemia or during ischemia[11,16]. Therefore, there is a good chance for α-KG to be used in combination with these approaches to achieve additive effectiveness.

The translation of approaches to recede I/R injury from bench to bedside has not been very successful. A lot of attempts failed and there are several underlying reasons. The animal models used for I/R injury differs from myocardial infarction patients from various aspects. The animal model uses young, healthy animals that receive external compression on their coronary artery to induce ischemia, and the duration is usually between 30 - 60 minutes. However, human patients with myocardial infarction are majorly elderly populations, and human myocardial infarction usually requires 90 minutes or more to be established and the potential ischemia time could be longer. These patients also have a higher chance of co-morbidities[10,16,17]. Our discovery of α-KG to confer

cardioprotection against I/R injury in the mouse model may help with the development of a novel cardioprotective strategy in clinical practice.

The following factors should be considered when designing the clinical study for this approach: (1) *Sex*. α-KG has a higher potency in male mice versus female mice, and the female mice showed more resistance against I/R injury compared to male mice. (2) *Route of administration*. α-KG is an endogenous metabolite and it may undergo vast metabolic changes depending on the route of administration. A careful bioavailability or metabolic profiling analysis will help better understand the best route of administration. (3) *Therapeutic window*. Previous clinical studies used α-KG at a concentration much higher than 800 μM[9]. Therefore, a higher dosage of α-KG may be tested in the clinical trial to optimize the treatment. (4) *Time of administration*. It is not clear whether administration of α-KG before ischemia or after reperfusion also confers cardioprotection. It was suggested that the mPTP opening usually occurs within a few minutes of reperfusion[12,18,19]. If the mechanism of α-KG is majorly through inhibiting the mPTP opening, α-KG should be administered within the 15 minutes of reperfusion or earlier.

In summary, we identified that a novel cardioprotective strategy that is promising in the translation from bench to bedside with advantages in efficacy, safety, and the therapeutic time window. We further identified that α-KG modulates the interaction between ATP synthase and CypD, and the cardioprotection by α-KG can be abolished by CypD deficiency. This suggests that α-KG may act through modulating the mPTP opening to confer cardioprotection. Further understanding of the mPTP composition and structure, the effects of α-KG on the mPTP opening, and the metabolic changes in the myocardium

treated with α-KG under I/R will help understand the mechanism(s) of α-KG to confer cardioprotection.

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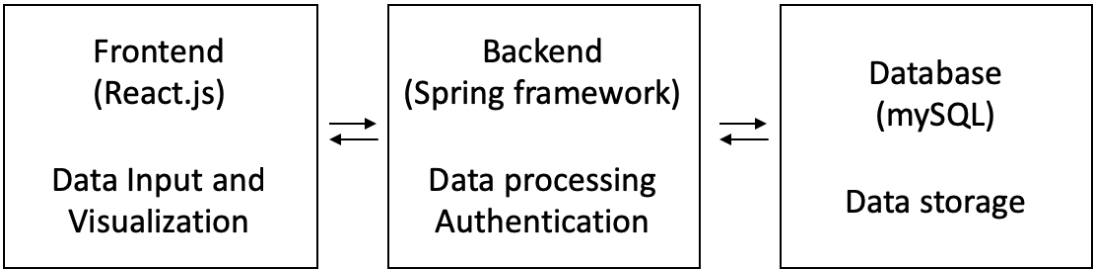
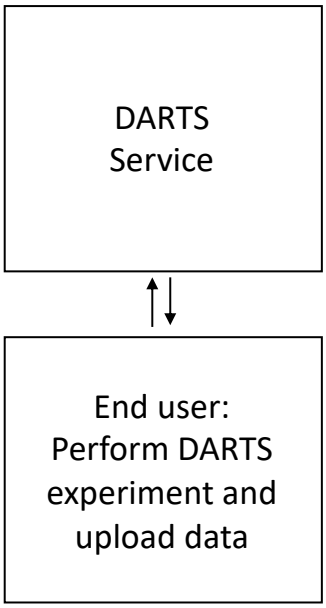
Appendix: A Software-as-a-service Drug Target Analysis Tool for Identification of Drug-protein Interactions using Drug Affinity Responsive Target Stability (DARTS)

Drug affinity responsive target stability (DARTS) has been proven advantageous as a small-molecule target identification method by leveraging the change in the digestion speed of target proteins against pronase when the target proteins are bound by small molecules[1]. Therefore, DARTS is able to identify thousands of proteins of which the digestion speeds were changed by the drug-protein interactions. The development of mass spectrometry method in analyzing the protein residuals generated by DARTS methods quantifies the changes in the proteins amount after digestion. However, due to the massive scale of data generated, an informatic tool to process, visualize and present these data is needed to allow for a broader application of the DARTS method in combination with mass spectrometry.

In this chapter, we briefly describe a software-as-a-service model for visualization of data generated from DARTS methods through mass spectrometry to allow for an in-depth analysis of DARTS results:



The frontend and backend service can be deployed on cloud platforms (e.g. Amazon web service or Google Cloud Platform). The service will allow for a broader accessibility of DARTS method by providing researchers access to data analysis and visualization tools through the cloud platform:



An example implementation is available at https://github.com/xiangyin701/darts.

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