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Critical role of glutamine metabolism in cardiomyocytes under oxidative stress

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Abbreviations: αKG, α-ketoglutarate; Gls, glutaminase; RNCMs, rat neonatal cardiomyocytes; GSH, glutathione; HF, heart failure; TCA cycle, tricarboxylic acid cycle

Abstract

Background: Metabolic remodeling in cardiomyocytes is deeply associated with the pathogenesis of heart failure (HF). Glutaminolysis is an anaplerotic pathway that incorporates α-ketoglutarate (αKG) derived from glutamine into the tricarboxylic acid (TCA) cycle. It is well known that cancer cells depend on glutamine for their increased energy demand and proliferation; however, the physiological roles of glutamine metabolism in failing hearts remain unclear.

Objective: To investigate the regulatory mechanisms and biological effects of glutamine metabolism in oxidative stress-induced failing myocardium.

Methods and results: The intracellular levels of glutamine, glutamate, and αKG were significantly decreased by H_2O_2 stimulation in rat neonatal cardiomyocytes (RNCMs). To better understand the metabolic flux in failing myocardium, we performed a stable isotope tracing study and found that glutaminolysis was upregulated in RNCMs under oxidative stress. Consistent with this, the enzymatic activity of glutaminase (Gls), which converts glutamine to glutamate, was augmented in RNCMs treated with H_2O_2. These findings suggest that glutamine anaplerosis is enhanced in cardiomyocytes under oxidative stress to compensate for the reduction of αKG. Furthermore, the inhibition of Gls reduced cardiac cell viability, ATP production, and glutathione (GSH) synthesis in

RNCMs with H_2O_2 stimulation. Finally, we evaluated the effects of αKG on failing myocardium and observed that dimethyl α-ketoglutarate (DMKG) suppressed oxidative stress-induced cell death likely due to the enhancement of intracellular ATP and GSH levels.

Conclusion: Our study demonstrates that under oxidative stress, glutaminolysis is upregulated to compensate for the loss of αKG and its replenishment into the TCA cycle, thereby exerting cardioprotective effects by maintaining ATP and GSH levels. Modulation of glutamine metabolism in failing hearts might provide a new therapeutic strategy for HF.

Keywords: glutaminolysis, α-ketoglutarate, glutaminase, metabolic remodeling, oxidative stress, glutathione

1. Introduction

Heart failure (HF) is a major public health problem worldwide because of its high morbidity and mortality. The prevalence is increasing with the growing aging population, resulting in an enormous economic burden in both developed and developing countries. Although it is well established that the underlying causes of HF are ischemic injury, valve dysfunction, several kinds of cardiomyopathies, and coexisting diseases such as hypertension and diabetes mellitus [1], the molecular mechanism of ventricular remodeling is complex and related to multiple factors including oxidative stress, inflammation, mitochondrial dysfunction, or activation of neurohormonal and sympathetic systems [2-4]. Among these factors, metabolic remodeling in cardiomyocytes is considered crucial in the pathophysiology of HF [5,6]. The heart is capable of utilizing various substrates for energy production, including fatty acids, glucose, and to a lesser extent, lactic acid, amino acids, and ketone bodies [7-12]. In particular, it is well known that a healthy heart mainly utilizes fatty acids for energy generation, whereas the failing heart shifts its dependence on substrate utilization from fatty acid oxidation towards glycolysis [13-15]. Such imbalanced substrate utilization causes energy deficit or accumulation of reactive oxygen species in the myocardium, leading to maladaptive cardiac remodeling and dysfunction [6].

Glutamine is the most abundant amino acid in the blood and the major carbon source to replenish TCA cycle intermediates through a mitochondrial metabolic pathway termed “glutaminolysis.” This anaplerotic pathway consists of two deamination reactions as the first two steps. Firstly, glutamine is converted to glutamate in the presence of the enzyme glutaminase (Gls), and secondly to α-ketoglutarate (αKG), which is incorporated into the TCA cycle, and finally catabolized to lactate [16]. Several studies on cancer pathology focused on glutaminolysis as glutamine is predominantly utilized by rapidly proliferating cancer cells for energy production and biosynthesis of nucleotides, proteins, and lipids [17]. Therefore, glutaminolysis has gained substantial interest as a new therapeutic approach targeting cancer metabolism.

However, the regulatory mechanisms and physiological roles of glutamine metabolism in cardiovascular disease are still unknown. In this study, we sought to identify the regulatory mechanisms of glutamine anaplerosis and its effects on cardiac energetics and physiology by using cardiomyocytes under oxidative stress which has a strong association with the pathogenesis of HF [18].

2. Methods and Materials

2.1. Cell culture

All animal studies were performed in accordance with the Institutional Guidelines of Kobe University and the Guide for the Care and Use of Laboratory Animals. Rat neonatal cardiomyocytes (RNCMs) were harvested from 1- to 3-day-old neonatal rat hearts, as described previously [19]. In brief, cells were cultured in Dulbecco's modified Eagle's medium/nutrient mixture F-12 Ham (DMEM/Ham's F-12) (FUJIFILM, #048-29785) supplemented with 5% calf serum and penicillin-streptomycin (100 U/mL and 100 μg/mL, respectively) (Sigma-Aldrich, #P4333) at 37°C and 5% CO_2. One day after seeding, the medium was changed to DMEM/Ham's F-12 containing 0.5% calf serum. All experiments were performed between the 2nd and 4th days after changing the medium.

2.2. Analysis of the metabolites with GC-MS

TCA cycle intermediates were analyzed using gas chromatography-mass spectrometry (GC-MS) (Shimadzu) as described previously [19]. RNCMs were rinsed with ice-cold phosphate-buffered saline (PBS) and quenched with cold methanol. After incubation for 30 min at 37°C, the cell lysate was centrifuged to remove the debris, and the aqueous phase was freeze-dried. Dried metabolites were derivatized and analyzed using the GC-MS system. Metabolite levels in RNCMs were normalized to protein content.

2.3. Stable isotope tracing study

Stable isotope tracing was performed as previously described [20]. In brief, RNCMs were cultured in glucose- and glutamine- depleted media (DMEM-F12, Biowest, #L0091) supplemented with 25 mM glucose and 4 mM [U-^{13}C]-glutamine (Cambridge Isotope Laboratories, #CLM-1822), or 25 mM [U-^{13}C]-glucose (Cambridge Isotope Laboratories, #CLM-1396) and 4 mM glutamine. The metabolites were extracted as described above at timepoints of 0.5, 1, or 2 h after stimulation with 100 μM H_2O_2. Lyophilized samples were dissolved in 30 μL of dimethylformamide and derivatized with 30 μL of N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) plus 1% trimethylchlorosilane (TMCS) (Sigma-Aldrich, #375934) at 85°C for 60 min. IsoCor® software was employed for the correction of data for naturally occurring isotopes [21].

