

Empagliflozins effect on molecular processes in hyperglycemia and failing cardiac myocytes

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Approved:

Donald M. Bers, Chair

Crystal Ripplinger

Jody L. Martin

Committee in Charge

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Abstract

In this dissertation I focus on a sodium glucose cotransporter-2 (SGLT2) inhibitor, empagliflozin (EMPA), which is used to treat type 2 diabetes mellitus (T2DM) and more recently heart failure (HF). In clinical trials SGLT2 inhibitors improved cardiovascular outcomes in HF patients regardless of their diabetes status. Preclinical studies demonstrated EMPA's effects also in *ex vivo* perfused hearts and isolated cardiomyocytes. However, cardiomyocytes do not express SGLT2, thus the mechanism of EMPA's direct effects in cardiac myocytes is unknown.

EMPA has been shown to inhibit the late sodium current (I_{NaL}) in a transverse aortic constriction (TAC) model of HF with reduced ejection fraction (HFrEF). Cardiomyocytes from a murine model of HF with preserved ejection fraction (HFpEF) also exhibited enhancement of the I_{NaL} , and importantly, was reversed with EMPA pre-treatment (~4hr), but interestingly this was not observed with acute (~3min) treatment. The same effects were also seen when a calcium/calmodulin dependent protein kinase II (CaMKII) inhibitor and reactive oxygen species (ROS) inhibitor was used. These findings point towards EMPA affecting upstream regulators of the I_{NaL} . CaMKII is an important upstream regulator of the I_{NaL} and it has increased activity in disease states such as HF. CaMKII can enhance ROS production via NADPH oxidase 2 (NOX2) and increased levels of ROS can oxidize an already active CaMKII to further enhance its activity. Moreover, a shift in cardiac fuel utilization in HF can increase glucose uptake into cardiomyocytes and can lead to an increase in ROS production. An additional modification of CaMKII that enhances its activity upon elevated glucose levels is O-linked-N-acetylglucosamine, or O-GlcNAcylation, where its attachment and detachment

are controlled by two enzymes, O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA), respectively. Thus, we hypothesized that EMPA is modulating one or more of these processes (CaMKII, ROS, NOX2, OGA, OGT), which have downstream effects on I_{NaL} .

Therefore, the work in this dissertation addressed this hypothesis by testing how EMPA affects hyperglycemia-induced myocyte ROS production in comparison to CaMKII inhibition in control and HFpEF myocytes, as well whether EMPA directly affects the activities of NOX2, OGA, and OGT. To test whether EMPA has effects on ROS we utilized isolated cardiomyocytes from transgenic mouse lines expressing the fluorescent ROS sensor Grx1-roGFP2 sensor, localized either to the cytosol or the mitochondria, allowing us to measure compartment-specific ROS. Acute hyperglycemia increases cytosolic ROS production and EMPA pretreatment (1 hr) was able to reduce the rate of production, however this was not seen in the mitochondria where hyperglycemia did not increase mitochondrial ROS. This was seen also in HFpEF mice and their aged-matched controls. We also tested whether CaMKII inhibition had similar effects to EMPA treatment and saw that CaMKII inhibition was able to attenuate the hyperglycemia-induced ROS production in the cytosol. Additional tests were done with heart tissue lysates to compare EMPA's effect on NOX2, O-GlcNAcase, and O-GlcNAc transferase with known inhibitors of these enzymes. No direct effect was seen on the activity of NOX2, O-GlcNAcase, and O-GlcNAc transferase by EMPA. This data suggests EMPA target works within this connected pathway, but not directly with NOX2, O-GlcNAcase, and O-GlcNAc inhibition, as seen by its ability to reduce the rate of hyperglycemia-induced ROS production, but not to the same degree of reduction as

CaMKII inhibition. Since ROS and CaMKII are altered in disease, this may provide insight into EMPA's SGLT2-independent and cardiomyocyte-specific targets.

EMPA has received considerable attention over recent years for its unexpected and direct cardiovascular effects. Hopefully, continued research discoveries contribute to better understanding of HF disease progression and elucidate EMPA's cardiac-specific target that ultimately translated into new therapies for HF patients.

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List of Symbols, Abbreviations, and Nomenclature

1. **AIP:** Autocamtide-2-related inhibitory peptide; CaMKII inhibitor
2. **AP:** Action potential
3. **APD:** Action potential duration
4. **CaMKII:** Calcium-calmodulin dependent protein kinase II
5. **DADs:** Delayed after depolarizations
6. **DM:** Diabetes Mellitus
7. **EADs:** Early after depolarizations
8. **ECC:** Excitation-contraction coupling
9. **EF:** Ejection fraction
10. **EMPA:** Empagliflozin; SGLT2 inhibitor
11. **GP91:** NOX2 inhibitor
12. **HF:** Heart failure
13. **HFD:** High fat diet
14. **HFpEF:** Heart failure with preserved ejection fraction
15. **HFrEF:** Heart failure with reduced ejection fraction
16. **HiGLU:** Hyperglycemia
17. **I_{NaL} :** Late sodium current
18. **L-NAME:** N-nitro-L-arginine methyl ester; NOS inhibitor
19. **NHE:** Sodium Hydrogen Exchanger
20. **NOS:** Nitric Oxide Synthase
21. **NOX2:** NADPH Oxidase 2
22. **OGA:** O-GlcNAcase

- 23. OGT:** O-GlcNAc transferase
- 24. O-GlcNAc:** O-linked N-acetylglucosamine
- 25. O-GlcNAcylation:** addition of O-GlcNAc to serine or threonine residues
- 26. OSMI-1:** OGT inhibitor
- 27. PVC:** premature ventricular contraction
- 28. ROS:** Reactive oxygen species
- 29. RyR:** Ryanodine Receptors
- 30. SCD:** Sudden cardiac death
- 31. SERCA:** Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase
- 32. SGLT2:** Sodium glucose cotransporter-2
- 33. SR:** Sarcoplasmic Reticulum
- 34. STV:** Short term variability of APD
- 35. T2DM:** Type 2 diabetes mellitus
- 36. TAC:** Transverse-aortic constriction
- 37. ThmG:** Thiamet G; OGA inhibitor

CHAPTER 1: INTRODUCTION AND BACKGROUND

Nearly 7 million Americans are currently living with heart failure (HF) (Tsao et al. 2023). Despite its prevalence, the underlying mechanisms of HF are not fully understood, and effective pharmacological treatments for patients remain limited, even more so for patients with heart failure with preserved ejection fraction (HFpEF). Mechanistic understanding, particularly of HFpEF, is further hampered by the heterogeneity of clinical phenotypes arising from diverse patient comorbidities. Common comorbidities include pulmonary and renal diseases, hypertension, and frequently metabolic disorders such as obesity and type II diabetes mellitus (T2DM). While HF and T2DM have distinct etiologies from one another, a pharmaceutical intervention, empagliflozin (EMPA), created for T2DM has been shown to improve cardiovascular outcomes in HF patients. However, because the molecular target of EMPA is not expressed in cardiomyocytes its cardiac-specific mechanism remains elusive. Thus, this project is designed to examine the overlapping cardiac processes in these diseases that EMPA may target.

This thesis will focus on EMPA's effect on reactive oxygen species in hyperglycemia and HFpEF as well as its effects on related processes. The subsequent sections in this dissertation begin with an overview of background including heart failure, diabetic cardiomyopathy, dysfunctional regulators of excitation-contraction coupling, and EMPA. In **Chapter 2**, the methodology of experiments is described, **Chapter 3** presents the results and interpretation of those studies, and **Chapter 4** discusses the implications of those results.

Disease Context

Heart Failure

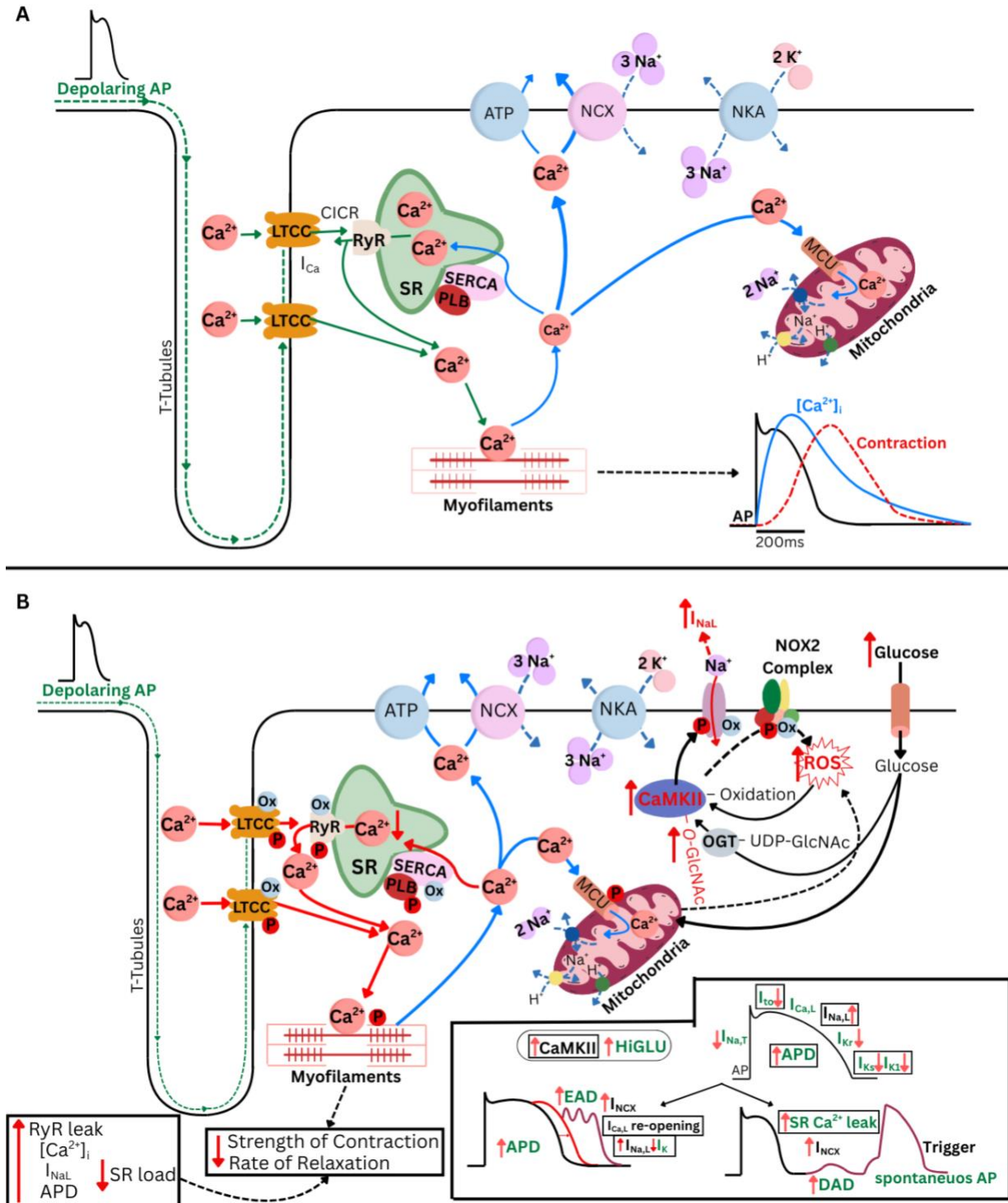
HF is typically categorized as either HF with reduced ejection fraction (HFrEF) or HF with preserved ejection fraction (HFpEF). HFrEF is characterized by *systolic* contractile dysfunction resulting from weakened myocardium and reduced ejection fraction (EF). HFpEF is defined by *diastolic* contractile dysfunction, and while relaxation is impaired, systolic contraction is maintained and thus the EF is preserved; however, the actual volume ejected may be reduced due to the reduction of the end diastolic volume. The impaired relaxation results in higher filling pressures, which becomes mechanically and energetically demanding on the heart and contributes to pulmonary congestion. Hallmark features of HF include contractile dysfunction, increased ryanodine receptor (RyR) calcium leak, prolongation of the action potential duration (APD), enhancement of the late sodium current (I_{NaL}), and an increased incidence of early and delayed after depolarizations (EADs & DADs).

Excitation-contraction coupling (ECC) is the most basic functional unit of the heart and is the process by which an electrical signal is linked to cardiomyocyte contraction and subsequent relaxation. The hallmark dysfunctional features seen in HF are key components of ECC. In healthy ventricular cardiomyocytes, an action potential (AP) propagates along the sarcolemma and t-tubules causing the depolarization of the membrane, and this triggers the opening of voltage-gated L-type calcium channels (Bers 2001; Eisner et al. 2017). This allows an influx of calcium into the cytosol. The cytosolic calcium influx then triggers additional calcium release from the sarcoplasmic reticulum (SR) via RyRs, a process known as calcium-induced calcium release (CICR) (Bers 2001).

The resulting rise in cytosolic calcium binds to troponin C, inducing conformational changes that permit actin and myosin filaments to slide, resulting in cell shortening and contraction (Solaro 2010). This process is illustrated by green arrows in **Figure 1A**. Contraction is followed by the lowering of cytosolic Ca^{2+} via reuptake into the SR and extrusion from the cell, allowing for relaxation. This process is illustrated by blue arrows in **Figure 1A**. Intracellular calcium levels are the primary determinants of contractile force and the rate of intracellular calcium clearance primarily determines relaxation (Bers 2001). Tight regulation of intracellular calcium is therefore essential for normal cardiac contraction and relaxation (Bers and Kranias 2005). Dysregulation in calcium handling can lead to both systolic and diastolic dysfunction and therefore is a central contributor to disease development.

In HF the diastolic calcium leak through RyRs has several detrimental consequences to ECC. First, calcium leak via the RyRs reduces SR calcium content. The reduction in SR calcium leads to weaker systolic contraction because there is a smaller than normal release of calcium from the SR in response to an AP. Second, the sensitized RyRs cause an increase in spontaneous calcium release events that contribute to the generation of proarrhythmic events, like EADs & DADs, in the cell, and this further reduces SR calcium content. Subsequently, RyR calcium leak contributes to diastolic dysfunction by elevating diastolic intracellular calcium concentrations ($[\text{Ca}^{2+}]_i$), impairing the relaxation of cardiomyocytes. Further ionic disturbances are seen in failing cardiomyocytes; in particular, intracellular sodium is increased due to enhanced late sodium current (I_{NaL}). This elevation in intracellular sodium reduces the driving force for the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX), impairing calcium extrusion, elevating diastolic $[\text{Ca}^{2+}]_i$ and

contributing to impaired relaxation. The increase seen in the I_{NaL} contributes to the prolongation of the APD, another key characteristic feature of HF (Makielski 2016). The I_{NaL} is a sustained sodium influx that contributes to the plateau phase of the cardiac AP (Makielski 2016). Under normal physiological conditions, the I_{NaL} contributes minimally to sodium influx. However, in HF the I_{NaL} is elevated leading to APD prolongation. Prolonged APDs cause a delayed repolarization which reduces diastolic filling times and promotes the potential development of proarrhythmic EADs. Together with impaired contractility and relaxation, these electrophysiological abnormalities substantially increase the heart's susceptibility to arrhythmias. Collectively, these mechanisms create a proarrhythmic state in which abnormal sodium and calcium handling converge to increase the risk of malignant arrhythmias in HF. **Figure 1B** illustrates changes seen in diseased cardiomyocytes and highlights downstream effects on ECC.