2.4. Analysis of the metabolites using LC-MS/MS

RNCMs were washed with ice-cold PBS and quenched with cold methanol. To quantify intracellular αKG, glutamate, and glutamine levels in RNCMs, internal standard αKG (1,2,3,4-13C4 αKG, Cambridge Isotope Laboratories, Inc. CLM-4442-0) and an internal standard mixture of amino acids (APDSTAG, FUJIFILM-WAKO, #293-73701) were added to the samples. After 1 h of incubation on ice, the samples were centrifuged, and

the supernatant was subjected to LC-MS/MS. The system consisted of a Q-Trap 6500 (Sciex) equipped with a Shimadzu LC-30AD HPLC system. For amino acid analysis, an Intrada Amino Acid column (100 mm × 3.0 mm, 3.0 μm, Imtakt Co, Kyoto, Japan) was used with an acetonitrile/formic acid/100mM ammonium formate gradient of 100:0.1:0 to 0:0:100 (v/v/v) at a flow rate of 0.6 mL/min. For organic acid analysis, an Intrada Organic Acid column (150 mm × 2.0 mm, 3.0 μm, Imtakt Co, Kyoto, Japan) was used with an acetonitrile/water/formic acid/100mM ammonium formate gradient of 10:90:0.1:0 to 10:0:0:90 (v/v/v/v) at a flow rate of 0.2 mL/min. To monitor and quantify the levels of αKG, glutamate, and glutamine, the multiple reaction monitoring (MRM) method was developed with signature ion pairs Q1 (parent ion)/Q3 (characteristic fragment ion) for each molecule.

2.5. Cell viability assay

One hundred thousand RNCM cells were placed into 24 well plates and incubated as described above. Subsequently, cells were stimulated with 100 μM H_2O_2 for 4 h, followed by crystal violet staining. When cells were treated with Gls inhibitor, compound 968 (Sigma-Aldrich, #SML1327), or dimethyl-α-ketoglutarate (DMKG) (Sigma-Aldrich, #349631), cells were incubated with 20 μM compound 968 or 4 mM DMKG before H_2O_2

stimulation. Cells were washed twice with PBS (-) and fixed for 5 min with 4% paraformaldehyde. After fixation, cells were stained with 0.1% crystal violet, washed twice with H_2O, and then lysed with 10% acetate. The lysate was used to measure absorbance at 595 nm.

2.6. Western blot analysis

Western blotting was performed as previously reported [19]. The following antibodies were used: Glutaminase (Abcam, #ab93434), IDH2 (Abcam, #ab131263), OGDH (Proteintech, #15212-1-AP), and GAPDH (Sigma-Aldrich, #G8795). The band intensity was quantified by densitometry analysis using ImageJ® software.

2.7. ATP measurement

Four hundred thousand RNCM cells were seeded into 12 well plates and incubated as described above. After treatment with the indicated reagents, intracellular ATP levels were quantified using an intracellular ATP assay kit (TOYO INK GROUP, #IC100) according to the manufacturer's protocol.

2.8. Quantification of Gls activity

Four hundred thousand RNCM cells were cultured in 12 well plates. After stimulation with 100 μM H_2O_2, Gls activity was measured with a commercially available kit (BioVision, # K455-100) according to the manufacturer's protocol.

2.9. Quantification of glutathione (GSH)

Four hundred thousand RNCM cells were seeded onto 12 well plates. After treatment with compound 968 or DMKG, cells were stimulated with 100 μM H_2O_2 for 1 h. Total glutathione (GSH) levels were determined using a commercially available kit (Cayman Chemical, #703002) according to the manufacturer's protocol.

2.10. Oxidative stress detection

Cells were pretreated with 4 mM DMKG and then stimulated with 100 μM H_2O_2 for 1 h. For the detection of ROS, cells were incubated in dihydroethidium (DHE) solution (2 μM) for 30 min at 37°C. After that, the nuclei and F-actin were counterstained with Hoechst and phalloidin (Thermo Fisher Scientific, #H1399, #A12379, respectively). Images were acquired with a confocal microscope (Carl Zeiss LSM700).

2.11. Statistical analysis

All statistical analyses were performed using GraphPad Prism software version 6.0 (GraphPad Software). Differences between two groups were obtained using an unpaired two-tailed Student's t-test. Differences between multiple groups were determined by one-way ANOVA with Tukey's or Dunnett's multiple comparisons test, as appropriate. All data are expressed as the mean ± SEM. P values < 0.05 were considered statistically significant.

3. Results

3.1. Glutaminolysis is upregulated in cardiomyocytes in response to oxidative stress

As a first step to determine the regulation of glutamine metabolism in failing myocardium, we measured the concentrations of metabolites involved in glutaminolysis in RNCMs under oxidative stress. Intracellular levels of αKG, glutamate, and glutamine were decreased by oxidative stress (Figure 1A).

To better understand the metabolic flux in the failing myocardium, we performed stable isotope tracing with [U-^{13}C]-glutamine (Figure 1B). As shown in Figure 1C-H, oxidative stress highly enhanced the anaplerotic flux of glutamine into the TCA cycle in RNCMs. In particular, the relative amounts of αKG, glutamate, glutamine, fumarate, and malate derived from [U-^{13}C]-glutamine were significantly increased in the short term; however,

glucose utilization, as measured by using [U-^{13}C]-glucose remained unaltered under the same conditions (Supplemental Figure 1). These findings indicate that glutaminolysis, but not glucose oxidation, is activated in cardiomyocytes as an acute response to oxidative stress to compensate for the loss of metabolites.

3.2. Gls is a key modulator of cardiac glutaminolysis

Next, to investigate the regulatory mechanism by which glutaminolysis is activated in cardiomyocytes under oxidative stress, we assessed the protein expression of glutaminolysis-related enzymes. The protein expression of Gls, isocitrate dehydrogenases (Idh2), and 2-oxoglutarate dehydrogenase (Ogdh) remained unchanged (Figure 2A, B). Conversely, the enzymatic activity of Gls was significantly upregulated in RNCMs stimulated with H_2O_2 (Figure 2C), suggesting that in pathological conditions, cardiomyocytes seek to compensate for the reduction of αKG and glutamate by enhancing Gls activity to increase the anaplerotic flux from glutamine to αKG.

3.3. Glutamine anaplerosis increases ATP and GSH synthesis, contributing to cardiac cell survival

Further, to evaluate the effect of glutamine anaplerosis on cell viability and ATP

production in cardiomyocytes, we inhibited the anaplerotic flux with a Gls inhibitor, compound 968. Interestingly, the inhibition of Gls significantly reduced cell viability (Figure 3A) and intracellular ATP content (Figure 3B) under oxidative stress. To compensate for the reduction of αKG due to oxidative stress, we treated RNCMs with DMKG, a membrane-permeable ester of αKG. Supplementation of DMKG protected cardiomyocytes from oxidative stress in a dose-dependent manner (Figure 3C, Supplemental Figure 2) and preserved intracellular ATP levels (Figure 3D). Finally, we confirmed that inhibition of Gls significantly decreased the total amount of GSH, an essential antioxidant consisting of glutamate, under both normal and oxidative stress conditions in RNCMs (Figure 4A). Meanwhile, pretreatment with DMKG significantly increased the total GSH levels in RNCMs with H_2O_2 stimulation (Figure 4B), followed by a decrease in intracellular ROS production (Figure 4C). These data indicate that under oxidative stress, glutamine anaplerosis is upregulated in cardiomyocytes, resulting in increased GSH synthesis through the shunt from glutamate to GSH. Additionally, supplementation of αKG also increases GSH synthesis, leading to an antioxidative effect.