A) Overview of physiological ECC. Green arrows represent elements related to contraction and blue arrows represent elements related to relaxation. **B)** Shows how excessive ROS and CaMKII activation disrupt calcium handling, leading to SR calcium leak, impaired relaxation, and arrhythmogenesis. Black arrows represent elements changed in disease, with red arrows representing downstream effects of those changes on ECC. Redrawn and adapted from Bers, 2002; Eisner et al., 2007; Hegyi et al., 2019; Hegyi et al., 2021; and Lu et al., 2020.

Diabetic Hyperglycemia

T2DM prevalence continues to increase, currently it is estimated that 451 million individuals worldwide have T2DM and this is projected to increase to 693 million by 2045 (Cho et al. 2018). Diabetes is strongly associated with increased *cardiovascular* morbidity and mortality (Al-Lawati 2017). Notably, T2DM is linked to a 2.5-fold increased incidence of developing HF (Ohkuma et al. 2019), even in the absence of coronary artery disease. Additionally, diabetes is disproportionately represented among patients with HFpEF (Gulsin et al. 2018; MacDonald et al. 2008; Nichols et al. 2004; Rosano, Vitale, and Seferovic 2017; Shindler et al. 1996). Severity of T2DM, independent of other risk factors, has been positively correlated with HF development (Thrainsdottir et al. 2005). Previous studies have shown diastolic and systolic dysfunction in diabetic patient hearts (Battiprolu et al. 2010). Diabetic cardiomyopathy is cardiac changes associated with diabetes that cannot be directly linked to other confounding factors (i.e. hypertension, coronary artery disease, valvular disease) and previous studies have identified cellular signaling pathways associated with disease progression (Battiprolu et al. 2010; Dillmann 2019; Jia, Hill, and Sowers 2018; Jubaidi et al. 2020). The

progression of cardiac dysfunction in T2DM has been associated with metabolic changes, mitochondrial dysfunction, elevated reactive oxygen species (ROS), and impaired calcium handling (Bhatt et al. 2015; Erickson et al. 2013; Kolwicz, Purohit, and Tian 2013).

Hyperglycemia is a defining feature of diabetes and imposes both acute and chronic metabolic stress on cardiomyocytes through alterations in cellular metabolism (Charlton et al. 2020). Elevated blood glucose causes the heart to shift its fuel utilization from partial glucose oxidation to free fatty acid (FFA) oxidation to produce adenosine triphosphate (ATP) (Mazumder et al. 2004; Wang et al. 2005). In parallel, mitochondrial uncoupling where FFA and oxygen consumption increase, through uncoupling proteins, without producing ATP (Skulachev 1999). The increased utilization of FFAs paired with mitochondrial uncoupling may lead to increases in mitochondrial ROS generation, which is seen in diabetic hearts. The accumulation of ROS, which will be discussed in depth later, has downstream consequences like mitochondrial damage, reductions in ATP production, and impaired contractility that could progress to HF.

Diabetic hyperglycemia also induces pathological changes in cardiomyocytes that impair calcium handling and disrupt ECC, contributing to the development of contractile dysfunction and cardiometabolic heart disease. Similar hallmark features of HF are seen in dysfunctional diabetic cardiomyocytes including contractile dysfunction, increased RyR calcium leak, enhancement of the I_{NaL} , prolongation of the APD, and an increased incidence of EADs & DADs (Dillmann 2019). In addition to increased SR calcium leak, cytosolic calcium transient abnormalities have also been observed in diabetic hearts, both of which contribute to decreased contractility and proarrhythmic

events (Bhatt et al. 2015). These maladaptive changes to calcium homeostasis can decrease mitochondrial calcium, which regulates enzymes along the Krebs cycle and electron transport chain, modulating ATP production (Wan et al. 1989). In addition to contraction and relaxation dysfunction, imbalances in calcium homeostasis can also reduce ATP production, causing further contractile dysfunction. The frequent coexistence of T2DM and HF paired with their shared underlying mechanisms highlights the need to better understand pathways linking metabolic dysfunction to cardiac pathology.

CaMKII and Redox Signaling in Diabetes and Heart Failure

Disruptions in cellular signaling pathways that regulate calcium handling and redox balance are central features of cardiac dysfunction in T2DM and HF. These processes are tightly interconnected and play critical roles in maintaining cardiomyocyte function under both physiological and pathological conditions. In particular, alterations in calcium-dependent signaling and oxidative stress responses contribute to impaired ECC, maladaptive remodeling, and disease progression. The following sections focus on key components of these pathways, with an emphasis on their roles in the development and progression of cardiac dysfunction.

CaMKII in Cardiac Function and Dysfunction

Calcium/calmodulin dependent protein kinase II (CaMKII) is typically a dodecamer with each subunit associated at the C-terminal to form a pair of parallel hexameric rings (Hoelz, Nairn, and Kuriyan 2003; Rellos et al. 2010). Individual monomers are made up of an association regulatory, and catalytic domain. The regulatory domain is where calcium/calmodulin binds. This domain also contains

posttranslational modifications by O-GlcNAcylation (Ser280), oxidation (Met281&282), autophosphorylation (Thr287), and nitrosylation (Cys290) (Erickson et al. 2008; Erickson et al. 2013; Erickson et al. 2015; Hudmon and Schulman 2002). At rest, when intracellular calcium remains low, CaMKII is inhibited and in a closed state where the regulatory domain acts as a pseudo-substrate and prevents other substrates from binding to the catalytic domain. Rising intracellular calcium binds to calmodulin, and calcium/calmodulin then activates CaMKII by binding to the regulatory domain. This induces a conformational change, opening up the catalytic domain to bind to substrates (Schulman and Anderson 2010), illustrated in **Figure 2**. This conformational change is reversible and typically becomes inactive as calcium levels drop in the cell. The direct regulation of CaMKII by intracellular calcium creates beat-to-beat changes in its activity (Schulman and Anderson 2010). However, CaMKII activity can become unlinked from the beat-to-beat changes in calcium when it becomes autonomously activated. Autonomous activation can occur through either autophosphorylation of CaMKII (site T287) or other posttranslational modifications (Erickson et al. 2008, 2013).

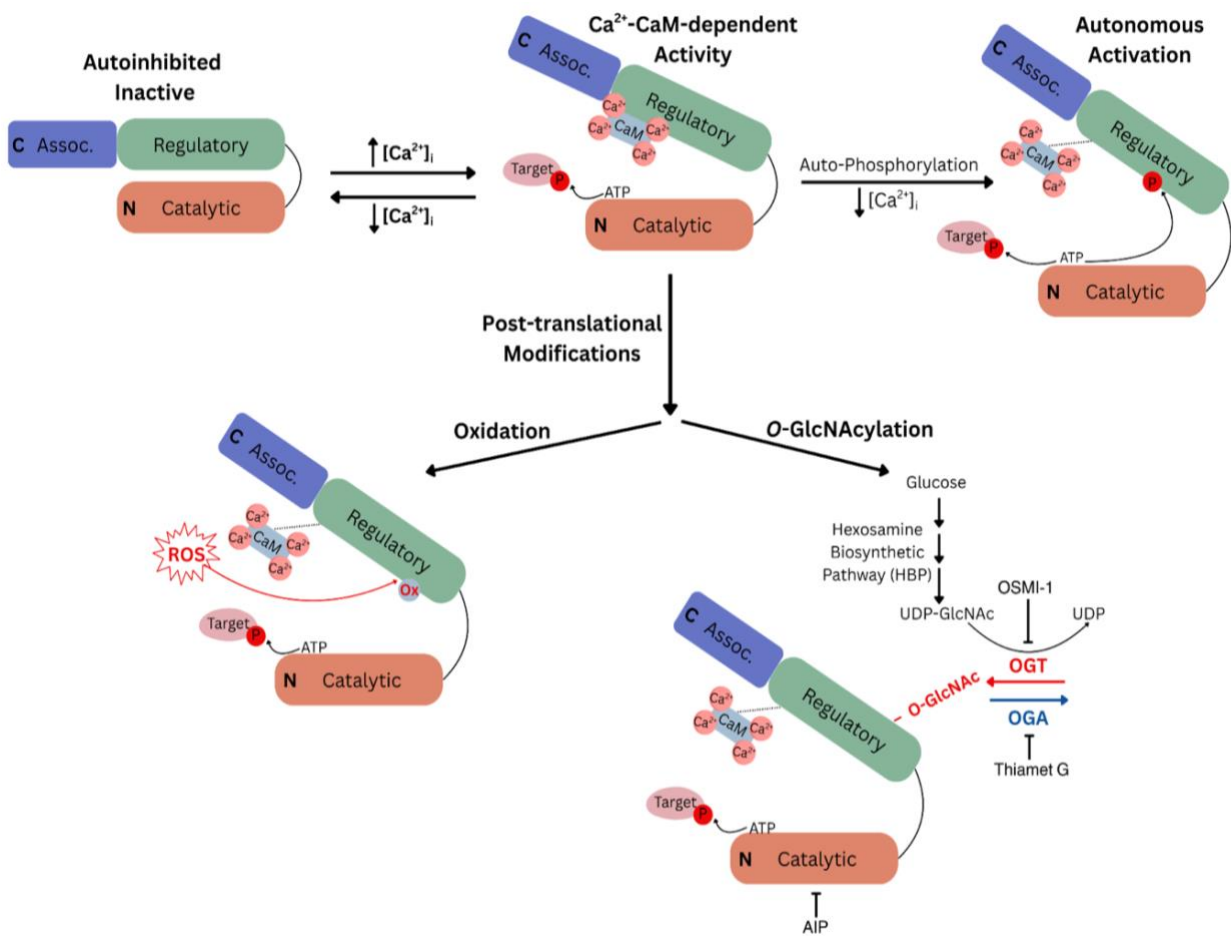


Figure 2. CaMKIIδ (Calcium/Calmodulin-dependent protein kinase II) activation.

Schematic of CaMKII structure and function. Autonomous activation and post-translational modifications by ROS and O-GlcNAcylation depicted. Redrawn and adapted from Hegyi et al., 2019; Erickson et al., 2008; Erickson, 2013; and Lu et al., 2020.

CaMKII is a kinase that orchestrates changes in key aspects of calcium handling, ECC, and contractility (Hegyi, Bers, and Bossuyt 2019; Luczak and Anderson 2014; Schulman and Anderson 2010). CaMKII phosphorylates critical ECC components, including L-type calcium channels, RyRs, and phospholamban (PLB) to modulate contraction. Phosphorylation of L-type calcium channels and RyRs increases their open

probability, enhancing calcium influx and SR calcium release (Maier and Bers 2007). As a result, the peak calcium current rises and the channel's inactivation slows, allowing greater calcium entry into the cytosol, while diastolic SR calcium leak can be proarrhythmic and elevate diastolic calcium. Phosphorylation of PLB relieves its inhibition of SERCA, enhancing calcium reuptake into the SR, accelerating relaxation, and increasing SR calcium load. CaMKII activity contributes to increased heart rate, contractility, and relaxation rate (Schulman and Anderson 2010). Together, these modifications enable the heart to meet increased functional demands during stress and exercise; however, when chronically activated it is known to contribute to disease development.

Importantly, CaMKII is known to be chronically activated in diabetic hearts and directly induces pathological changes in calcium handling (Anderson 2007; Anderson, Brown, and Bers 2011; Curran et al. 2014; Lu et al. 2020; Luo et al. 2013; Tian et al. 2010). Additionally, expression of CaMKII is increased in HF along with its activity (Kirchhefer et al. 1999). Diabetic mouse hearts (leptin receptor-deficient *db/db* – *Lepr^{db/db}*) have been shown to have increased CaMKII activity that enhanced phosphorylation of RyRs at CaMKII-specific sites (Hegyi, Bers, and Bossuyt 2019), leading to increased spontaneous calcium release events and a higher prevalence of ventricular arrhythmias (Kadosaka et al. 2023). In parallel, CaMKII-dependent phosphorylation of voltage-gated sodium channels enhanced I_{NaL} , contributing to APD prolongation (Wagner et al. 2006). Moreover, autonomous, or calcium-independent, activation of CaMKII can become dysregulated, resulting in autonomous, calcium-independent activation, a phenomenon commonly observed in cardiovascular disease.

Autonomous activation of CaMKII can result from different post-translational modifications, as discussed previously. Multiple studies have demonstrated that attachment of *O*-linked-*N*-acetylglucosamine (*O*-GlcNAc) to CaMKII can promote CaMKII's autonomous, calcium-independent activation (Erickson et al. 2013; Hegyi, Fasoli, et al. 2021). *O*-GlcNAcylation is increased under hyperglycemic conditions and serves as a fuel-sensitive post-translational modification in cardiomyocytes (Lima, Moreira, and Sakamoto-Hojo 2022). There are only two enzymes that control *O*-GlcNAcylation cycling, *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase (OGA), which catalyze the attachment and removal of *O*-GlcNAc (Dias, Cheung, and Hart 2012) (**Figure 2**). Elevated *O*-GlcNAcylation exerts widespread effects on ECC by modifying key signaling proteins involved in calcium handling. *O*-GlcNAc-modified CaMKII increases SR calcium leak through enhanced RyR activity and is associated with increased arrhythmia susceptibility under hyperglycemic conditions (Erickson et al. 2013; Hegyi, Fasoli, et al. 2021). In parallel with increased SR calcium leak, calcium reuptake into the SR is slowed in diabetic (*db/db*) cardiomyocytes, further reducing SR calcium load (Dillmann 2019). This impairment may be attributed to *O*-GlcNAcylation of phospholamban (PLB), the inhibitory regulator of the SERCA, or increased PLB expression, both of which would enhance SERCA inhibition and have been reported in T2DM (Hegyi, Fasoli, et al. 2021). Oxidation is an additional mechanism by which CaMKII becomes autonomously active in disease (Erickson et al. 2008). Oxidative modifications of CaMKII also sustain the kinase's activity, prolonging phosphorylation of ECC components such as RyRs, voltage-gated sodium channels (Luczak and Anderson 2014), as well as multiple potassium channels, as demonstrated by Wagner et al.

(2009) and others and reviewed extensively in the CaMKII literature. At the cellular level, hyperglycemia has been shown to activate CaMKII through O-GlcNAcylation, which promotes increased ROS production (Lu et al. 2020). This feed-forward environment favors additional activating post-translational modifications of CaMKII, including oxidation and autophosphorylation, alongside O-GlcNAcylation. As a result, hyperglycemic conditions may drive multiple activating modifications of CaMKII simultaneously, positioning the kinase as a key integrator of metabolic and redox stress signals in the diabetic heart. **Figure 2** illustrates CaMKII's inactive, active, and autonomously active states including the two post-translational modifications discussed. **Figure 1B** illustrates important points in ECC that chronic CaMKII phosphorylation leads to dysfunction. Together these alterations, exacerbate diastolic calcium accumulation and impair relaxation.

Reactive Oxygen Species in Cardiac Function and Dysfunction

Reactive oxygen species (ROS) are highly reactive oxidizing molecules containing one or more oxygen atoms and unpaired electrons, including species such as hydrogen peroxide (H_2O_2), superoxide ('O_2^-), nitric oxide (NO'), and hydroxyl radicals ('OH) (Ghodeswar and Bandyopadhyay 2025). In cardiomyocytes, ROS are generated across multiple subcellular compartments and participate in localized signaling pathways that influence ECC. Superoxide is primarily produced within the mitochondria at Complexes I and III of the electron transport chain and is rapidly converted to H_2O_2 by superoxide dismutase (Phaniendra, Jestadi, and Periyasamy 2015). Hydrogen peroxide, which can also arise in the cytosol and nucleus, is more stable and diffusible than superoxide and is metabolized to water and oxygen, mainly by catalase and other

antioxidant systems. Due to these properties H_2O_2 serves as an important redox signaling molecule capable of modifying proteins involved in ECC (Steinhorn et al. 2018).

Additional reactive species contribute to redox dysregulation in cardiomyocytes. Nitric oxide (NO^*), a reactive nitrogen species is often grouped within the broader ROS signaling network, is produced by nitric oxide synthase (NOS) isoforms localized to specific cellular compartments including the SR, sarcolemma, t-tubules, and mitochondria, enabling spatially restricted regulation of ECC and redox balance (Ziolo, Kohr, and Wang 2008). In contrast, the hydroxyl radical is an extremely short-lived and highly reactive species generated locally, primarily in the mitochondria and cytosol, where it rapidly reacts with nearby lipids, proteins, and nucleic acids and can initiate lipid peroxidation reactions (Vásquez-Vivar, Kalyanaraman, and Kennedy 2000). Because of its extremely short half-life and limited diffusion, $\bullet\text{OH}$ causes highly localized oxidative damage that can disrupt membrane integrity and modify key ECC proteins, impairing Ca^{2+} handling and contractile function.

At physiological levels, ROS function as important signaling molecules that modulate cardiomyocyte function (Ghodeswar and Bandyopadhyay 2025). In particular, ROS can influence ECC by oxidizing key calcium channels, RyRs, and SERCA (Köhler, Sag, and Maier 2014; Lim et al. 2008, 2014; Zima and Blatter 2006). Oxidation of L-type calcium channels and RyRs increases their open probability, enhancing calcium influx and SR calcium leak (Brown and Griending 2015; Lim et al. 2008; Tian et al. 2021). In contrast, oxidation of SERCA reduces its activity, slowing calcium reuptake into the SR. Through these mechanisms, moderate ROS signaling can transiently

elevate intracellular calcium and augment contractile strength during increased functional demand (Zima and Blatter 2006). However, sustained elevations in ROS can overwhelm antioxidant defenses and promote maladaptive oxidative modification of calcium-handling proteins, ultimately contributing to impaired ECC and pathological remodeling.

Pathological levels of ROS contribute to disruptions in calcium and sodium handling, impaired ECC, and arrhythmogenesis (Brown and Griendling 2015; Hegyi, Bers, and Bossuyt 2019; Köhler, Sag, and Maier 2014; Tian et al. 2021; Wagner et al. 2006; Zima and Blatter 2006). The increased open probability of L-type calcium channels and RyRs from chronic oxidation ultimately produces persistently higher intracellular calcium concentrations, impairing relaxation of the myofilament (Cooper et al. 2013; Zima and Blatter 2006). Oxidized RyRs exhibit increased sensitivity to luminal calcium concentrations and shortened refractory periods, leading to enhanced SR calcium leak and a higher frequency of spontaneous calcium release events (Brown and Griendling 2015; Cooper et al. 2013; Liang et al. 2020; Lim et al. 2008; Tian et al. 2021). These events reduce SR calcium content, weaken systolic calcium release, and increase susceptibility to arrhythmias (Q. Li et al. 2015; Oda et al. 2015). SERCA oxidation reduces its activity and in disease that leads to elevated intracellular calcium concentrations and impaired relaxation. Chronic oxidation ultimately leads to elevated diastolic cytosolic calcium which impairs myofilament relaxation causing mechanical dysfunction that is seen in both diabetes and HF (González et al. 2023; Oda et al. 2015; Schiattarella et al. 2019). Collectively, dysregulated redox balance is a central mechanism linking metabolic stress to impaired ECC.

Both diabetes and HF are characterized by chronic metabolic stress that disrupts intracellular redox signaling pathways critical for ECC (Mengstie et al. 2022). Diabetic hyperglycemia is associated with increased intracellular ROS production (Hegyi, Bers, and Bossuyt 2019), and even acute elevations in glucose have been shown to rapidly increase production of ROS in cardiomyocytes (Brown and Griendling 2015; Giacco and Brownlee 2010). In both T2DM and HF, ROS accumulation has been shown to be central to disease progression, contributing to maladaptive remodeling of ion channels and signaling proteins (Akki et al. 2009; Guo et al. 2007; Heymes et al. 2003; Köhler, Sag, and Maier 2014; Yano et al. 2005).

As mentioned, different types of ROS can originate from multiple intracellular sources, including mitochondrial metabolism and enzymatic oxidases (Laddha and Kulkarni 2020). Common sources of ROS are in the mitochondria's electron transport chain (ETC) at complex I and III where a majority of electrons are transported to molecular oxygen as the terminal electron acceptor. In cardiomyocytes exposed to hyperglycemia, ROS production arises predominantly from the mitochondrial ETC and NADPH oxidases (NOX), particularly NOX2 (González et al. 2023; Lima, Moreira, and Sakamoto-Hojo 2022; Lu et al. 2020). Enhanced ATP synthesis and excess substrate availability (NADH & FADH₂) increases the mitochondrial membrane potential and electron pressure within the ETC, promoting electron leak and increasing mitochondrial ROS production (Jubaidi et al. 2020; Laddha and Kulkarni 2020; Q. Li et al. 2015). The increased energy demand of the heart seen in disease puts a strain at the mitochondria which can drive ROS production, as well as the shift in fuel utilization discussed previously.

In the cytosol primary ROS generation is attributed to NADPH oxidase 2 (NOX2). In the cardiomyocyte along the sarcolemma and t-tubules is the transmembrane protein, NOX2, which forms a complex upon activation (Rastogi et al. 2017). NOX2 activation complex forms around the heterodimerization of gp91^{phox} and p47^{phox} proteins (Rastogi et al. 2017). CaMKII-dependent activation of NOX2 has been shown to amplify ROS generation (Heymes et al. 2003; Laddha and Kulkarni 2020; Rastogi et al. 2017). While CaMKII can increase ROS production via NOX2, ROS also oxidizes kinases, including CaMKII (Erickson et al. 2008; Lu et al. 2020). Thus, oxidation of CaMKII promotes autonomous CaMKII activation, which subsequently activates NOX2 and increases ROS production, creating a feed-forward signaling loop between oxidative stress and kinase activation. Figure 1B illustrates important points in ECC that, when chronically oxidized, leads to dysfunction as well as the dynamic relationship that exists between ROS and CaMKII.

The Late Sodium Current and Ionic Dysregulation in Cardiomyocytes

The I_{NaL} is the influx of sodium that contributes to the plateau phase of the cardiac AP (Philippaert et al. 2021). Under physiological conditions it appears the I_{NaL} has small effects, however in pathological conditions increases in the I_{NaL} can significantly disrupt intracellular sodium homeostasis and have further downstream effects (Hegyi, Pölönen, et al. 2021; Hegyi and Bers 2022; Makielski 2016; Viatchenko-Karpinski et al. 2014).

The I_{NaL} is consistently elevated in HF and contributes to proarrhythmic changes seen (Hegyi, Mira Hernandez, Shen, et al. 2022; Makielski 2016). The enhanced I_{NaL} increases intracellular sodium concentrations, which reduces the electrochemical

driving force for the sodium-calcium exchanger (NCX), which normally accounts for ~30% of calcium removal from the cytosol at each beat in human and rabbit ventricular myocytes (Bers 2001). Impaired NCX activity reduces calcium extrusion, resulting in intracellular calcium overload that compromises relaxation and promotes diastolic dysfunction (Hegyi, Pölönen, et al. 2021). Elevations in intracellular calcium, due to spontaneous SR calcium release, increases inward NCX current and depolarizes the resting membrane potential (DAD) closer to the threshold of sodium channel activation and can trigger spontaneous AP and extrasystoles, which may initiate arrhythmias (Hegyi, Ko, et al. 2021; M. B. Liu et al. 2015). The pathological I_{NaL} disrupts intracellular sodium homeostasis, triggering downstream consequences such as prolonged APDs, intracellular calcium overload, impaired relaxation, and heightened susceptibility to arrhythmias (Hegyi, Pölönen, et al. 2021; Hegyi and Bers 2022). APD prolongation promotes EAD formation by permitting reactivation of L-type Ca^{2+} channels during repolarization. In combination with impaired contractility and relaxation, these electrophysiological abnormalities substantially increase the heart's susceptibility to arrhythmias (Hegyi, Mira Hernandez, Ko, et al. 2022; Hegyi, Mira Hernandez, Shen, et al. 2022).

Some relevant regulators of sodium channel function are phosphorylation and oxidation. Notably, ROS can augment the I_{NaL} through oxidation of methionine residues in the sodium channels α subunit which impairs the channels inactivation (Kassmann et al. 2008). Additionally, CaMKII is known to alter sodium channel inactivation via phosphorylation at multiple sites which increases the I_{NaL} (Wagner et al. 2006). A vicious cycle is formed with the elevation in intracellular sodium leading to elevated

[Ca²⁺]_i which allows for prolonged CaMKII activation that can phosphorylate sodium channels to enhance the I_{NaL}. Further complicating this cycle, sustained CaMKII activation increases ROS production which can oxidize both sodium currents and CaMKII contributing to increases in the I_{NaL} and autonomous CaMKII activation, all of which promotes APD prolongation and arrhythmogenesis (Hegyi, Pölönen, et al. 2021; Hegyi and Bers 2022; Wagner et al. 2006). The pathological I_{NaL} represents a critical downstream effector through which CaMKII and ROS signaling converge to destabilize ECC in cardiometabolic disease and HF.

Hyperglycemia–ROS–CaMKII–I_{NaL} Pathway Convergence

Hyperglycemia and HF are associated with increased production of ROS (González et al. 2023; Heymes et al. 2003; Lima, Moreira, and Sakamoto-Hojo 2022; Lu et al. 2020; Luo et al. 2013; Schiattarella et al. 2019), enhanced CaMKII activity (Hegyi, Bers, and Bossuyt 2019; Kushnir et al. 2010; Schulman and Anderson 2010), and elevated protein O-GlcNAcylation, including O-GlcNAc modification of CaMKII (Erickson et al. 2013; Hegyi, Fasoli, et al. 2021). These stress-activated pathways are closely interconnected, as mentioned above, ROS can promote CaMKII activation (Erickson et al. 2008; Luczak and Anderson 2014) and CaMKII can enhance pro-oxidant signaling, and O-GlcNAcylation and ROS can sustain autonomous CaMKII activity under pathological conditions (Erickson et al. 2013; Lu et al. 2020). Dysregulation of these signaling pathways converge on key regulators of calcium and sodium handling, including RyRs, SERCA, and voltage-gated sodium channels (Hegyi, Pölönen, et al. 2021).

Pathological activation of CaMKII and oxidative modification of calcium handling proteins promote increased SR calcium leak, reduced SERCA activity, diminished SR calcium load, and impaired intracellular Ca^{2+} cycling (Hegyi, Bers, and Bossuyt 2019; Kushnir et al. 2010). In parallel, pathological enhancement of the I_{NaL} disrupts sodium homeostasis, prolongs the APD, and exacerbates intracellular calcium overload through impaired NCX (Hegyi, Pölönen, et al. 2021; Makielski 2016; Njégic, Wilson, and Cartwright 2020). Collectively, these ionic and signaling disturbances contribute to impaired relaxation, diastolic dysfunction, reduced contractile reserve, and heightened susceptibility to arrhythmias, all of which are hallmark features of both diabetic cardiomyopathy and HF (Njégic, Wilson, and Cartwright 2020). Thus, interventions that modulate nodes in this pathway may normalize sodium loading, calcium handling, and repolarization in stressed cardiomyocytes and help to reduce proarrhythmic events. This pathway and downstream consequences are illustrated in **Figure 3**.

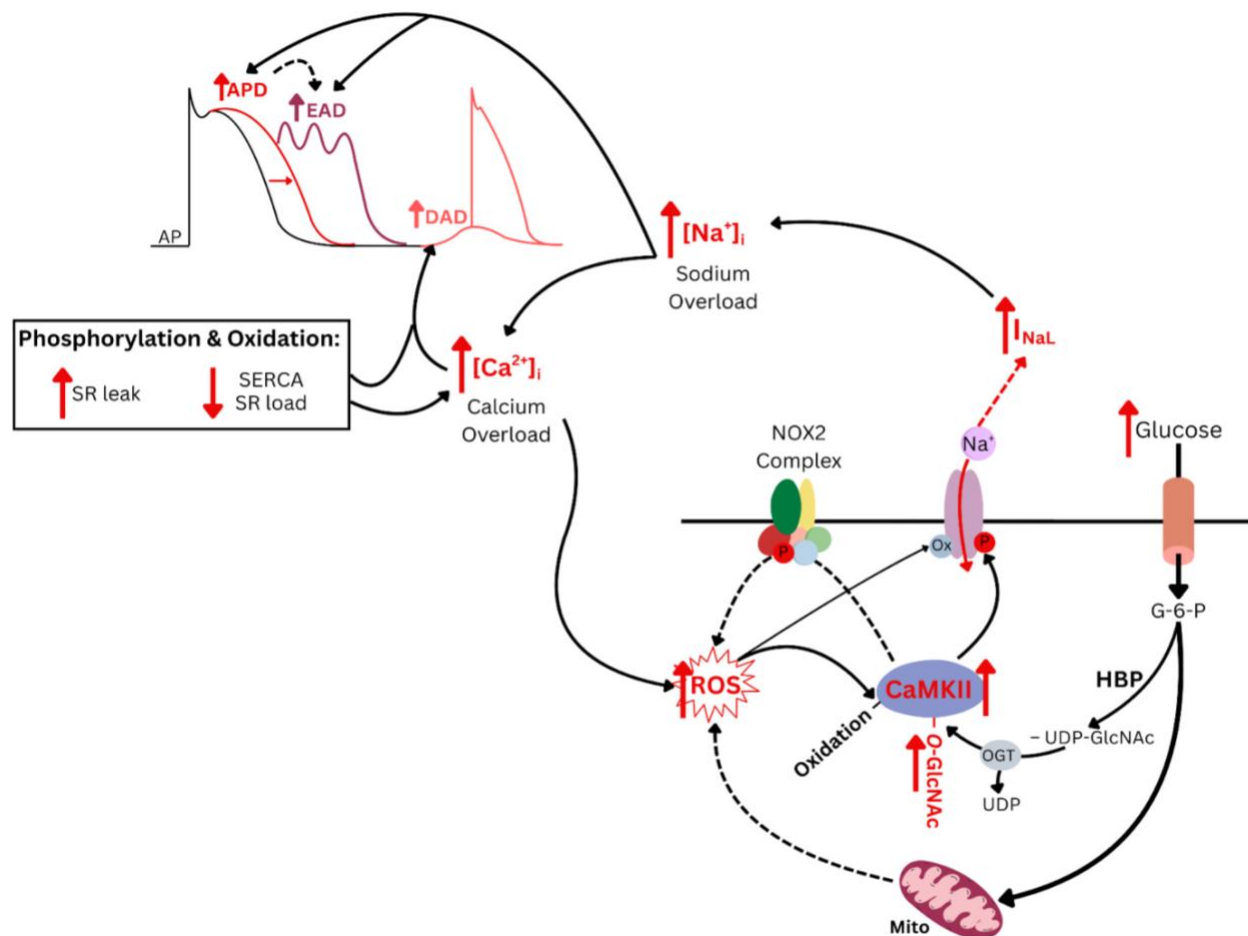


Figure 3. Feed-forward amplification between ROS and CaMKII in diabetic and failing myocardium.

Hyperglycemia and metabolic stress increase mitochondrial and NOX2-derived ROS, promoting oxidation-dependent autonomous activation of CaMKII at Met281/282.