4. Discussion

In the present study, we found that oxidative stress reduced intracellular levels of αKG,

glutamate, and glutamine in cardiomyocytes. In addition, to counteract the reduction of these metabolites, glutaminolysis was upregulated because of the increased enzymatic activity of Gls. Blocking the anaplerotic flux with Gls inhibitor exacerbated oxidative stress-induced cell death, ATP production, and GSH synthesis in cardiomyocytes, while DMKG treatment improved cell viability against oxidative stress, possibly due to enhanced ATP and GSH synthesis.

4.1. Glutaminolysis is a key pathway that affects various pathologies

Glutamine directly supports the biosynthesis of amino acids, fatty acids, and de novo purine and pyrimidine for cell growth and division [22]. Therefore, it has been established that enhanced glutaminolysis has a negative impact on cancer development, and the suppression of the anaplerotic flux is thought to be a new therapeutic target in cancer [17]. Indeed, Gls inhibitor, CB-839, decreased glutamine consumption and glutamate production in breast cancer cell lines, and inhibited cancer cell proliferation [23]. In addition, alanine-serine-cysteine transporter 2 (ASCT2), a primary transporter of glutamine, is another candidate for cancer therapy. Schulte et al. showed that the ASCT2 antagonist, v-9302, attenuated cancer cell growth and proliferation, and inversely increased cell death, contributing to antitumor effects [24].

Interestingly, these interventions involving glutaminolysis to treat cancer may have different effects on the heart. Here, we found that the Gls inhibitor deteriorated oxidative stress-induced cardiac cell death in contrast to the beneficial effect observed in cancer, where it inhibits unregulated cell growth and division. This might be because, on one hand, glutamine anaplerosis has negative impacts represented by cell proliferation and migration on cancer development, on the other hand, it mediates pro-survival effect in cardiomyocytes, as shown in human endothelial cells [25].

4.2. Role of glutamine metabolism in cardiac energetics

Glutamine metabolism and its regulation in the failing heart are not fully understood. Our study with stable isotope tracing indicated that cardiomyocytes activated glutaminolysis, but not glucose oxidation, for mitochondrial oxidative phosphorylation immediately after stimulation with H_2O_2. Although these results seem to be inconsistent with the known substrate shift from fatty acid to glucose in failing heart, it has been reported that glucose is preferentially utilized for biomass synthesis rather than ATP synthesis in an in vivo murine model of pressure-overloaded heart [26]. Similarly, Tran et al. demonstrated that increased shunting from glucose to the hexosamine biosynthetic pathway results in cardiac hypertrophy [27]. Ritterhoff et al. also reported that anabolic

aspartate synthesis from glucose leads to cardiac hypertrophy by providing nitrogen for nucleotide synthesis [28]. Thus, the heart utilizes glucose for biomass synthesis, not energy production in mitochondrial oxidative phosphorylation, under stress conditions. In this regard, our data seem to be consistent with these previous studies. More importantly, glutamine utilization in cardiomyocytes was significantly upregulated by oxidative stress. One possibility is that the enzymatic activity of Gls is enhanced in the myocardium to compensate for decreased ATP production by oxidative stress. In fact, we confirmed that the inhibition of Gls reduced intracellular ATP levels, whereas αKG exogenously supplemented to cardiomyocytes maintained the ATP levels. Considering the metabolic dynamics of pathological myocardium, as shown in the present study, the heart might rely on glutamine oxidation for its energy requirement under pathological conditions.

4.3. Effects of αKG on failing heart

There might be other underlying mechanisms by which glutamine anaplerosis directly affects cell survival regardless of energy supply. Numerous studies have reported multiple effects of αKG on several organisms and tissues. It has been revealed that αKG extended the lifespan of nematodes and drosophila by interacting with the target of

rapamycin [29,30] and then affected gene expression by regulating histones and DNA demethylases [31]. In addition, dietary supplementation of αKG extends lifespan of mice by suppressing chronic inflammation and regulating secretion of interleukin-10 in T cells [32]. Regarding the heart, αKG has been shown to improve insulin sensitivity and glucose uptake in cardiac mesenchymal cells isolated from diabetic donors and in the heart of diabetic model animals by restoring the DNA demethylation cycle [33]. In addition, Salabei et al. demonstrated that glutamine and αKG promoted the proliferation of cardiac progenitor cells in adult mice via the mammalian target of rapamycin (mTOR) signaling pathway [34]. Thus, αKG acts as an essential signaling molecule or a key modulator of epigenetic changes.

Apart from these roles, the present study further revealed the novel aspects of αKG in failing myocardium. We found that supplementary treatment with αKG increased the production of GSH in cardiomyocytes. Because the interconversion between αKG and glutamate occurs bidirectionally, the enhanced glutamine anaplerosis induced by oxidative stress might also contribute to promoting GSH synthesis in failing hearts through the increased flux from glutamate to GSH. In support of this notion, the activation of Nrf2, a key transcription factor involved in antioxidant defense, promoted GSH synthesis due to the utilization of glutaminolysis-derived glutamate as a source in murine

heart [35], and then αKG protected neuronal cells against oxidative stress by decreasing ROS production [36]. Taken together, glutamine anaplerosis might not only contribute to cardiac mitochondrial energy generation, but also enhance antioxidant synthesis, further contributing to cardiac protection.

4.4. Study limitations

This study has several limitations. First of all, we did not investigate the biological role of glutamine metabolism in in vivo HF models and the regulatory mechanisms of glutaminolysis in different pathological states such as cardiac hypertrophy or ischemia. Moreover, we could not demonstrate how Gls activity was regulated in oxidative stress-induced cardiomyocytes. Lastly, the detailed metabolic pathway leading to the conversion of supplemented αKG into glutamate for GSH synthesis, was not identified.

5. Conclusions

Here, we demonstrate that glutaminolysis is a critical pathway to rescue cardiomyocytes against oxidative stress. Cardiac glutaminolysis ameliorates maladaptive metabolic remodeling and increases antioxidant activity during heart failure. To our knowledge, this is the first study to provide new insights into cardiac metabolism involving glutamine

anaplerosis for energy production and antioxidant synthesis in response to oxidative stress. Further elucidation of cardiac glutaminolysis in various animal models or human samples of cardiovascular diseases will promote our understanding of the underlying mechanisms of glutamine metabolism in failing heart as well as provide a novel strategy for HF treatment.

Disclosure

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References

[1] M. Jessup, S. Brozena, Heart failure, N Engl J Med 348 (2003) 2007-2018. 10.1056/NEJMra021498.

[2] D. Mancini, D. Burkhoff, Mechanical device-based methods of managing and treating heart failure, Circulation 112 (2005) 438-448. 10.1161/CIRCULATIONAHA.104.481259.

[3] S.A. Dick, S. Epelman, Chronic Heart Failure and Inflammation: What Do We Really Know?, Circ Res 119 (2016) 159-176. 10.1161/CIRCRESAHA.116.308030.