Activated CaMKII enhances NOX2 activity and downstream Ca²⁺-handling abnormalities, creating a feed-forward loop that sustains redox signaling and electrophysiological remodeling. Redrawn and adapted from Erickson et al., 2008; Hegyi et al., 2021; and Lu et al., 2020.

SGLT2 Inhibitor: Empagliflozin

Sodium glucose co-transporter 2 (SGLT2) inhibitors are a class of drugs developed for T2DM treatment. SGLT2 inhibitors included in this drug class are bexagliflozin, canagliflozin, dapagliflozin, empagliflozin, and ertugliflozin all of which work relatively in the same way (Padda, Mahtani, and Parmar 2025). These drugs prevent glucose reuptake in the nephron by inhibiting SGLT2, causing an increased excretion of glucose and a reduced blood glucose level. Additionally, there is sotagliflozin which is a dual inhibitor of both SGLT2 and SGLT1 that has been specifically FDA-approved to reduce heart failure risk, without any indication of glycemic control (Padda, Mahtani, and Parmar 2025). While foundational in modern diabetes care, most drugs within the SGLT2 inhibitor class have additional indications in cardiovascular disease risk and death reduction, as well as heart failure hospitalization reduction. However, bexagliflozin and ertugliflozin remain solely approved for glycemic control and have no formal indications of cardiovascular protections (Corbin et al. 2023; Hadd et al. 2023). While findings show favorable trends in cardiovascular outcomes these observations did not reach statistical significance. Canagliflozin has FDA approval for risk reduction of major adverse cardiovascular events including cardiovascular death and myocardial infarction in patients with T2DM and established cardiovascular disease (Neal et al. 2017). Dapagliflozin and empagliflozin are both FDA approved for treatment in patients with HFrEF and HFpEF and provide risk reduction of cardiovascular death and hospitalization regardless of diabetes status (Curtain et al. 2021; Packer, Anker, et al. 2021; Packer, Butler, et al. 2021; Pellicori et al. 2020). Importantly, empagliflozin and dapagliflozin show cardiovascular improvements across the spectrum of phenotypes

and irrespective of T2DM and are now recognized as a foundational therapy for HF (Padda, Mahtani, and Parmar 2025).

Empagliflozin (EMPA) has emerged as a therapy with robust cardioprotective effects across multiple patient populations, including individuals with diabetes as well as those with HF, independent of glycemic status (Anker et al. 2021; Fitchett et al. 2016; Lopaschuk and Verma 2020; Zinman et al. 2015). While originally developed as a glucose-lowering agent, accumulating clinical and experimental evidence indicates that EMPA exerts direct effects on cardiac structure, function, and electrophysiology that cannot be fully explained by systemic metabolic changes alone. Indeed, cardiac myocytes do not express SGLT2, implying that there are likely cardiomyocyte off-target EMPA effects which may be beneficial (see below). This section reviews the clinical and preclinical evidence supporting EMPA's cardioprotective actions, highlighting the apparent dissociation between these effects and glucose lowering effects, and summarizes candidate intracellular signaling pathways through which EMPA may act in cardiomyocytes.

Clinical and Preclinical Evidence

EMPA is a SGLT2 inhibitor originally developed for the treatment of T2DM to improve glycemic control. Large randomized clinical trials have demonstrated that EMPA significantly improves cardiovascular outcomes in patients with diabetes (EMPA-Reg Outcome trial) (Anker et al. 2021; Fitchett et al. 2019), HFrEF (EMPEROR-Reduced trial) (Packer, Anker, et al. 2021), and HFpEF (EMPEROR-Preserved trial) (Packer, Butler, et al. 2021). EMPA reduced HF-related hospitalizations and mortality in both HFrEF and HFpEF patients regardless of their diabetes status, a benefit not seen

with other glucose lowering agents (Anker et al. 2021; Fitchett et al. 2016; Lopaschuk and Verma 2020; Zinman et al. 2015). In addition to its metabolic effects, EMPA has demonstrated both positive renal and hemodynamic effects that may contribute to improved cardiovascular outcomes (Cowie and Fisher 2020). While the antiarrhythmic effects of SGLT2 inhibitors in patients with HFpEF are yet to be determined, dapagliflozin, a structurally related SGLT2 inhibitor, reduced the risk of ventricular arrhythmias and sudden cardiac death in patients with HFrEF (Curtain et al. 2021). Notably, the cardiovascular benefits of EMPA occur rapidly, often within weeks of treatment initiation, and cannot be fully explained by improvements in glycemic control alone, suggesting mechanisms distinct from long-term metabolic remodeling (Packer, Butler, et al. 2021; Pellicori et al. 2020).

Preclinical studies demonstrated that EMPA has direct effects on *ex vivo* perfused hearts and isolated cardiac myocytes, providing further evidence that there is a direct effect of EMPA on the heart (Cowie and Fisher 2020). However, the major issue that arises is that SGLT2 expression is virtually absent in cardiac myocytes, thus pointing to off-target beneficial effects in the heart itself (J. Chen et al. 2010; Di Franco et al. 2017). Given this lack of expression, the molecular mechanism by which EMPA directly acts in the heart remains elusive. Together, these findings indicate that EMPA exerts direct cardiac effects through mechanisms independent of SGLT2, motivating investigation into alternative intracellular targets.

Potential Candidates for Empagliflozin's Cardiac-Specific Targets

EMPA exerts robust cardioprotective effects and several intracellular signaling pathways, that are dysregulated in T2DM and HF, have emerged as potential mediators

of EMPA's direct cardiac actions (Hegyi and Bers 2023). These pathways converge on key determinants of ECC, including sodium handling, calcium homeostasis, redox signaling, and CaMKII activation. Specific cellular and/or subcellular interactions remain unknown, but based on functional evidence EMPA may act through the following pathways to modulate cardiac electrophysiology and contractile function.

Sodium-Hydrogen Exchanger (NHE1)

The sodium-hydrogen exchanger-1 (NHE1) has been proposed as a potential target of EMPA in cardiomyocytes, particularly under pathological conditions such as ischemia. Initial studies, including work by Uthman et al. (2018), reported that SGLT2 inhibitors exert a class effect to inhibit NHE1, leading to reduced intracellular sodium concentrations and subsequent improvements in calcium handling. However, this proposed mechanism remains highly controversial. More recent work directly challenged these findings, demonstrating that empagliflozin does not inhibit NHE1 nor reduce intracellular sodium in cardiomyocytes (Chung et al., 2021). This discrepancy has been highlighted in subsequent discussions and editorials (Murphy and Eisner 2021), emphasizing the lack of consensus in the field.

In diabetic cardiomyocytes, NHE1 inhibition has been shown to improve calcium extrusion, but does not rescue SR calcium leak or SERCA dysfunction (Kadosaka et al. 2023). Therefore, while modulation of NHE1 may influence sodium handling, the lack of consensus regarding direct inhibition by EMPA suggests that NHE1 is unlikely to be the primary mediator of EMPA's effects on SR calcium regulation and redox signaling, supporting the involvement of alternative intracellular targets.

O-GlcNAcylation and CaMKII

EMPA has been associated with reduced glucose uptake, CaMKII autonomous activation, and O-GlcNAc post-translational protein modifications in db/db mouse hearts (Kadosaka et al. 2023). Chronic EMPA treatment in diabetic (db/db) mice led to improvements in diastolic function including decreases in CaMKII activity, RyR phosphorylation, and cardiac glucose levels (Moellmann et al. 2020). When compared with EMPA, the CaMKII inhibitor KN-93 produced a similar reduction in SR calcium leak in *db/db* cardiomyocytes, but failed to improve SR calcium reuptake (Kadosaka et al. 2023). A study looking at both a HFrEF mouse model and human failing ventricular myocytes saw reduced CaMKII activity, CaMKII-dependent RyR phosphorylation, SR calcium leak, and spontaneous calcium release events in cardiomyocytes with a 24-hr pretreatment with EMPA, but acute (30min) EMPA treatment had no effect on CaMKII activity or calcium handling (Mustroph et al. 2018). This suggests EMPA may reduce spontaneous diastolic activity by attenuating CaMKII activation; however, this effect likely occurs indirectly since acute treatment did evoke the same effects. Modulation of O-GlcNAc and CaMKII signaling is associated with improvements in calcium handling in diabetic cardiomyocytes and supports the possibility that EMPA acts at a molecular target upstream of both pathways.

Reactive Oxygen Species (ROS)

Emerging evidence suggests that EMPA attenuates oxidative stress in cardiomyocytes through mechanisms independent of glucose lowering and SGLT2 inhibition. Experimental studies have demonstrated reductions in intracellular and mitochondrial ROS levels following EMPA treatment in models of diabetes and heart

failure (Cai et al. 2024; S. Chen et al. 2022; X. Li et al. 2021). These effects have been linked to improvements in mitochondrial function, reduced activation of redox-sensitive kinases, and suppression of pro-oxidant signaling pathways. Importantly, EMPA-mediated reductions in ROS have been observed on time scales inconsistent with transcriptional remodeling, suggesting modulation of acute signaling or enzymatic activity (Benedetti R et al. 2025; Cai et al. 2024; X. Li et al. 2021). Given the concerted relationship between ROS and CaMKII activation, attenuation of oxidative stress represents a plausible upstream mechanism by which EMPA indirectly suppresses CaMKII activity and downstream electrophysiological remodeling. These redox-dependent alterations may represent a mechanistic bridge between metabolic stress and downstream electrophysiological and contractile dysfunction observed in diabetic hearts.

The Late Sodium Current (I_{NaL})

Pathological enhancement of the I_{NaL} is a well-established contributor to prolonged APD, impaired relaxation, and arrhythmogenesis in HF (Makielski 2016). EMPA was reported to inhibit the I_{NaL} in a TAC-induced HFrEF murine model (Philippaert et al. 2021). EMPA treatment has also demonstrated potential antiarrhythmic effects that could be associated with attenuation of the I_{NaL} (Hegyi, Mira Hernandez, Ko, et al. 2022; Hegyi, Mira Hernandez, Shen, et al. 2022).

A clinically relevant concentration of EMPA (1 μ M) has been associated with the reversal of both the upregulated I_{NaL} and arrhythmogenic APD changes seen in a translational HFpEF mouse model (Hegyi, Mira Hernandez, Shen, et al. 2022). The attenuation of the I_{NaL} , reductions in APD prolongation, and decreased short-term

variability of APD (STV) effects were observed only following prolonged cellular pretreatments (approx 4hrs) (Hegyi, Mira Hernandez, Ko, et al. 2022; Hegyi, Mira Hernandez, Shen, et al. 2022). Notably, EMPA's effects on the I_{NaL} appear to be context-dependent and indirect. Unlike classical sodium channel blockers, EMPA does not acutely suppress peak sodium current and does not rapidly inhibit I_{NaL} (Hegyi, Mira Hernandez, Shen, et al. 2022; Schneider et al. 2024). Instead, EMPA-induced reductions in I_{NaL} emerge following prolonged exposure, consistent with modulation of upstream signaling pathways rather than direct channel blockade (Hegyi, Mira Hernandez, Shen, et al. 2022). Strikingly, EMPA's delayed effect was mimicked by CaMKII inhibition as well as by suppression of ROS levels (Hegyi, Mira Hernandez, Shen, et al. 2022; Lu et al. 2020). Since CaMKII both activates *and* is activated by ROS production (Erickson et al. 2008; Lu et al. 2020), inhibition of the I_{NaL} could be mediated by a reduction in oxidative stress and/or CaMKII activation. This indicates that EMPA may act in cardiac myocytes via the pathway illustrated in **Figure 4**. Together, an interconnected signaling network linking metabolic stress to electrophysiological remodeling, hyperglycemia–ROS–CaMKII– I_{NaL} pathway, provides a possible point in where EMPA could be acting and having the widespread cardiac effects seen.

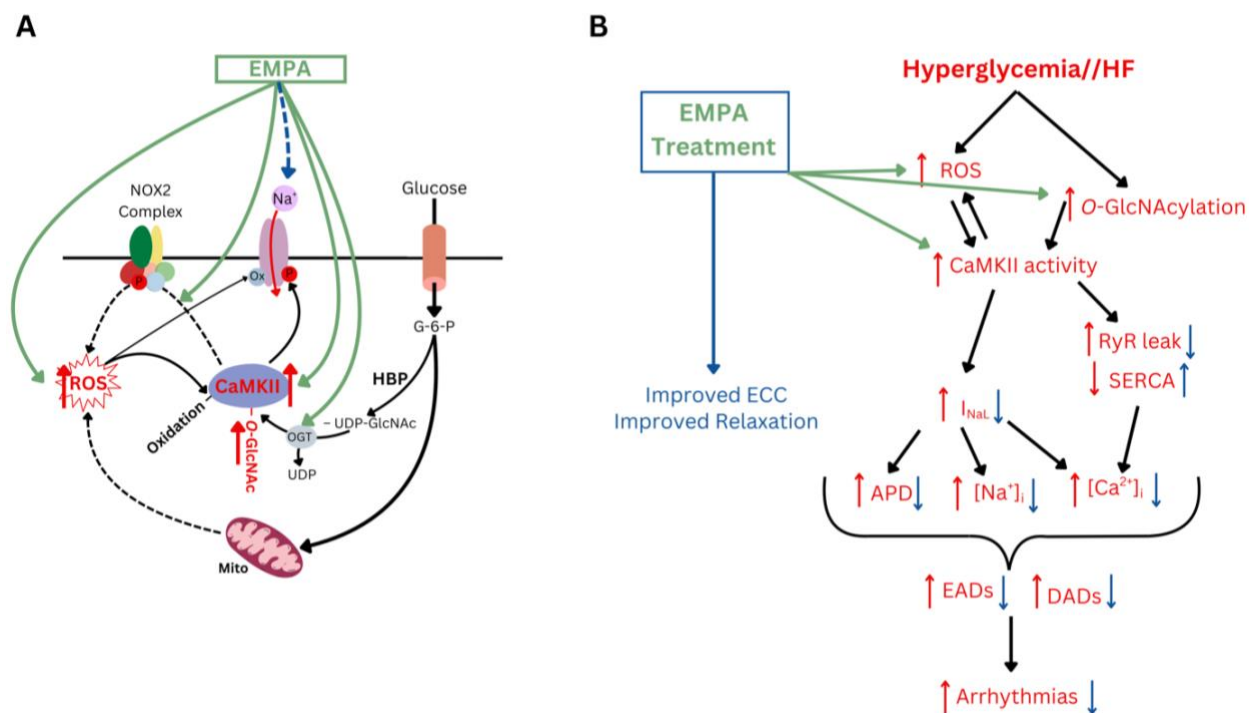


Figure 4. Schematic of molecular changes in heart failure and the proposed pathway where empagliflozin may intervene.

A) Within the CaMKII—ROS— I_{NaL} pathway where we propose EMPA may directly target to have its downstream effects on the I_{NaL} . **B)** Schematic illustrating key ECC changes seen in disease (in red), known improvements seen with EMPA-tx (illustrated by blue arrows), and the pathway proposed being upstream of these outcomes (green). Redrawn and adapted from Hegyi et al., 2019; Hegyi et al., 2021; Hegyi & Bers, 2023.

Scope of the Dissertation

SGLT2 inhibitors, particularly EMPA, have emerged as effective therapies for reducing HF risk and improving cardiovascular outcomes in patients with and without diabetes. These benefits occur rapidly and cannot be fully explained by improvements in glycemic control alone, suggesting direct cardiac or cellular mechanisms. Emerging

evidence indicates that EMPA may modulate oxidative stress, intracellular ion handling, and signaling pathways central to cardiomyocyte dysfunction.

A key proposed mechanism underlying hyperglycemia-associated cardiac dysfunction is the ROS–CaMKII– I_{NaL} signaling axis. In this pathway, hyperglycemia increases ROS, which activates CaMKII and enhances I_{NaL} (Hegyi, Pölönen, et al. 2021; Hegyi and Bers 2023). This cascade promotes intracellular calcium dysregulation and contributes to impaired contractile function and arrhythmogenesis. In HF, this signaling axis is further amplified by feed-forward interactions involving sodium and calcium overload, mitochondrial stress, and RyR instability.

Within this framework, EMPA has been shown to improve calcium handling and reduce arrhythmia susceptibility in diabetic cardiomyocytes (Kadosaka et al. 2023). It has also been associated with reduced ROS levels (Cowie and Fisher 2020) and decreased CaMKII activity in cardiac disease (Mustroph et al. 2018). In HF models, EMPA reduces I_{NaL} , although with delayed kinetics consistent with indirect upstream modulation rather than direct channel blockade (Hegyi, Mira Hernandez, Shen, et al. 2022). Together, these findings support a model in which EMPA acts primarily through redox and CaMKII-dependent signaling pathways that converge on I_{NaL} regulation.

Collectively, these data support a unified mechanism in which EMPA disrupts a feed-forward ROS–CaMKII– I_{NaL} signaling network that links metabolic stress to electrical and calcium-handling abnormalities in cardiomyocytes. This axis integrates redox signaling, ion homeostasis, and electrophysiological remodeling and is relevant to both diabetes and HF associated cardiomyopathy.

Accordingly, the central hypothesis of this dissertation is that EMPA attenuates hyperglycemia-induced cytosolic ROS production in cardiomyocytes that involves CaMKII activation and downstream enhancement of late sodium current in both control and HFpEF hearts. This effect is hypothesized to arise through coordinated modulation of upstream metabolic and redox pathways. EMPA may reduce cellular glucose uptake and thereby limit metabolic drive into stress-responsive pathways such as the hexosamine biosynthetic pathway, reducing O-GlcNAc dependent regulation of CaMKII. In parallel, reduced CaMKII activation may limit stimulation of NOX2, while EMPA may also directly or indirectly suppress NOX2-derived ROS production. In addition, enhancement of endogenous antioxidant systems, including superoxide dismutase activity and broader ROS scavenging capacity, may further contribute to restoration of redox balance.

To test this framework, this dissertation uses the genetically encoded redox biosensor Grx1-roGFP2 to measure real-time cytosolic redox dynamics in isolated adult mouse cardiomyocytes from control and HFpEF models under acute hyperglycemic conditions, with and without EMPA. These studies are complemented by pharmacological interventions and enzyme activity assays to interrogate key redox-regulatory and metabolic pathways. Together, this approach defines how EMPA modulates oxidative stress signaling and downstream functional consequences in cardiomyocytes under metabolic challenge.

CHAPTER 2: MATERIALS AND METHODS

Animal Models

All cells were obtained from adult mice (25 wks), including Grx1-roGFP2 (targeted either to the cytosol or mitochondria) (Swain et al. 2016) and WT C57BL/6J (Jackson Lab) mice. For redox measurements all cells were obtained from mouse Grx1-roGFP2 transgenic mouse lines with sensors localized either to the mitochondria or the cytosol (Swain et al. 2016; Lu et al. 2020). For the HFpEF mouse model mice were fed a high fat diet (HFD; 60% kcal fat) and administered N^ω-nitro-L-arginine methyl ester (L-NAME; 0.5g/L) via water starting at 10 wks of age for 15 wks to induce HFpEF (HFD+L-NAME) (Schiattarella et al. 2019; Hegyi et al., 2026; Roh et al. 2022). The HFpEF model was utilized in the Grx1-roGFP2 mice (Swain et al. 2016) and both mitochondrial- and cytosolic-targeted Grx1-roGFP2 mice were used for all HF measurements. These were compared to the appropriate controls [normal chow (16 kcal% fat)-fed] and vehicle-treated. All surgical procedures, echocardiographic follow-ups, morphometric analysis, and cell isolation were performed by experienced technicians and senior researchers in the laboratory. For enzyme activity assays wild type (C57BL/6J) mouse whole hearts were used to make tissue lysates (described in Enzyme Activity Assay Kits section). All lysates were made according to each specific kit.

Cardiomyocyte Isolation

Cardiac ventricular myocytes were isolated using previously described methods (Pereira et al. 2013) which was approved by the UC Davis Institutional Animal Care and Use Committee (IACUC). Cardiac myocytes were isolated at 25 wks of age from Grx1-

roGFP2 mice were obtained by enzymatic digestion of mouse left ventricles with type II collagenase (89 mg) (Worthington Biochemical Company, Cat#LS004177; 290 units/mg dry weight) and type XIV protease (4 mg) (from streptomyces griseus; Sigma-Aldrich, Cat#P5147; ≥ 3.5 units/mg powder). Heparin injected mice (400 U/kg body weight) were briefly anesthetized with isoflurane (for induction 5% and for maintenance 3–3.5%) to achieve verified deep insensate anesthesia prior to heart excision. Following excision, the heart is cannulated via the aorta and then perfused on a constant flow Langendorff apparatus (37°C) for 15-20 min with 50 mL MEM (135 mM NaCl, 4.7 mM KCl, 0.6 mM KH_2PO_4 , 0.6 mM Na_2HPO_4 , 1.2 mM MgSO_4 , 20 mM HEPES-free acid, 30 mM taurine, 10 μM CaCl_2 with enzymes and 100% O_2 at pH = 7.4). Then gentle pipetting was used to disaggregate myocytes. Subsequently, myocytes were filtered through nylon mesh and sedimented repeatedly following step increases in free $[\text{Ca}^{2+}]$ (0.125, 0.25 and 0.5 mmol/L). Prior to initiation of experiments, myocytes are kept in normal Tyrode (NT) solution (ingredients listed in **Table 1**; pH = 7.4) at room temperature (22–23 °C). All experiments were then performed at room temperature (RT) (22–23°C) with pH 7.4.

Redox Sensor Measurements

Changes in intracellular redox state were measured using the genetically encoded biosensor Grx1-roGFP2. This construct consists of glutaredoxin-1 (Grx1) fused to a redox-sensitive green fluorescent protein (roGFP2), allowing reversible detection of the glutathione redox potential (GSH/GSSG) in living cells (Swain et al. 2016). The roGFP2 moiety contains engineered cysteine residues that undergo disulfide bond formation upon oxidation, resulting in a shift in excitation properties. The Grx1 domain facilitates rapid equilibration between the sensor and the endogenous glutathione redox

couple, enabling specific reporting of glutathione redox balance rather than nonspecific ROS accumulation (Swain et al. 2016). This approach allows for sensitive detection of dynamic changes in redox homeostasis in response to acute metabolic perturbations and pharmacological treatment.

Cardiomyocytes expressing Grx1-roGFP2 were imaged using dual-excitation fluorescence at 405 nm and 488 nm, and emission was detected at >530 nm. Isolated, intact cardiac myocyte redox measurements were performed using a confocal 2-dimensional scanning microscopy with (Nikon, x40 objective) ≈ 20 nW laser power to minimize laser induced artifacts. Images were acquired every 30 seconds for 30 minutes with constant perfusion that switched between solutions. The ratio of fluorescence intensity (405 nm/488 nm) was calculated to provide a ratiometric measure of cellular redox state, independent of sensor expression level, photobleaching, or cell morphology.

To gain a better mechanistic understanding of EMPA's advantageous effects in cardiac myocytes we tested how EMPA affects ROS levels at baseline (normal glucose 5.5 mM glucose) and in response to acute hyperglycemia (HiGLU; 30 mM glucose), which is known to increase cytosolic ROS levels in cardiomyocytes (Lu et al. 2020). As the hyperglycemia is used to model the diabetic heart, we also performed these experiments in a two-hit HFpEF mouse model, using a nitric oxide synthase inhibitor, L-NAME, in combination with a HFD. We also tested changes in baseline and HiGLU cytosolic ROS levels when cardiomyocytes were treated with a CaMKII inhibitor, autocamtide-2-related inhibitory peptide that is myristoylated to allow cellular uptake

(AIP; Anaspec Cat# AS-64929; 1 μ M for 1hr), to test if EMPA treatment had similar effects to CaMKII inhibition.

Freshly isolated intact myocytes were first equilibrated with the normal glucose normal tyrodes (NT) solution (Table 1) and incubated for ~1 hour with either dimethyl sulfoxide (DMSO; 0.01%) as a control, EMPA (1 μ M), or AIP (1 μ M). Measurements began with cells being perfused at 1 mL/min with the normal glucose NT solution, to gather a baseline measurement of ROS levels, this was followed by the HiGLU NT solution to measure the rate of increase of ROS and the difference between the treatment groups. This was followed by H₂O₂ (100 μ M) and DTT (1 mM) perfusion, to achieve maximal and minimal signals for calibrations. The timeline of experiments is represented in Figure 5. All experiments were done at room temperature (22-23°C) with a pH 7.4.

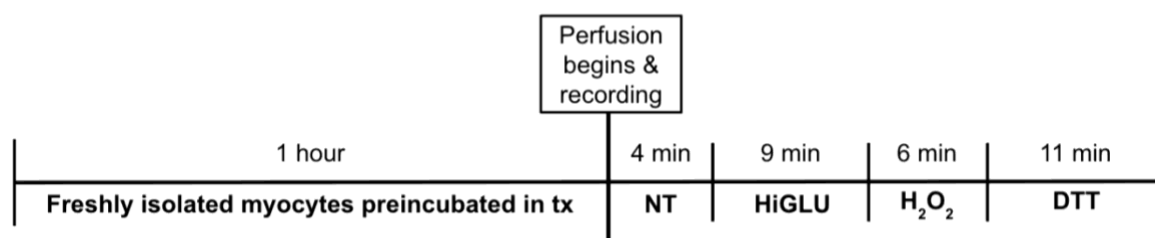


Figure 5. Timeline of incubation and perfusion of redox experiments.

Freshly isolated myocytes get preincubated in NT and its treatment (EMPA or control) for 60 minutes. Perfusion with NT starts and then the 30 minute recording begins. Changes in perfusion occur according to the timeline.

Solutions

NT contained (mmol/L) 140 NaCl, 6 KCl, 10 HEPES, 1 MgCl₂, 1.8 CaCl₂, 5.5 glucose, and 24.5 mannitol (Table 1). NT with 5.5mmol/L glucose was used to mimic physiological normal glucose levels. The normal glucose NT contains 24.5mmol/L of mannitol to account for osmolality differences between the glucose solutions. This is consistent with prior observations from our lab (Lu et al. 2020). The HiGLU solution is the NT with changes in the glucose and mannitol content. The HiGLU NT has 30 mmol/L glucose and no mannitol added. Both solutions have a pH 7.4 and were used at room temperature (22-23°C).

Table 1. Normal Tyrode (NT) components and concentrations.

Components	Normal Glucose Concentrations (mM)	High Glucose Concentrations (mM)
NaCl	140.0	140.0
KCl	4.0	4.0
CaCl₂ x 2H₂O	1.8	1.8
MgCl₂ x 6H₂O	1.0	1.0
HEPES	5.0	5.0
Na-HEPES	5.0	5.0
Glucose	5.5	30.0
Mannitol	24.5	0.0

E_{GSH} Calculation

Sensor calibration was performed according to previous studies with these sensors (Gutscher et al. 2008). Oxidation difference (OxD) and redox potential (E_{GSH}) calculations were done as described in previous studies, and are also provided below (Gutscher et al. 2008).

Equation 1. Oxidation difference

OxD_{roGFP2}:

$$\text{OxD}_{\text{roGFP2}} = \frac{I_{405} \times I_{488\text{red}} - I_{405\text{red}} \times I_{488}}{I_{405} \times I_{488\text{red}} - I_{405} \times I_{488\text{ox}} + I_{405\text{ox}} \times I_{488} - I_{405\text{red}} \times I_{488}}$$

Where I_{405} and I_{488} are the intensities of fluorescence measured at these excitation wavelengths with the subscripts _{red} and _{ox} referring to the maximally reduced (DTT) and oxidized (H₂O₂) values measured at the end of each experimental run.

Equation 2. Glutathionine redox potential

E_{GSH}:

$$E_{\text{GSH}} = E_{\text{roGFP2}} = E^{\circ}_{\text{roGFP2}} - \frac{RT}{2F} \ln \left(\frac{1 - \text{OxD}}{\text{OxD}} \right)$$

Where OxD is the OxD_{roGFP2} calculated in Equation 1, F is the Faraday constant (96,485 C/mol), R is the universal ideal gas constant (8.314 J/mol*K), T is the absolute temperature in kelvins (293.15 K). OxD is used to calculate the probe redox potential, where E_{roGFP2} is -280 mV (or -0.280 V). Assuming that the probe and the glutathione redox couple are in equilibrium, the E_{GSH} = E_{roGFP2}.

Analysis of the fluorescence intensities at the excitation intensities (405 nm and 488 nm) after stimulation with H₂O₂ (maximum oxidation response) and dithiothreitol

(DTT; maximum reduction response) in basal conditions is needed to determine E_{GSH} . To calculate OxD, emission intensities (I) are obtained from the measurements at 405 nm and 488 nm. OxD is then used in the probe redox potential calculation where E'_{roGFP2} is -280 mV. Under the assumption that the glutathione redox couple and the probe are in equilibrium then E_{GSH} should equal E_{roGFP2} ($E_{\text{GSH}}=E_{\text{roGFP2}}$).

Pharmacological Treatments and Assay Kits

Enzyme activity assay kits were used with pharmacological interventions to measure whether EMPA had a direct effect on the enzyme activity of NADPH oxidase 2 (NOX2), O-GlcNAc transferase (OGT), and O-GlcNAcase (OGA). EMPA's effects were compared to change in activity with inhibitors known to specifically inhibit those enzymes.

The O-GlcNAcase (OGA) assay kit was purchased from Biomedical Research Service Center at SUNY Buffalo (Lee, T; Cat.# E-130). The UDP-GlcNAc 6-phosphate (UDP-GlcNAc6P) Glycosyltransferase assay kit, with UDP-sugar substrate GlcNAc, was purchased from Promega (Promega; Cat.# V6971). NADH Oxidase (NOX) Activity assay kit was purchased from Elabscience (Elabscience®; Cat.# E-BC-K806-MNADH). Pierce BCA Protein assay kit was purchased from ThermoFisher Scientific (Thermo Scientific™; Cat.# 23225). All kits were used according to the manufacturer instructions.

Freshly excised hearts were used as tissue lysates following the protocol of each specific kit. Dose response curves were done for all enzyme activity kits. Tissue lysates were pre-treated with either EMPA or an enzyme-specific inhibitor and incubated for 1-hour. The inhibitor used was based on which enzyme activity kit was being used.

NADPH Oxidase (NOX)

For NADPH oxidase (NOX) GP91-ds-tat (Anaspec; Cat.# AS-63818) was the inhibitor used. NOX measurements were done with a variety of EMPA concentrations, a maximum NOX2 inhibitor concentration (10 μ M GP91-ds-tat), and a combination of EMPA concentrations with the maximum GP91 concentration. We found that maximum inhibition of NOX2 was seen at a GP91 concentration of 10 μ M. This kit measures NOX activity and is not specific to NOX2 so we designed experiments to determine whether EMPA has a similar effect to a NOX2 inhibitor. The treatment combining both EMPA with maximum concentration of NOX2 inhibitor (GP91-ds-tat) was used to see if there was any additional inhibition with EMPA which would point to EMPA having effects on NOX isoforms other than NOX2.