[4] B. Zhou, R. Tian, Mitochondrial dysfunction in pathophysiology of heart failure, J Clin

Invest 128 (2018) 3716-3726. 10.1172/JCI120849.

[5] S. Neubauer, The failing heart--an engine out of fuel, N Engl J Med 356 (2007) 1140-1151. 10.1056/NEJMra063052.

[6] E. Bertero, C. Maack, Metabolic remodelling in heart failure, Nat Rev Cardiol 15 (2018) 457-470. 10.1038/s41569-018-0044-6.

[7] H. Taegtmeyer, P. McNulty, M.E. Young, Adaptation and maladaptation of the heart in diabetes: Part I: general concepts, Circulation 105 (2002) 1727-1733. 10.1161/01.cir.0000012466.50373.e8.

[8] K. Ichihara, J.R. Neely, D.L. Siehl, et al., Utilization of leucine by working rat heart, Am J Physiol 239 (1980) E430-436. 10.1152/ajpendo.1980.239.6.E430.

[9] H. Sun, K.C. Olson, C. Gao, et al., Catabolic Defect of Branched-Chain Amino Acids Promotes Heart Failure, Circulation 133 (2016) 2038-2049. 10.1161/CIRCULATIONAHA.115.020226.

[10] K.C. Bedi, Jr., N.W. Snyder, J. Brandimarto, et al., Evidence for Intramyocardial Disruption of Lipid Metabolism and Increased Myocardial Ketone Utilization in Advanced Human Heart Failure, Circulation 133 (2016) 706-716. 10.1161/CIRCULATIONAHA.115.017545.

[11] B. Bartelds, H. Knoester, G.C. Beaufort-Krol, et al., Myocardial lactate metabolism

in fetal and newborn lambs, Circulation 99 (1999) 1892-1897. 10.1161/01.cir.99.14.1892.

[12] J.F. Dhainaut, M.F. Huyghebaert, J.F. Monsallier, et al., Coronary hemodynamics and myocardial metabolism of lactate, free fatty acids, glucose, and ketones in patients with septic shock, Circulation 75 (1987) 533-541. 10.1161/01.cir.75.3.533.

[13] H. Ashrafian, M.P. Frenneaux, L.H. Opie, Metabolic mechanisms in heart failure, Circulation 116 (2007) 434-448. 10.1161/CIRCULATIONAHA.107.702795.

[14] G.D. Lopaschuk, J.R. Ussher, C.D. Folmes, et al., Myocardial fatty acid metabolism in health and disease, Physiol Rev 90 (2010) 207-258. 10.1152/physrev.00015.2009.

[15] A. Nickel, J. Loffler, C. Maack, Myocardial energetics in heart failure, Basic Res Cardiol 108 (2013) 358. 10.1007/s00395-013-0358-9.

[16] R. Curi, C.J. Lagranha, S.Q. Doi, et al., Molecular mechanisms of glutamine action, J Cell Physiol 204 (2005) 392-401. 10.1002/jcp.20339.

[17] B.J. Altman, Z.E. Stine, C.V. Dang, From Krebs to clinic: glutamine metabolism to cancer therapy, Nat Rev Cancer 16 (2016) 619-634. 10.1038/nrc.2016.71.

[18] V. Lubrano, S. Balzan, Role of oxidative stress-related biomarkers in heart failure: galectin 3, alpha1-antitrypsin and LOX-1: new therapeutic perspective?, Mol Cell Biochem 464 (2020) 143-152. 10.1007/s11010-019-03656-y.

[19] M. Nagao, R. Toh, Y. Irino, et al., beta-Hydroxybutyrate elevation as a compensatory

response against oxidative stress in cardiomyocytes, Biochem Biophys Res Commun 475 (2016) 322-328. 10.1016/j.bbrc.2016.05.097.

[20] Y. Takeuchi, Y. Nakayama, E. Fukusaki, et al., Glutamate production from ammonia via glutamate dehydrogenase 2 activity supports cancer cell proliferation under glutamine depletion, Biochem Biophys Res Commun 495 (2018) 761-767. 10.1016/j.bbrc.2017.11.088.

[21] P. Millard, F. Letisse, S. Sokol, et al., IsoCor: correcting MS data in isotope labeling experiments, Bioinformatics 28 (2012) 1294-1296. 10.1093/bioinformatics/bts127.

[22] A.N. Lane, T.W. Fan, Regulation of mammalian nucleotide metabolism and biosynthesis, Nucleic Acids Res 43 (2015) 2466-2485. 10.1093/nar/gkv047.

[23] M.I. Gross, S.D. Demo, J.B. Dennison, et al., Antitumor activity of the glutaminase inhibitor CB-839 in triple-negative breast cancer, Mol Cancer Ther 13 (2014) 890-901. 10.1158/1535-7163.MCT-13-0870.

[24] M.L. Schulte, A. Fu, P. Zhao, et al., Pharmacological blockade of ASCT2-dependent glutamine transport leads to antitumor efficacy in preclinical models, Nat Med 24 (2018) 194-202. 10.1038/nm.4464.

[25] K.J. Peyton, X.M. Liu, Y. Yu, et al., Glutaminase-1 stimulates the proliferation, migration, and survival of human endothelial cells, Biochem Pharmacol 156 (2018) 204-

214. 10.1016/j.bcp.2018.08.032.

[26] Y. Umbarawan, M. Syamsunarno, N. Koitabashi, et al., Glucose is preferentially utilized for biomass synthesis in pressure-overloaded hearts: evidence from fatty acid-binding protein-4 and -5 knockout mice, Cardiovasc Res 114 (2018) 1132-1144. 10.1093/cvr/cvy063.

[27] D.H. Tran, H.I. May, Q. Li, et al., Chronic activation of hexosamine biosynthesis in the heart triggers pathological cardiac remodeling, Nat Commun 11 (2020) 1771. 10.1038/s41467-020-15640-y.

[28] J. Ritterhoff, S. Young, O. Villet, et al., Metabolic Remodeling Promotes Cardiac Hypertrophy by Directing Glucose to Aspartate Biosynthesis, Circ Res 126 (2020) 182-196. 10.1161/CIRCRESAHA.119.315483.

[29] R.M. Chin, X. Fu, M.Y. Pai, et al., The metabolite alpha-ketoglutarate extends lifespan by inhibiting ATP synthase and TOR, Nature 510 (2014) 397-401. 10.1038/nature13264.

[30] Y. Su, T. Wang, N. Wu, et al., Alpha-ketoglutarate extends Drosophila lifespan by inhibiting mTOR and activating AMPK, Aging (Albany NY) 11 (2019) 4183-4197. 10.18632/aging.102045.

[31] I. Martinez-Reyes, N.S. Chandel, Mitochondrial TCA cycle metabolites control

physiology and disease, Nat Commun 11 (2020) 102. 10.1038/s41467-019-13668-3.

[32] A. Asadi Shahmirzadi, D. Edgar, C.Y. Liao, et al., Alpha-Ketoglutarate, an Endogenous Metabolite, Extends Lifespan and Compresses Morbidity in Aging Mice, Cell Metab 32 (2020) 447-456 e446. 10.1016/j.cmet.2020.08.004.