Concentrations used were as follows:

EMPA: 3, 1, 0.3, 0.1, 0 μ M

GP91-ds-tat (NOX2 inhibitor): 10 μ M

EMPA + 10 μ M GP91: 3, 1, 0.3, 0.1 μ M

O-GlcNAc Transferase (OGT)

For O-GlcNAc transferase (OGT) activity OSMI-1 (MedChemExpress; Cat.# HY-119738) was the inhibitor used. Per the Promega OGT activity kit recommended recombinant Human O-GlcNAcTransferase/OGT Protein, CF (R&D Systems; Cat.# 8446-GT) and O-linked GlcNAc transferase (OGT) Substrate (Anapec; Cat.# AS-63726) were used for the OGT protein and OGT substrate for all experiments. All experiments followed Promega protocol and for the dose response curve the 'Determining IC₅₀

Values of Glycosyltransferase Inhibitors' protocol was used (UDP-Glo™

Glycosyltransferase Assay; Cat.# V6971).

Concentrations used were as follows:

EMPA: 500, 166.7, 56.6, 18.5, 6.2, 2.1, 0.7, 0.2, 0.08, 0.025, 0 μ M

OSMI-1: 500, 166.7, 56.6, 18.5, 6.2, 2.1, 0.7, 0.2, 0.08, 0.025, 0 μ M

O-GlcNAcase (OGA)

For O-GlcNAcase (OGA) activity ThiametG (MedChemExpress; Cat.# HY-12588) was used as the inhibitor. Freshly excised hearts were homogenized and prepared as tissue lysates using a detergent-based lysis buffer. OGA activity was measured using a colorimetric assay adapted from a commercially available kit (Biomedical Research Service Center, University at Buffalo). This assay is based on the enzymatic cleavage of the synthetic substrate *p*-nitrophenyl- β -N-acetylglucosaminide by OGA, producing *p*-nitrophenol. Upon addition of NaOH to terminate the reaction, *p*-nitrophenol is ionized, generating a yellow chromophore that can be quantified by absorbance at 405 nm. Briefly, tissue lysates were incubated with assay solution containing the substrate at 37°C for 30-60 minutes. The reaction was terminated by addition of NaOH, and absorbance was measured at 405 nm using a plate reader. OGA activity was calculated from the rate of *p*-nitrophenol formation and normalized to protein concentration. OGA activity experiments were performed after lysates were incubated for 1hr with inhibitors.

Concentrations used were as follows:

EMPA: 3, 1, 0.3, 0.1, 0.03, 0.01, 0 μ M

ThiametG (OGA inhibitor): 10, 3, 1, 0.3, 0.1, 0.03, 0.01, 0 μ M

Statistical Analysis

Data is presented as mean $\pm$ SEM. Normality of the data was assessed using the Shapiro-Wilk test. Statistically significant differences of normally distributed data were determined using 2 tailed student t-test (paired or unpaired) or one-way ANOVA, when applicable. One-way ANOVA was followed by Šídák's multiple comparisons test. Calibrations of the redox sensor data were fit using a one-site binding (Michaelis-Menten-type) model in GraphPad Prism to relate fluorescence ratio to sensor oxidation state. Dose response curves were performed on GraphPad and used a non-linear regression curve fit. Differences were considered significant if $P < 0.05$. Exact P-values are noted in figures or results section. Redox experiments used ImageJ for image processing. Data analysis was performed on GraphPad Prism 10. In cell experiments N represents mouse numbers while n represents cell numbers. For assay kits n represents the number of mouse hearts used and is the average of triplicates for each mouse heart.

CHAPTER 3: RESULTS

Hyperglycemia-Induced ROS

To measure hyperglycemia-induced ROS production, we used transgenic mice with cardiac-specific expression of the genetically encoded ratiometric redox sensor Grx1-roGFP2 targeted to either the cytosol (cyto-Grx1-roGFP2) or mitochondria (mito-Grx1-roGFP2). In cardiomyocytes, cytosolic expression appears diffuse throughout the cell, whereas mitochondrial targeting produces a striated, punctate pattern consistent with mitochondrial localization. These distinct subcellular localization patterns have been previously validated in cardiomyocytes expressing these sensors (Lu et al., 2020). Representative images of cyto- and mito-Grx1-roGFP2-expressing cardiomyocytes during solution perfusion are shown in **Figure 6A**.

The Grx1-roGFP2 sensors were calibrated in our imaging system (**Figure 6B**). The calibration of the sensors (**Figure 6B**) demonstrated similar EC_{50} of both cyto-roGFP and mito-roGFP (23.81 and 43.62 μ M H_2O_2 , respectively). Traces seen in **Figures 6C and 6D** show intact cardiomyocytes perfused with normal glucose followed by HiGLU perfusion in both mito- and cyto-Grx1-roGFP2. HiGLU conditions had no significant effect on mitochondrial ROS production (**Figure 6C**), while a significant increase in cytosolic ROS production occurred (**Figure 6D**).

Dynamic and specific glutathione redox potential (E_{GSH}) information can be provided by the Grx1-roGFP2 sensor for intact cardiomyocytes during the HiGLU treatment. The mitochondrial glutathione redox potential Mean $\pm$ SEM was -285 ± 4.79 mV at baseline measurements and by the end of HiGLU perfusion it was -289 ± 7.52 mV (**Figure 6C**). While baseline cytosolic E_{GSH} Mean $\pm$ SEM was -310 ± 6.15 mV and after

HiGLU it was $-263 \pm 2.69 \text{ mV}$ (**Figure 6D**), further confirming no significant change between normal and HiGLU for the mito-Grx1-roGFP2 sensor. Further, the cyto-Grx1-roGFP2 sensor showed a significant increase in E_{GSH} levels from baseline to HiGLU ($P < 0.0001$) (**Figure 6D**).

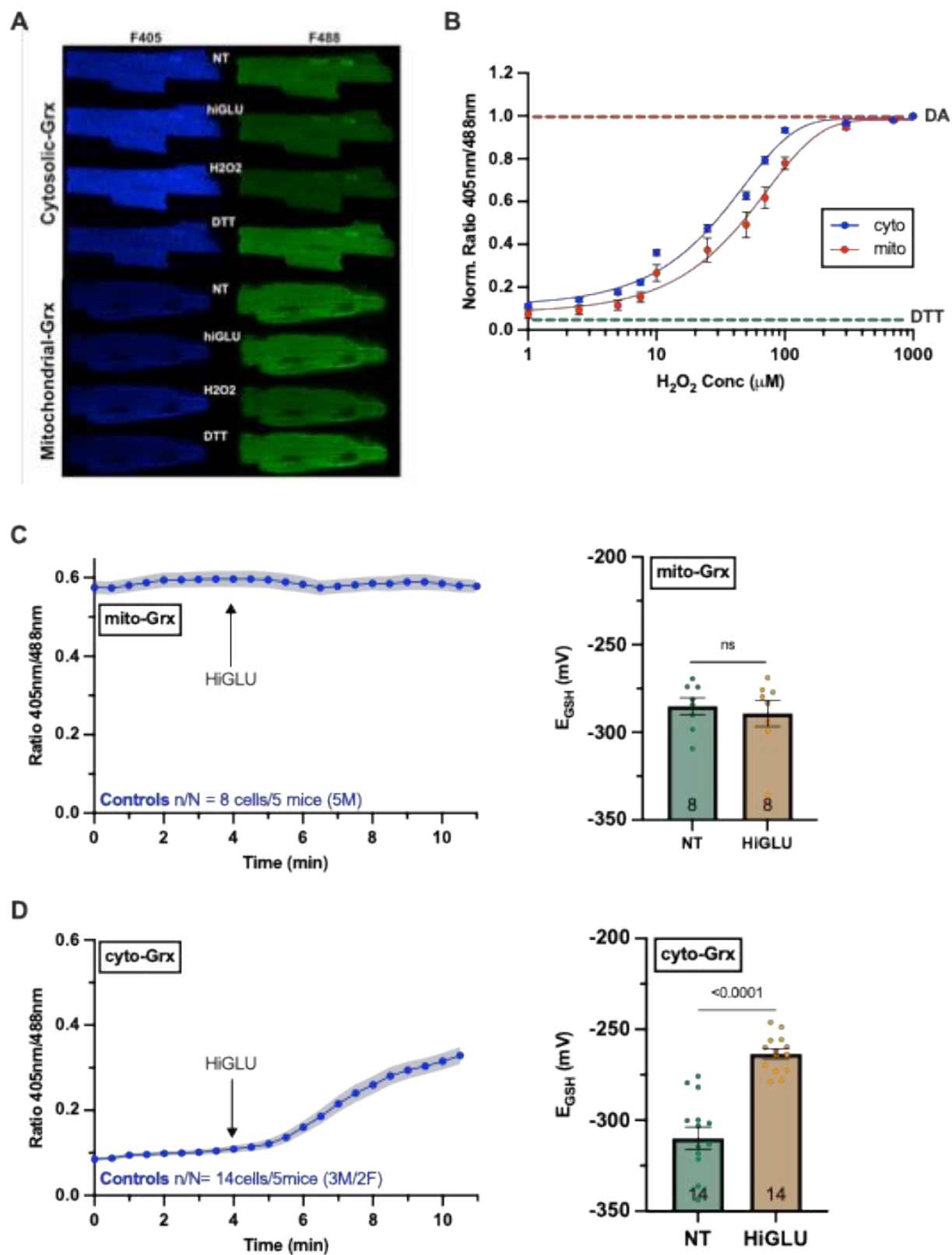


Figure 6. Example of GRX1-roGFP2 sensor.

A) Representative confocal images of myocytes expressing cytosolic and mitochondrial-Grx redox sensors. **B)** In situ redox sensors calibration. **C)** Representative traces of mito-Grx ratiometric and converted E_{GSH} in response to HiGLU. **D)** Representative traces of cyto-GRX ratiometric signals and after conversion to E_{GSH} in response to HiGLU. (ns: $P > 0.05$). One-way ANOVA comparing all NT groups to each other, all HiGLU groups to each other, and each NT groups to its corresponding HiGLU. Unpaired t-test was used for E_{GSH} comparisons. Animal and cell numbers are shown in figure.

Empagliflozin and Hyperglycemia-Induced ROS

To test whether EMPA had an effect on hyperglycemia-induced ROS production we preincubated both mito- and cyto-Grx1-roGFP2 mouse cardiomyocytes in a physiological concentration of 1 μ M EMPA for 1 hour and compared them to cells untreated during that time period.

Figure 7A-B shows that in mito-Grx1-roGFP2 cardiomyocytes acute hyperglycemia showed no significant increase in ROS production regardless of treatment. The mitochondrial glutathione redox potential confirmed that there was no significant change between normal and high glucose in the mito-Grx1-roGFP2 sensor in both treatment types (**Figure 7C**). **Figure 8A-B** shows that in cyto-Grx1-roGFP2 cardiomyocytes acute hyperglycemia increased ROS production and that pretreatment with EMPA (1 μ M for 1-hr) was able to significantly reduce the rise of hyperglycemia-induced ROS. Notably, EMPA pretreatment did not alter the signal of either sensor in control 5.5 mM glucose condition.

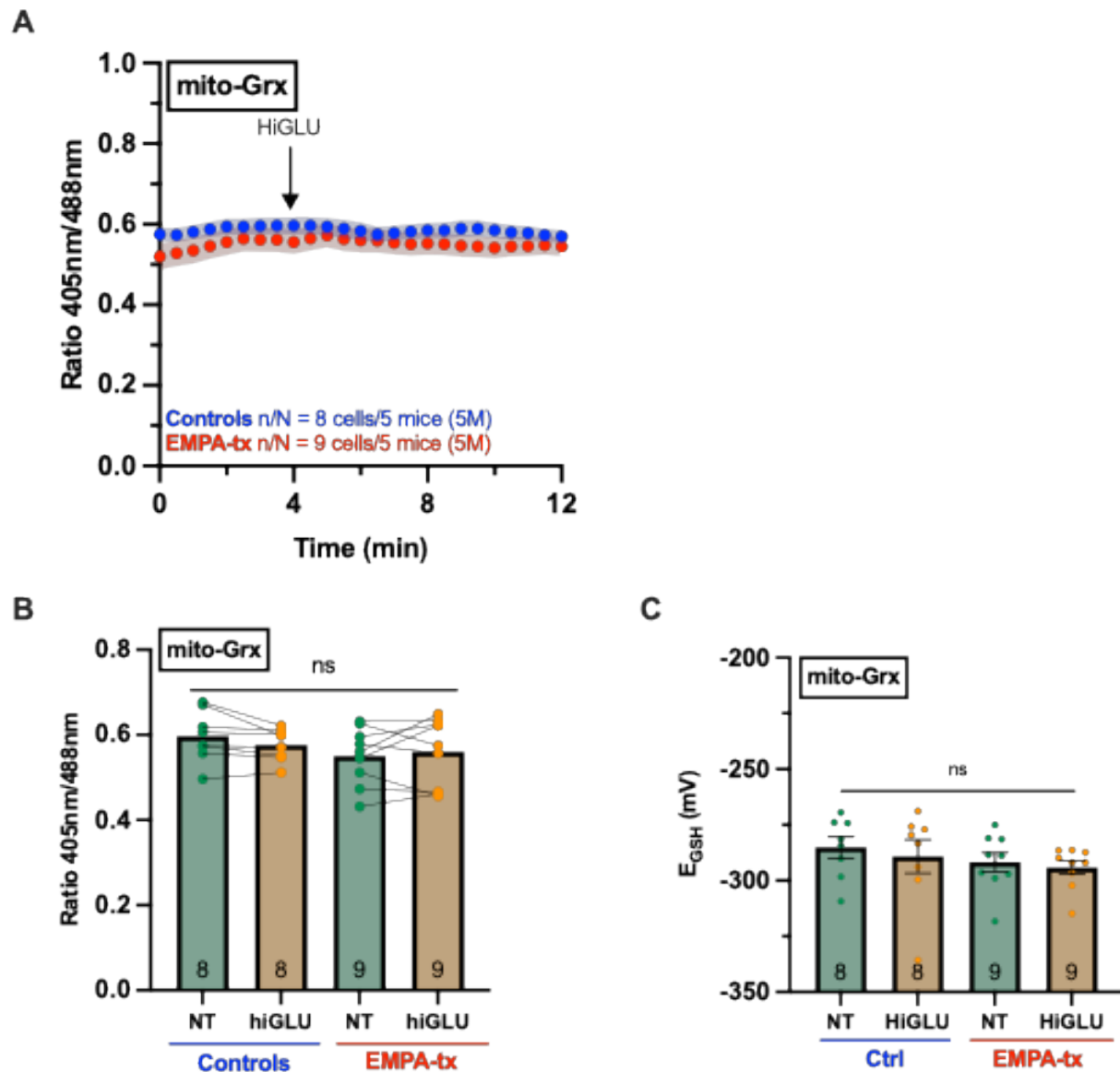


Figure 7. Mitochondrial ROS levels for controls vs. empagliflozin-treated cardiac myocytes exposed to acute hyperglycemia.

A) Time course trace comparison of control vs. empagliflozin-treated cardiomyocytes.

B) Final mitochondrial ROS levels for normal and HiGLU perfusion in control and

empagliflozin-treated myocytes. **C)** Redox potential of each treatment type at baseline

and HiGLU. ($P < 0.05$). One-way ANOVA comparing all NT groups to each other, all

HiGLU groups to each other, and each NT groups to its corresponding HiGLU. One-way ANOVA was followed by Šídák's multiple comparisons test. Animal numbers are shown in figure panel A and cell numbers are shown in panels B&C.

There was a significant difference in both cytosolic treatment groups between baseline and high glucose exposure ($P < 0.0001$ controls; $P = 0.0002$ EMPA-tx) and the EMPA treated myocytes had significantly lower ROS levels under high glucose in comparison to the controls ($P < 0.0001$) (**Figure 8B**). The cytosolic glutathione redox potential confirmed that there was significant change between normal and high glucose in the cyto-Grx1-roGFP2 sensor in both treatment types (Controls $P < 0.0001$; EMPA $P = 0.0216$) and the hyperglycemia-induced E_{GSH} was significantly lower in the EMPA treated myocytes compared to controls ($P = 0.0348$) (**Figure 8C**).

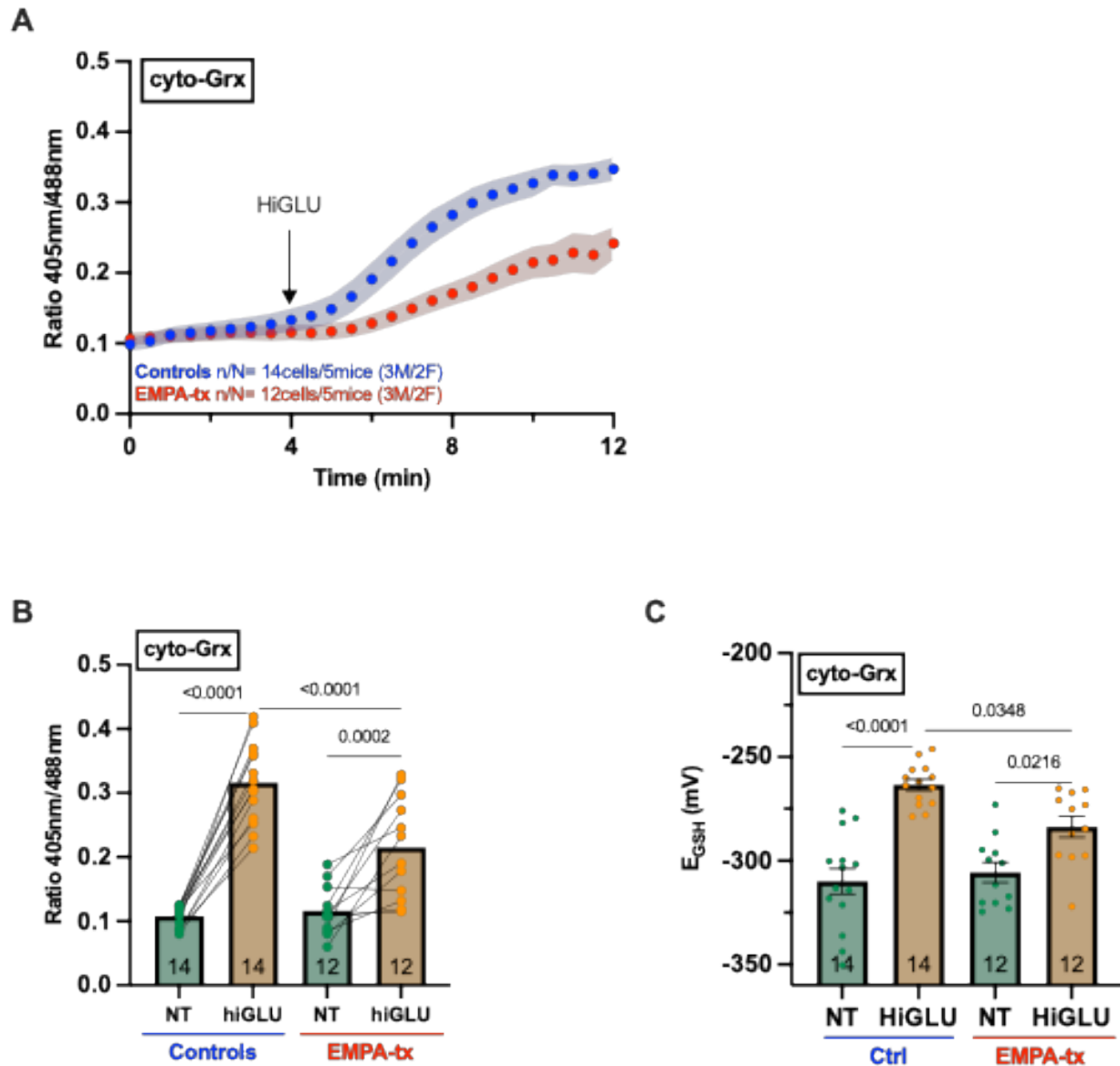


Figure 8. Cytosolic ROS levels for control vs. empagliflozin-treated cardiac myocytes exposed to acute hyperglycemia.

A) Time course trace comparison of control vs. empagliflozin-treated cardiomyocytes.

B) Final cytosolic ROS levels for normal and HiGLU perfusion in control and

empagliflozin-treated myocytes. **C)** Redox potential of each treatment type at baseline

and HiGLU. ($P < 0.05$). One-way ANOVA comparing all NT groups to each other, all

HiGLU groups to each other, and each NT groups to its corresponding HiGLU. One-way ANOVA was followed by Šídák's multiple comparisons test. Animal numbers are shown in figure panel A and cell numbers are shown in panels B&C.

Heart Failure and Redox

We also tested EMPA's effects on ROS in a two-hit model (HFD+L-NAME) of heart failure with preserved ejection fraction (HFpEF) in both cyto- and mito-Grx1-roGFP2 mice. An illustration of the induction of HFpEF via HFD+L-NAME methodology can be seen in **Figure 9A**. Echocardiographs of the HFpEF and their age-matched controls (25-weeks of age) in both mouse lines were done (**Figure 9**), and representative images are shown in **Figure 9B**. Ejection fraction (EF) was shown to be preserved (EF>50%) via echocardiographic evaluation across all groups (**Figure 9C**). In the HFD+L-NAME mice evidence of cardiac hypertrophy was seen from significantly increased left ventricular (LV) remodeling index (LVRI), a ratio of LV mass (LVM) and the LV internal diameter (LVID) (**Figure 9D**). A significant difference in diastolic dysfunction between HFpEF and control groups was evident in the diastolic E/A ratio, with the HFpEF groups having significantly higher ratios indicative of diastolic dysfunction (**Figure 9E**). Further, HFpEF groups had significantly higher LV filling pressures (diastolic E/e' ratio) in comparison to the control groups (**Figure 9F**). Overall, HFpEF groups had marked diastolic dysfunction with preserved systolic function (**Figure 9C-F**), confirming the development of HFpEF.

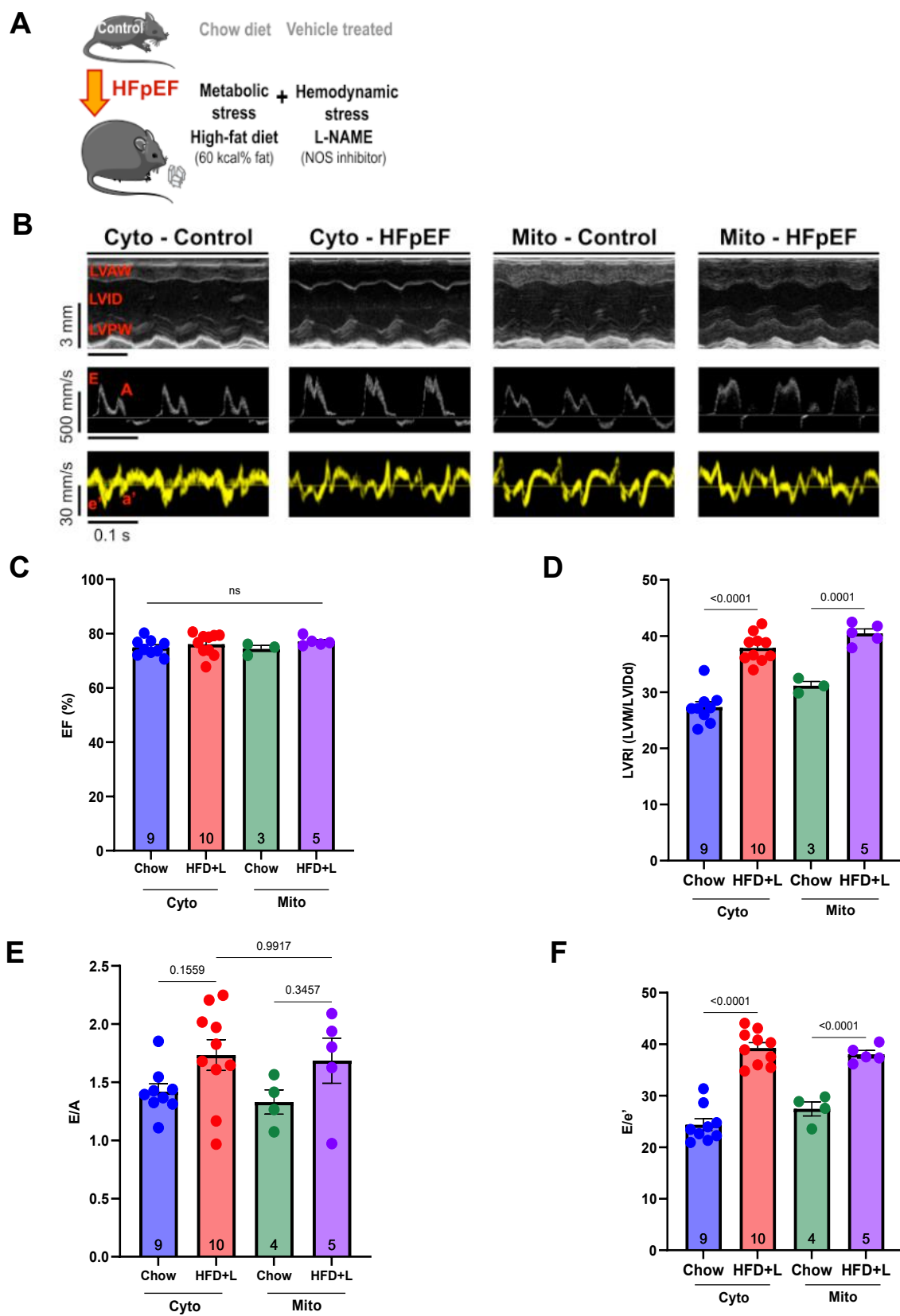


Figure 9. Echocardiographs and corresponding data of heart failure and age-

matched control mice both cyto- and mito-Grx1-roGFP2 lines.

Marked diastolic dysfunction with preserved systolic function in HFD+L-NAME induced HFpEF. **A)** Illustration of methodology of inducing HFpEF with HFD+L-NAME. **B)** Representative LV M-mode, flow, and tissue Doppler echocardiographic images taken at 25-wks of age. **C)** Preserved EF (Mean $\pm$ SEM: Cyto-chow 74.9 $\pm$ 1.0; Cyto-HFD+L 76.1 $\pm$ 1.3; Mito-chow 74.4 $\pm$ 1.2; Mito-HFD+L 77.1 $\pm$ 0.7) and **D)** significantly increased LVRI in HFD+L-NAME, or HFpEF, groups (Mean $\pm$ SEM: Cyto-chow 27.3 $\pm$ 0.1; Cyto-HFD+L 37.9 $\pm$ 0.8; Mito-chow 31.1 $\pm$ 0.8; Mito-HFD+L 40.5 $\pm$ 0.8). **E)** Significant differences between HFpEF and control groups, with increased diastolic dysfunction (E/A Mean $\pm$ SEM: Cyto-chow 1.4 $\pm$ 0.1; Cyto-HFD+L 1.9 $\pm$ 0.1; Mito-chow 1.3 $\pm$ 0.1; Mito-HFD+L 1.9 $\pm$ 0.1) and **F)** LV filling pressures in HFpEF groups (E/e' Mean $\pm$ SEM: Cyto-chow 24.4 $\pm$ 1.2; Cyto-HFD+L 39.3 $\pm$ 1.0; Mito-chow 27.4 $\pm$ 1.4; Mito-HFD+L 38.1 $\pm$ 0.7). One-way ANOVA was followed by Šídák's multiple comparisons test. Animal numbers are shown in figure. LVAW: left ventricular atrial wall; LVID: left ventricular internal diameter; LVPW: left ventricular posterior wall; HFD: high fat diet; HFpEF: heart failure with preserved ejection fraction; EF: ejection fraction; LV: Left ventricle; LVRI: LV remodeling index LVM: left ventricular mass; E/A: ratio between mitral E wave and A wave (diastolic dysfunction); E/e': ratio between mitral E wave and e' wave (LV filling pressure).

Mitochondrial Redox

We performed further redox experiments, utilizing the mito-Grx1-roGFP2 mice, to study changes in mitochondrial ROS production in HFpEF and observe EMPA's effects. In myocytes expressing mitochondrial roGFP2, neither HiGLU nor HFpEF (in combination with HiGLU) caused a significant increase in mitochondrial ROS levels

(HiGLU Mean $\pm$ SEM: Ctrl-ctrls 0.577 $\pm$ 0.01; Ctrl-EMPA 0.561 $\pm$ 0.03; HF-ctrls 0.524 $\pm$ 0.02; HF-EMPA 0.490 $\pm$ 0.02). (**Figure 10A-B**). Notably, a comparison between the HFpEF and age-matched controls revealed that baseline (NT) ROS is significantly ($P=0.0006$) reduced in the untreated HFpEF mice (NT Mean $\pm$ SEM: HF-ctrls 0.466 $\pm$ 0.02) (**Figure 10B**). Additionally, HFpEF mouse myocytes that were treated with EMPA also had a significant ($P=0.0072$) (NT Mean $\pm$ SEM: HF-EMPA 0.442 $\pm$ 0.01) reduction in baseline ROS levels when compared to the baselines of EMPA-treated age-matched controls (NT Mean $\pm$ SEM: Ctrl-EMPA 0.551 $\pm$ 0.02) (**Figure 10B**). There was also a significant reduction ($P<0.0001$) in baseline ROS levels in EMPA treated HFpEF cardiomyocytes when compared to untreated control myocytes (NT Mean $\pm$ SEM: Ctrl-ctrls 0.597 $\pm$ 0.02) (**Figure 10B**). However, there was no significant difference in glutathione redox potential across normal or high glucose, EMPA-treated or untreated, and HFpEF or age-matched controls (NT Mean $\pm$ SEM: Ctrl-ctrls -285 $\pm$ 4.79; Ctrl-EMPA -292 $\pm$ 4.29; HF-ctrls -301 $\pm$ 4.05; HF-EMPA -304 $\pm$ 3.65) (HiGLU Mean $\pm$ SEM: Ctrl-ctrls -289 $\pm$ 7.52; Ctrl-EMPA -294 $\pm$ 3.10; HF-ctrls -292 $\pm$ 3.42; HF-EMPA -297 $\pm$ 4.36), (**Figure 10C**). This further confirmed that mitochondrial ROS still did not increase in cardiomyocytes when exposed to HiGLU nor HFpEF, regardless of EMPA treatment.