[33] F. Spallotta, C. Cencioni, S. Atlante, et al., Stable Oxidative Cytosine Modifications Accumulate in Cardiac Mesenchymal Cells From Type2 Diabetes Patients: Rescue by alpha-Ketoglutarate and TET-TDG Functional Reactivation, Circ Res 122 (2018) 31-46. 10.1161/CIRCRESAHA.117.311300.

[34] J.K. Salabei, P.K. Lorkiewicz, C.R. Holden, et al., Glutamine Regulates Cardiac Progenitor Cell Metabolism and Proliferation, Stem Cells 33 (2015) 2613-2627. 10.1002/stem.2047.

[35] J. Song, Y. Meng, M. Wang, et al., Mangiferin activates Nrf2 to attenuate cardiac fibrosis via redistributing glutaminolysis-derived glutamate, Pharmacol Res 157 (2020) 104845. 10.1016/j.phrs.2020.104845.

[36] K. Sawa, T. Uematsu, Y. Korenaga, et al., Krebs Cycle Intermediates Protective against Oxidative Stress by Modulating the Level of Reactive Oxygen Species in Neuronal HT22 Cells, Antioxidants (Basel) 6 (2017). 10.3390/antiox6010021.

Figure legends

Figure 1. The modulation of glutaminolysis in cardiomyocytes under oxidative stress

A) RNCMs were stimulated with 100 μM H_2O_2, and then levels of α-ketoglutarate (αKG), glutamate (Glu), and glutamine (Gln) were determined using LC-MS (n=5). **B)** Schematic depiction of the metabolites derived from [U-^{13}C]-glutamine in the TCA cycle. **C-H)** RNCMs were incubated in media containing [U-^{13}C]-glutamine. The metabolites were extracted at timepoints of 0.5, 1, or 2 h after stimulation with 100 μM H_2O_2, and their levels were determined using GC-MS (n=3). The quantification of M+4 and/or M+5 labeling of the metabolites was performed using IsoCor software. *$P < 0.05$, **$P < 0.01$. †$P < 0.0001$. Data analyzed by unpaired Student's t-test. M+, mass plus.

Figure 2. Oxidative stress affects the activity, but not the protein expression of glutaminase, in cardiomyocytes.

A) RNCMs were treated with 100 μM H_2O_2 and western blot analysis was performed to estimate the protein expression levels of glutaminase (Gls), isocitrate dehydrogenases (Idh2), and 2-oxoglutarate dehydrogenase (Ogdh) (n=3). **B)** A schema to show where Gls, Idh2, and Ogdh function in the TCA cycle. **C)** RNCMs were treated with 100 μM

H_2O_2 for indicated hours, and then, Gls activity was measured by a commercially available kit (n=5-6). **P＜0.01, ****P＜0.0001. NS; not significant. Data analyzed by unpaired Student's t-test [A] or one-way ANOVA with Dunnett's multiple comparisons test [C].

Figure 3. Glutaminolysis is associated with cell death and ATP synthesis in cardiomyocytes under oxidative stress.

A) RNCMs were cultured with or without Gls inhibitor, compound 968 (20 μM), and then stimulated with 100 μM H_2O_2. Cell viability was determined by crystal violet staining (n=3). **B)** Intracellular ATP content was determined in RNCMs under the same condition as [A] (n=3). **C, D)** RNCMs were treated with 4 mM dimethyl-α-ketoglutarate (DMKG). After that, cells were stimulated with 100 μM H_2O_2, followed by crystal violet staining or ATP measurement with the same methodology as (A) or (B) (n=3). *P＜0.05. Data analyzed by one-way ANOVA with Tukey's multiple comparisons test.

Figure 4. Glutamine anaplerosis contributes to GSH synthesis, and DMKG counteracts oxidative stress by increasing intracellular GSH levels.

A, B) Total glutathione (GSH) levels in RNCMs stimulated with 100 μM H_2O_2 after

treatment of 20 μM compound 968 (A) or 4 mM DMKG (B) (n=5-6). *P＜0.05, **P＜0.01, ****P＜0.0001. Data analyzed by one-way ANOVA with Tukey's multiple comparisons test. **C)** Representative images of ROS production in RNCMs. Cells were incubated with 4 mM DMKG and then stimulated with 100 μM H_2O_2. ROS is detected by fluorescent staining with DHE reagent, and photographs were acquired with a confocal microscope. Scar bar; 50 μm. Blue; Hoechst, Green; phalloidine, Red; DHE. Three independent experiments were performed.

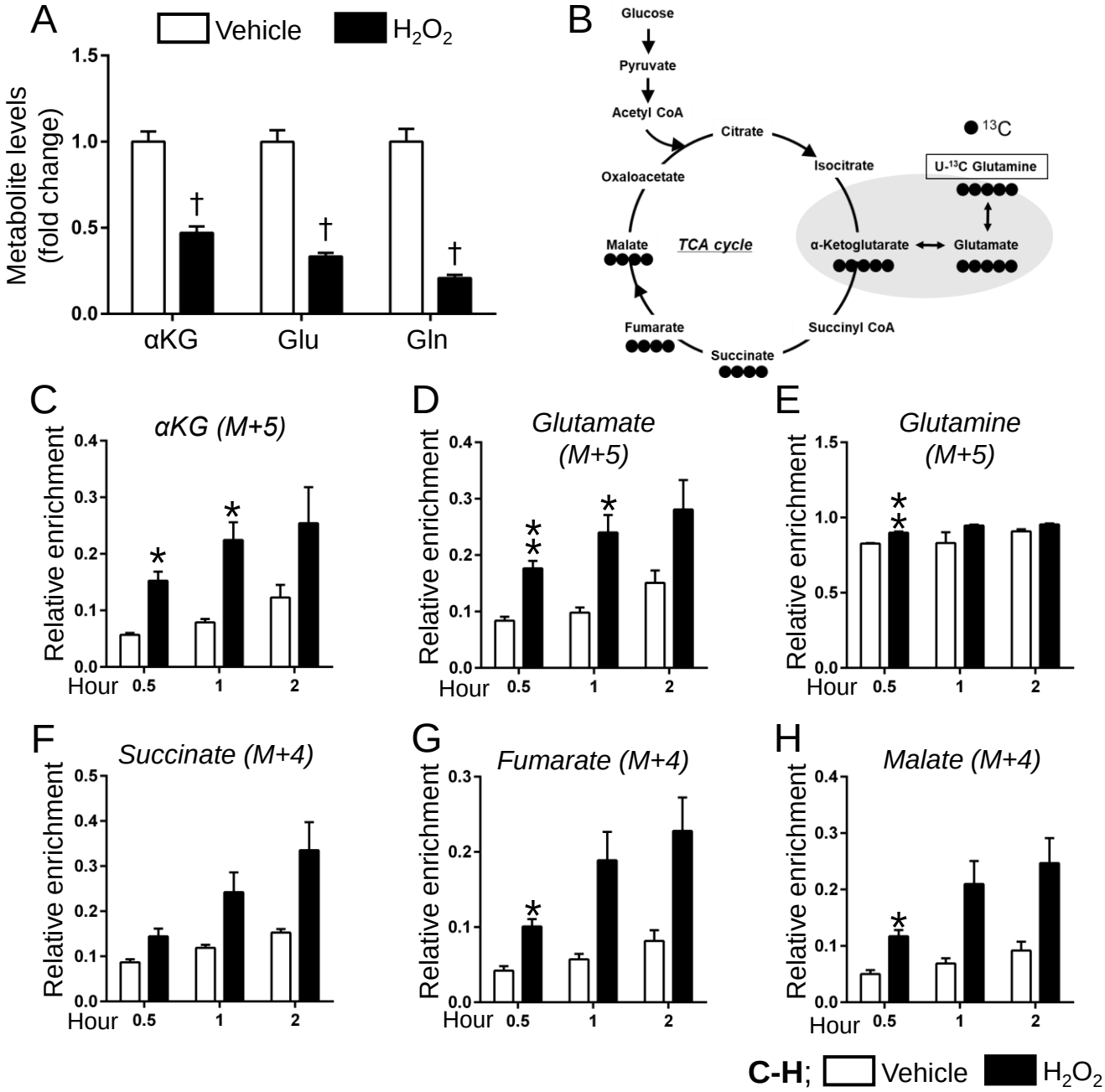

A
Vehicle
H2O2
Metabolite levels (fold change)
1.5
1.0
0.5
0.0
†
†
†
αKG
Glu
Gln
B
Glucose
Pyruvate
Acetyl CoA
Citrate
Isocitrate
Oxaloacetate
Malate
TCA cycle
α-Ketoglutarate
Glutamate
Fumarate
Succinyl CoA
Succinate
13C
U-13C Glutamine
C
αKG (M+5)
Relative enrichment
Hour 0.5 1 2
D
Glutamate (M+5)
E
Glutamine (M+5)
F
Succinate (M+4)
G
Fumarate (M+4)
H
Malate (M+4)
C-H; Vehicle H2O2

Figure 1.

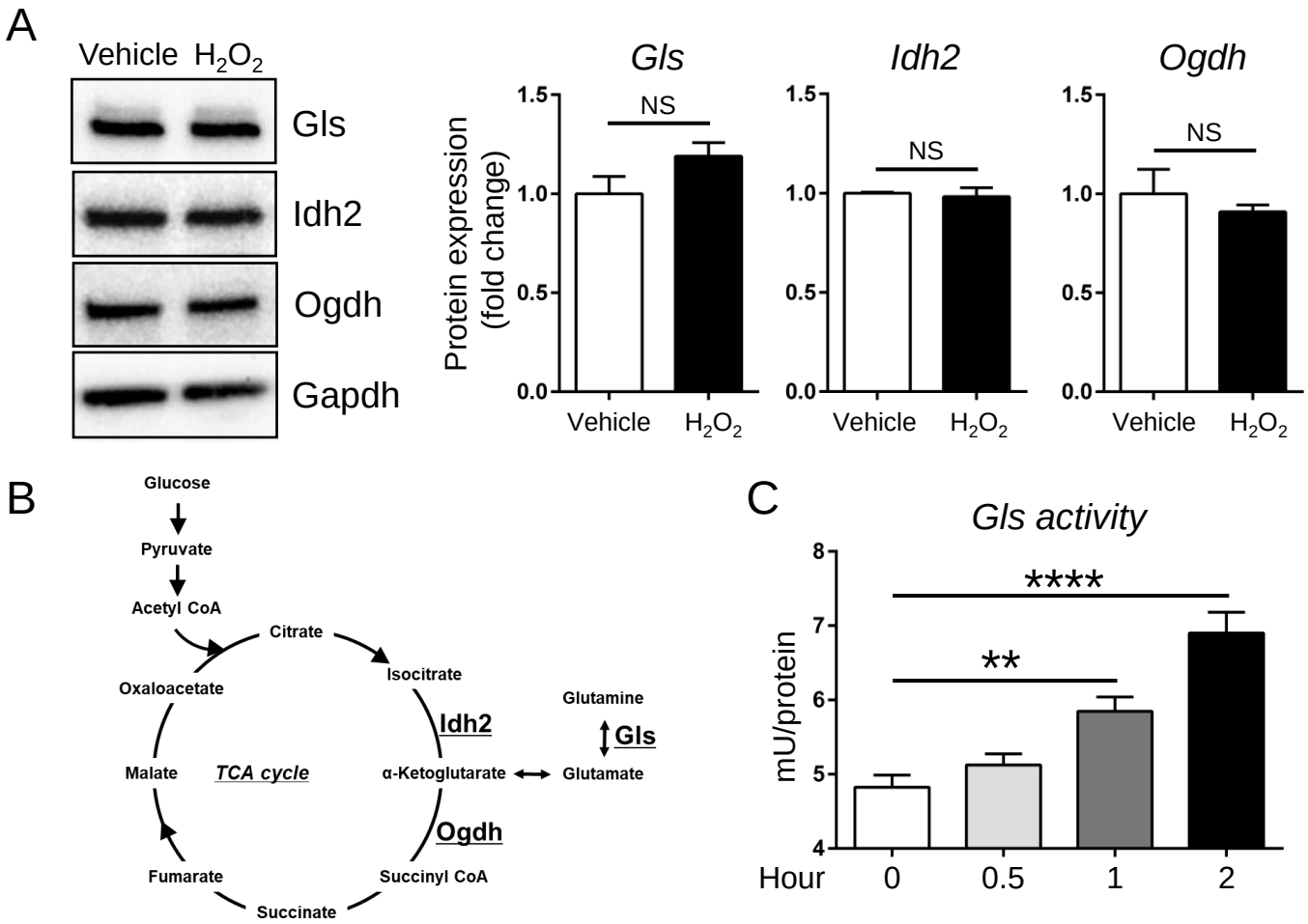

A
Vehicle
H_2O_2
Gls
Idh2
Ogdh
Gapdh
Gls
Idh2
Ogdh
Protein expression (fold change)
NS
NS
NS
1.5
1.0
0.5
0.0
Vehicle
H_2O_2
B
Glucose
Pyruvate
Acetyl CoA
Citrate
Isocitrate
Oxaloacetate
Idh2
Glutamine
Gls
Malate
TCA cycle
α-Ketoglutarate
Glutamate
Ogdh
Fumarate
Succinyl CoA
Succinate
C
Gls activity
mU/protein
8
7
6
5
4
****
**
Hour
0
0.5
1
2

Figure 2.

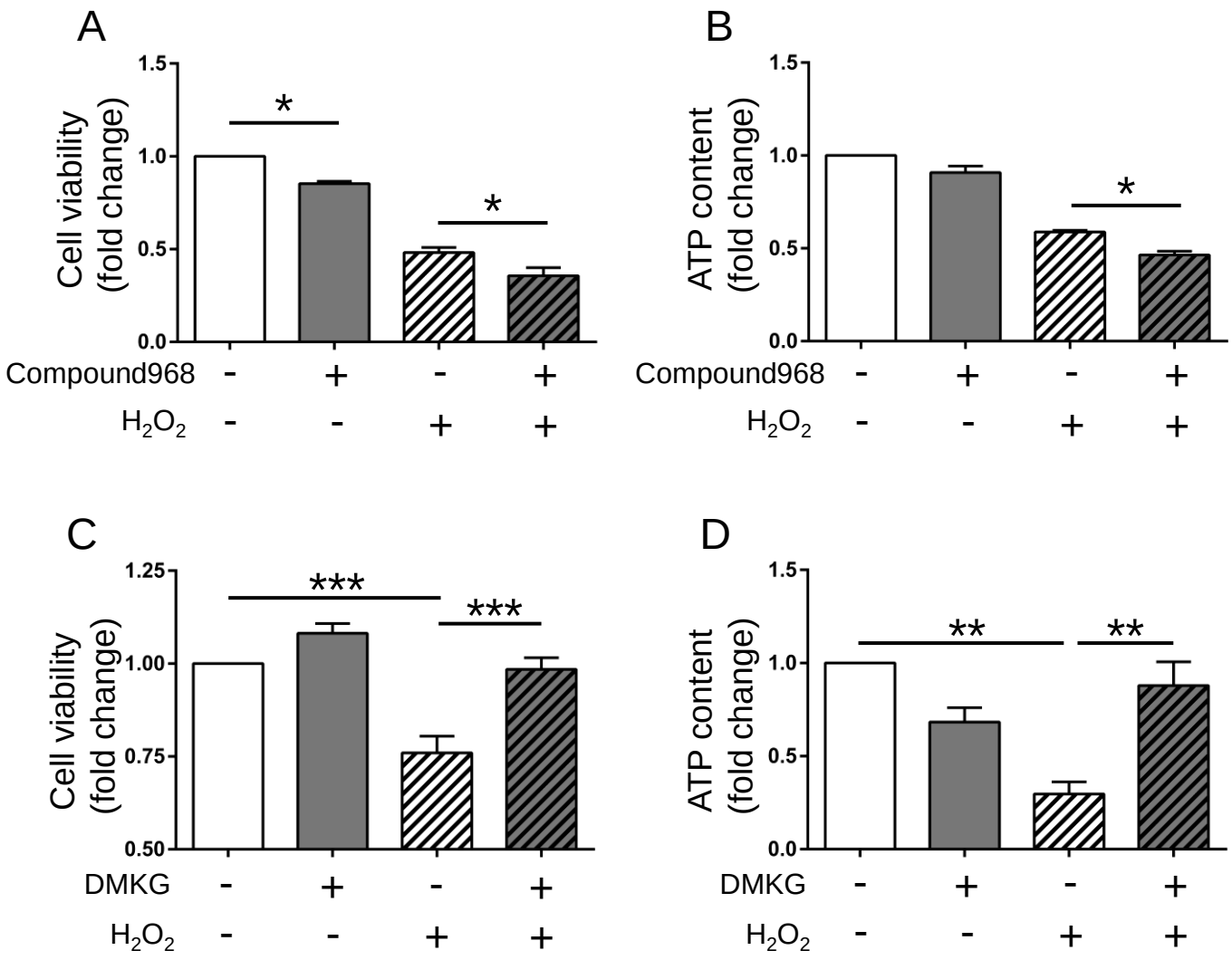

A
Cell viability (fold change)
1.5
1.0
0.5
0.0
*
*
Compound968 - + - +
H2O2 - - + +
B
ATP content (fold change)
1.5
1.0
0.5
0.0
*
Compound968 - + - +
H2O2 - - + +
C
Cell viability (fold change)
1.25
1.00
0.75
0.50
***
***
DMKG - + - +
H2O2 - - + +
D
ATP content (fold change)
1.5
1.0
0.5
0.0
**
**
DMKG - + - +
H2O2 - - + +

Figure 3.

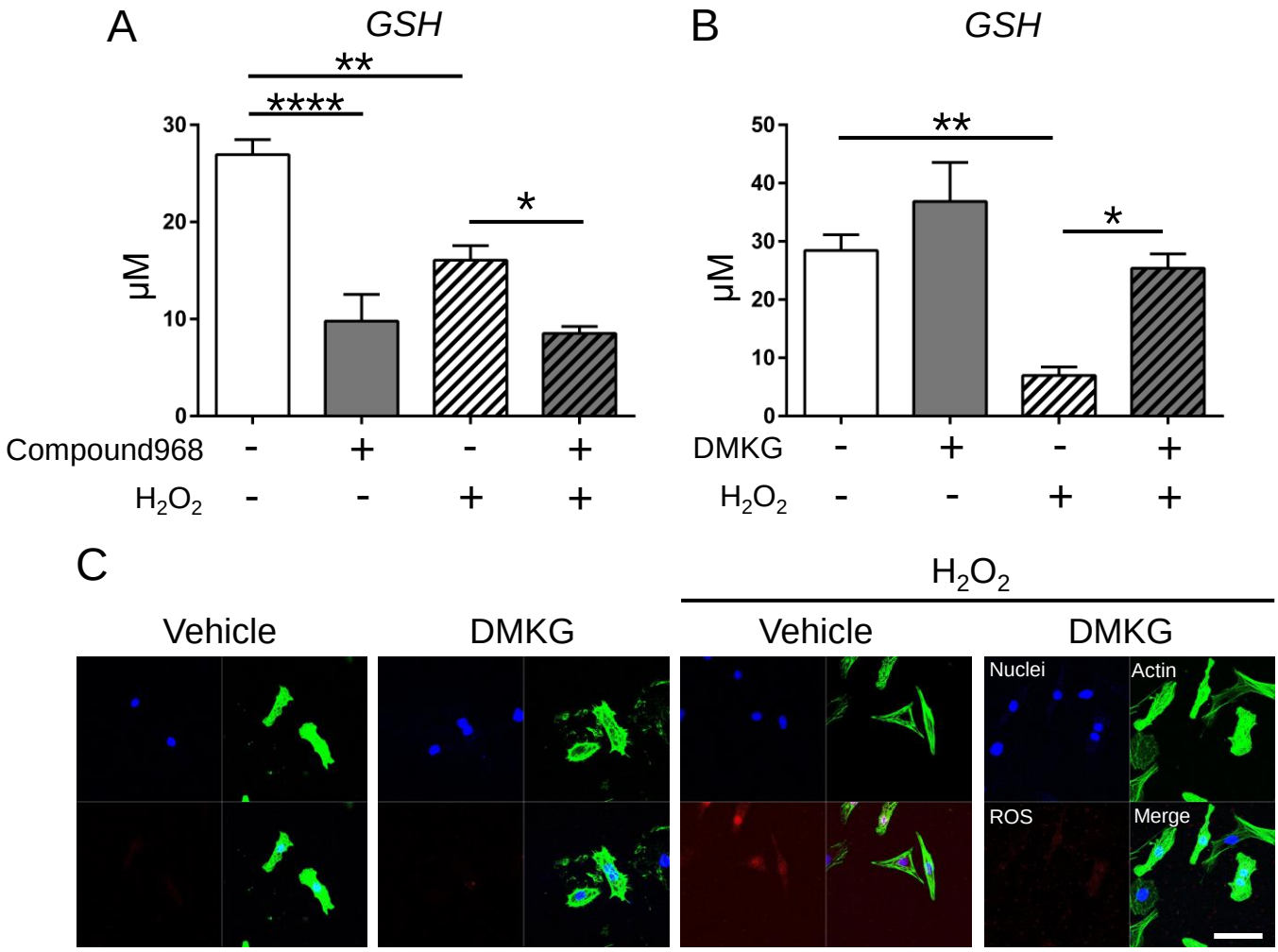

A
GSH
**
****
*
30
20
10
0
µM
Compound968 - + - +
H2O2 - - + +
B
GSH
**
*
50
40
30
20
10
0
µM
DMKG - + - +
H2O2 - - + +
C
H2O2
Vehicle
DMKG
Vehicle
DMKG
Nuclei
Actin
ROS
Merge

Figure 4.

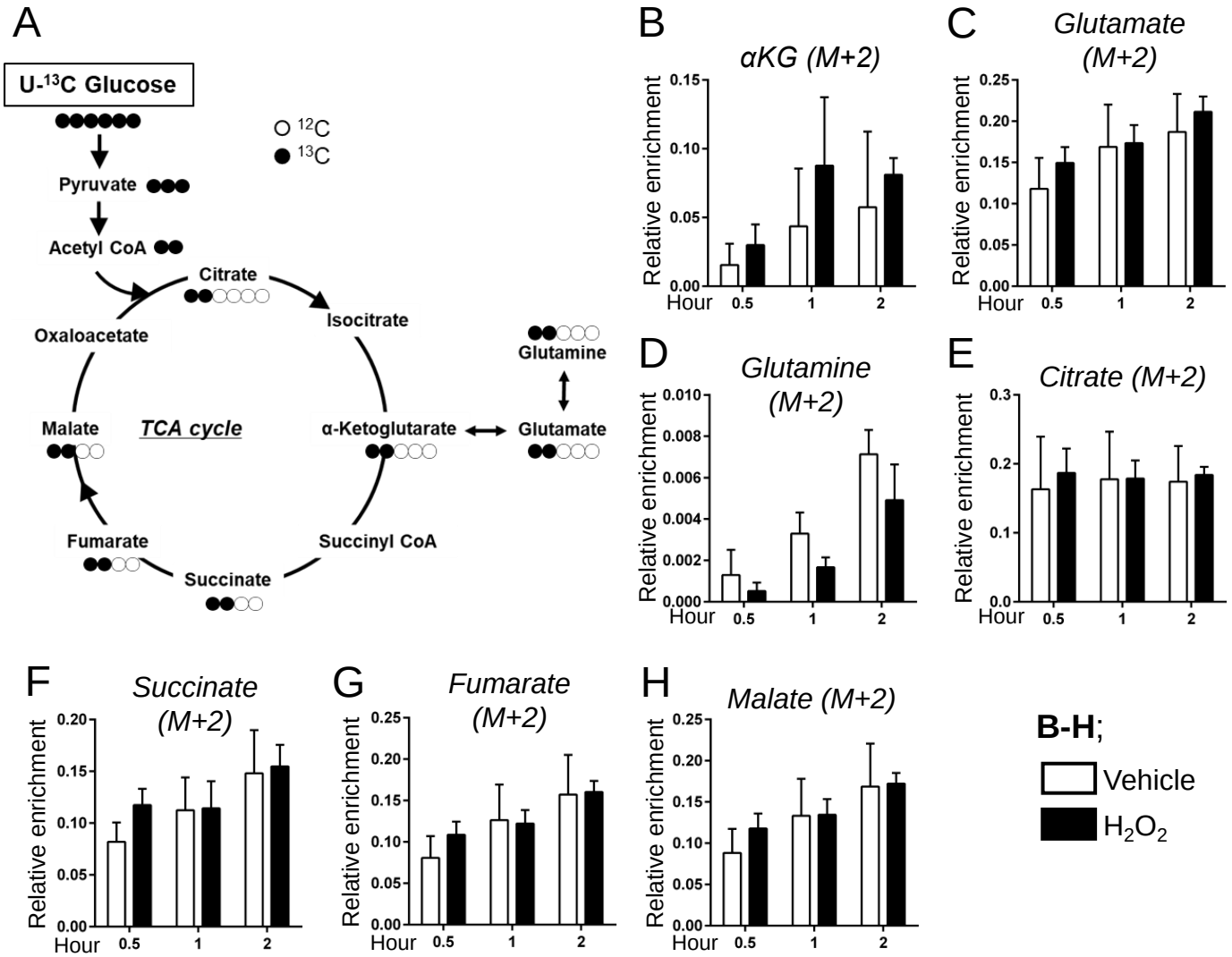
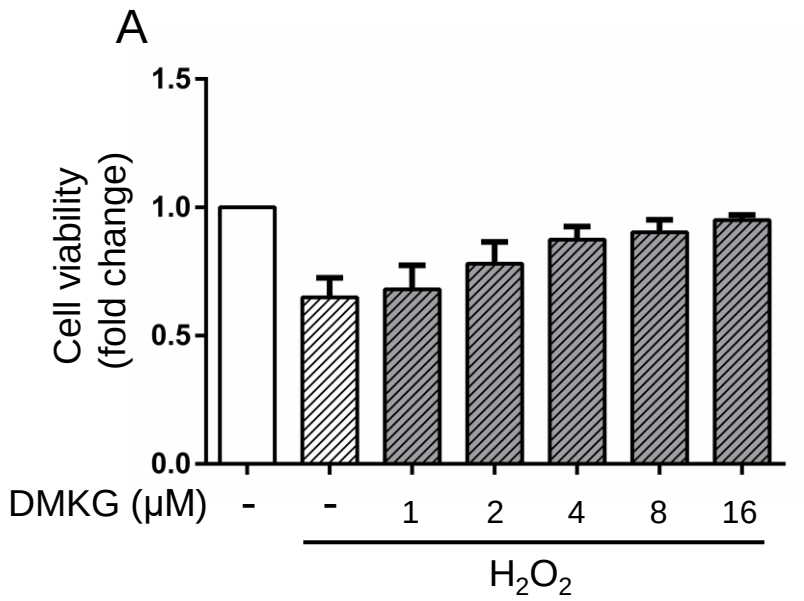


Figure 1. Glucose utilization in cardiomyocytes remains unaffected by oxidative stress in the short term.

A) Schematic depiction of the metabolites derived from [U-^{13}C]-glucose in the TCA cycle. **B-H)** Stable isotope tracing was performed in RNCMs, using [U-^{13}C]-glucose with the same methodology as Figure 1 (n=3). *P＜0.05, **P＜0.01. Data analyzed by unpaired Student's t-test. M+, mass plus.

Supplemental Figure 1.



Supplemental Figure 2. DMKG shows dose dependency in cardiac cell viability under oxidative stress. A) RNCMs were cultured with DMKG at indicated concentration and then stimulated with 100 μM H_2O_2. Cell viability was determined by crystal violet staining.