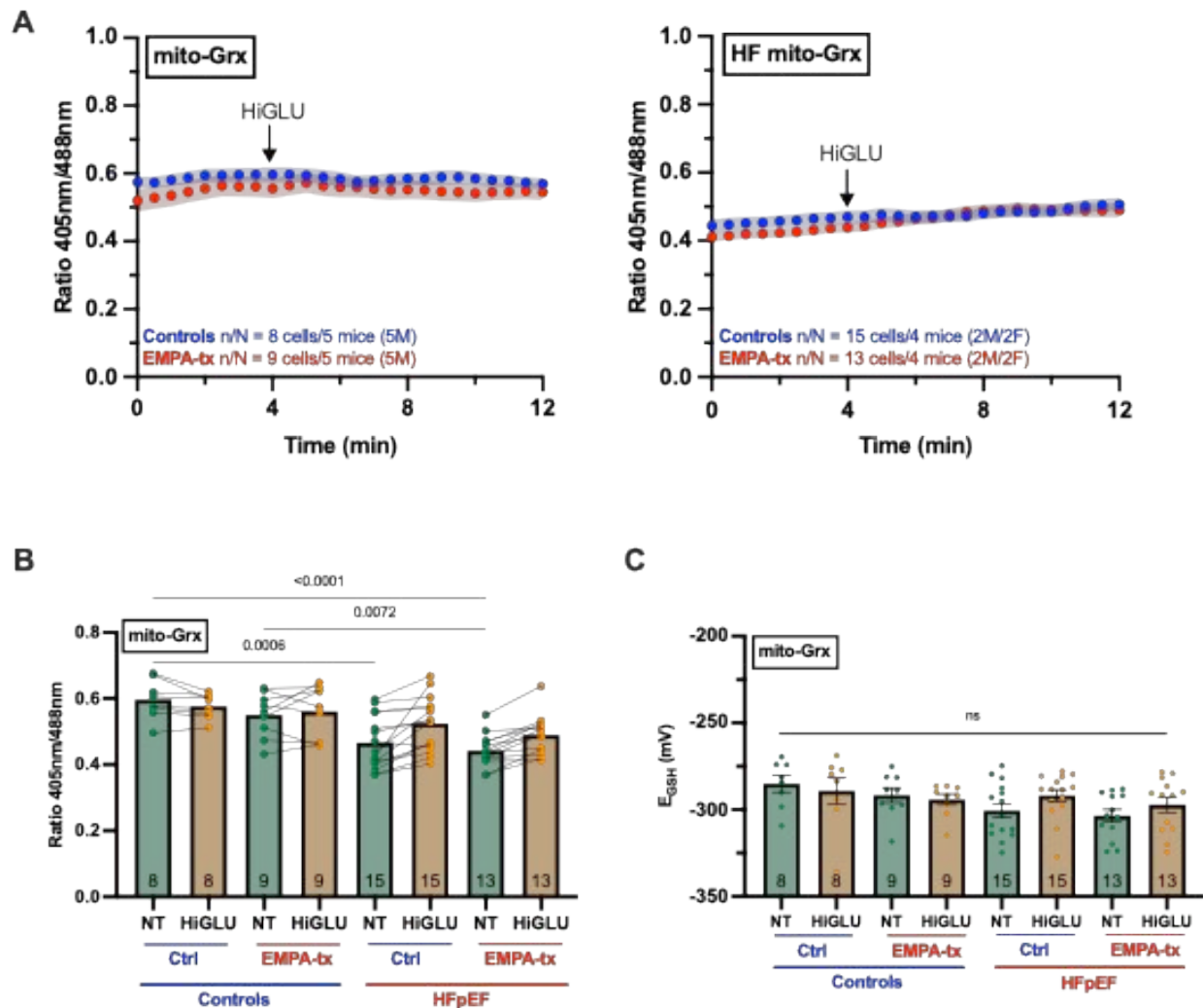


Figure 10. HFpEF vs. age-matched control groups in mito-Grx1-roGFP2 mice.

ROS levels for empagliflozin-treated vs. control cardiac myocytes exposed to acute hyperglycemia. **A)** Normal to HiGLU trace for both empagliflozin-treated and untreated cardiomyocytes in control and HFpEF mice. **B)** Control and HFpEF mice pairwise comparison of treatment type in NT vs. HiGLU. Comparisons made with one-way ANOVA comparing all NT groups to each other, all HiGLU groups to each other, and each NT group to its corresponding HiGLU. **C)** Age-matched control and HFpEF mice redox potentials in each treatment type at baseline and hyperglycemia. One-way

ANOVA comparing all NT groups to each other, all HiGLU groups to each other, and each NT group to its corresponding HiGLU. One-way ANOVA was followed by Šídák's multiple comparisons test. Animal numbers are shown in figure panel A and cell numbers are shown in panels B&C.

Cytosolic Redox

We further studied cytosolic ROS production and its changes under HFpEF and EMPA treatment. Utilizing myocytes expressing cytosolic roGFP2, we observed increases in cytosolic oxidation levels upon HiGLU exposure in both HFpEF and their age-matched controls (**Figure 11A**). There was no difference in baseline levels of ROS in either HFpEF and controls, and no difference across treatments (**Figure 11B**). Significant HiGLU-induced increases were seen in all groups, regardless of heart failure status (Ctrl-ctrls $P<0.0001$; Ctrl-EMPA $P=0.0026$; HF-ctrls $P<0.0001$; HF-EMPA $P<0.0001$) (**Figure 11B**). While there was no difference in baseline ROS levels (NT Mean $\pm$ SEM: Ctrl-ctrls 0.108 ± 0.004 ; Ctrl-EMPA 0.116 ± 0.011 ; HF-ctrls 0.105 ± 0.005 ; HF-EMPA 0.0904 ± 0.003), HiGLU-induced ROS levels were significantly lower in EMPA-treated myocytes compared to the non-EMPA controls (HiGLU Mean $\pm$ SEM: Ctrl-ctrls 0.316 ± 0.02 ; Ctrl-EMPA 0.215 ± 0.02 ; HF-ctrls 0.285 ± 0.02 ; HF-EMPA 0.213 ± 0.02) (**Figure 11B**). No significant differences were observed between HiGLU-induced ROS levels when comparing the EMPA-treated controls and HFpEF groups. Additionally, no significant differences were seen in HiGLU-induced ROS levels between non-treated controls and HFpEF groups. Interestingly, HiGLU-induced ROS levels in the EMPA-treated HFpEF cardiomyocytes were significantly lower ($P<0.001$) in comparison to the non-EMPA age-matched controls and EMPA-treated controls HiGLU-induced ROS

levels were significantly lower in comparison to the untreated HFpEF myocytes ($P=0.0239$) (**Figure 11B**). Overall, EMPA was able to reduce hyperglycemia-induced ROS levels in both controls and HFpEF cardiomyocytes.

Examining the glutathione redox potential revealed significant differences between baseline and HiGLU counterparts in controls and EMPA-treated myocytes (Ctrl-ctrls $P<0.0001$; Ctrl-EMPA $P=0.0216$; HF-ctrls $P<0.0001$; HF-EMPA $P<0.0001$) (**Figure 11C**). There were no significant differences in baseline redox potentials seen between HFpEF or control mice myocytes regardless of treatment (NT Mean $\pm$ SEM: Ctrl-ctrls -310 ± 6.15 ; Ctrl-EMPA -306 ± 4.74 ; HF-ctrls -306 ± 3.56 ; HF-EMPA -316 ± 2.62). Hyperglycemic redox potentials were significantly different between groups (HiGLU Mean $\pm$ SEM: Ctrl-ctrls -263 ± 2.69 ; Ctrl-EMPA -284 ± 5.05 ; HF-ctrls -265 ± 2.70 ; HF-EMPA -288 ± 3.86). Untreated myocytes had significantly higher E_{GSH} than those treated with EMPA in both HFpEF ($P<0.0001$) and age-matched controls ($P=0.0384$). There was also significant differences in redox potential observed when comparing EMPA-treated control myocytes to non-treated HFpEF myocytes ($P=0.0184$) and EMPA-treated HFpEF myocytes to non-treated control myocytes ($P=0.0003$) (**Figure 11C**). Therefore, EMPA had a reducing effect on both hyperglycemia-induced ROS levels and E_{GSH} in the cytosol, regardless of HF status.

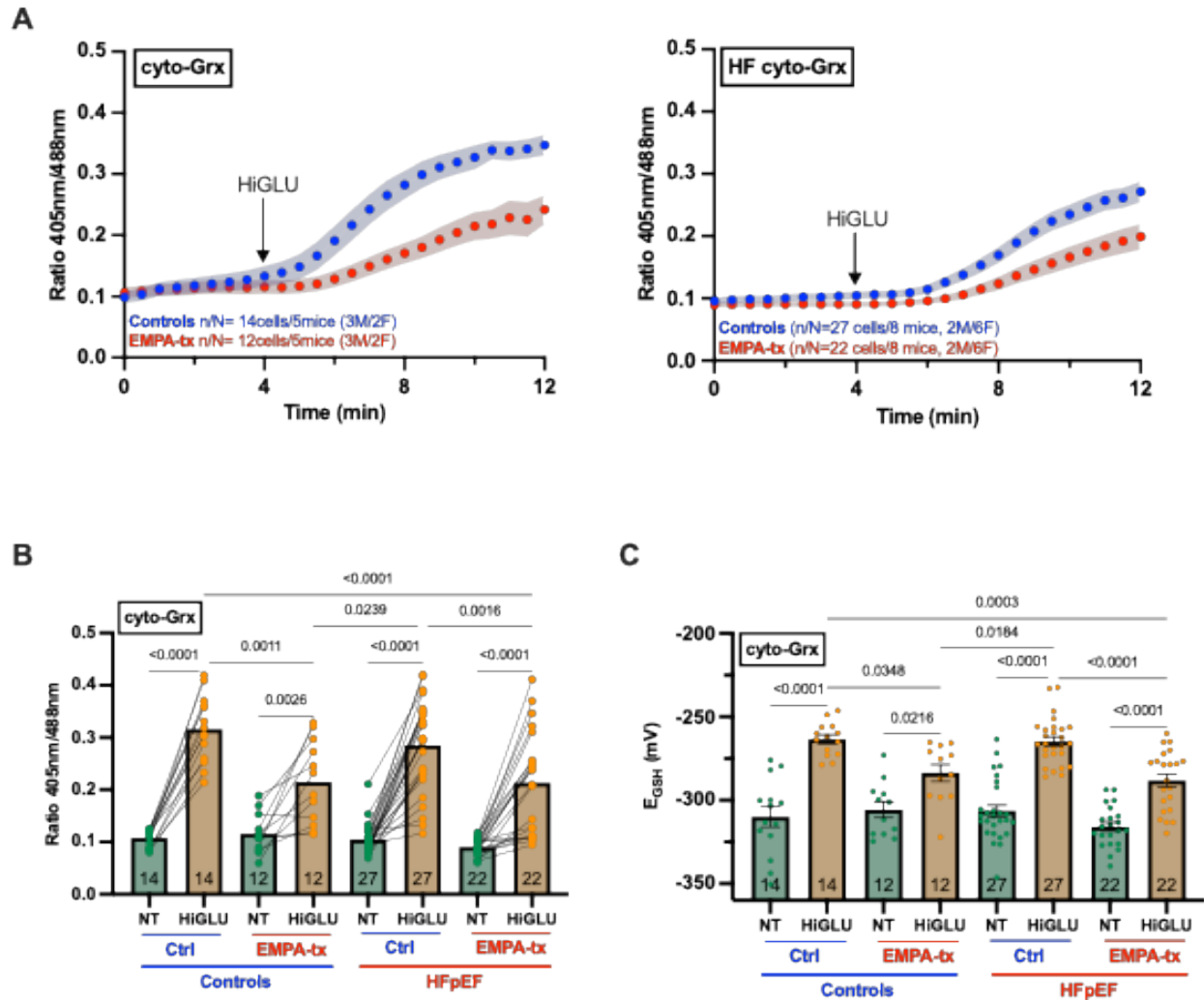


Figure 11. HFpEF vs. age-matched control groups in cyto-Grx-roGFP2 mice.

ROS levels for empagliflozin-treated vs. control cardiac myocytes exposed to acute hyperglycemia. **A)** Normal to HiGLU trace for both empagliflozin-treated and untreated cardiomyocytes in control and HFpEF mice. **B)** Control and HFpEF mice pairwise comparison of treatment type in NT vs. HiGLU. Comparisons made with one-way ANOVA comparing all NT groups to each other, all HiGLU groups to each other, and each NT group to its corresponding HiGLU. **C)** Age-matched control and HFpEF mice redox potentials in each treatment type at baseline and hyperglycemia. One-way ANOVA comparing all NT groups to each other, all HiGLU groups to each other, and

each NT group to its corresponding HiGLU. One-way ANOVA was followed by Šídák's multiple comparisons test. Animal numbers are shown in figure panel A and cell numbers are shown in panels B&C.

Empagliflozin Effects on Enzyme Activity

Based on prior work (Shan Lu et al., 2020; 2021), hyperglycemia-induced ROS production is mediated through the following pathway: increased extracellular glucose leads to enhanced glucose influx and elevated intracellular glucose availability, promoting flux through the hexosamine biosynthetic pathway (HBP) and increased production of UDP-GlcNAc. This metabolite serves as a substrate for OGT, resulting in increased O-GlcNAcylation of target proteins, including CaMKII. O-GlcNAc mediated modification of CaMKII enhances its activation, which in turn stimulates NOX2, leading to increased ROS production.

Based on this framework, we investigated whether EMPA attenuates hyperglycemia-induced ROS production through modulation of key nodes within this pathway. To do so, we assessed enzyme activities involved in O-GlcNAc cycling and ROS generation and employed pharmacological inhibitors targeting components of this signaling cascade, including CaMKII. These approaches allowed us to determine whether EMPA acts upstream at the level of metabolic flux and O-GlcNAc signaling, or downstream through effects on CaMKII activation and NOX2 dependent ROS production.

Empagliflozin Effects vs. CaMKII Inhibition

Prior work has demonstrated that hyperglycemia-induced increases in cytosolic ROS production require O-GlcNAcylation dependent activation of CaMKII δ , including

modification at S280, leading to downstream activation of NOX2. Based on this mechanism, we tested whether inhibition of CaMKII recapitulates the effects of EMPA on cytosolic ROS production. Using the cyto-Grx1-roGFP2 mice from 25-wk-old mice, we compared EMPA treated myocytes to those treated with the CaMKII inhibitor, autocamtide-2-related inhibitory peptide (AIP). AIP (1 μ M) significantly reduced cytosolic ROS production under high glucose conditions to near baseline levels (**Figure 12A**). Of note, that level of AIP inhibition was insufficient to abolish the HiGLU-induced increase in cytosolic ROS production previously reported in CaMKII δ knockout mice or knock-in mice in which the O-GlcNAcylation site was mutated (CaMKII δ -S280A; Lu et al. 2020), but confirmed the role of CaMKII in this process. Moreover, there was no significant difference between baseline and high glucose levels of ROS in AIP treated cells (**Figure 12B**). As previously seen, there was a significant difference in ROS levels between baseline and hyperglycemia in controls ($P<0.0001$) and EMPA treated ($P<0.0001$) myocytes (**Figure 12B**). Between treatment groups all baseline levels were similar (Mean $\pm$ SEM: Controls 0.109 $\pm$ 0.007; EMPA 0.104 $\pm$ 0.005; AIP 0.108 $\pm$ 0.004), while high glucose ROS levels were all significantly different from one another ($P<0.0001$). The controls had the highest ROS levels (Mean $\pm$ SEM: 0.345 $\pm$ 0.01), followed by EMPA treated cells (Mean $\pm$ SEM: 0.249 $\pm$ 0.01), and with AIP treated cell having the lowest ROS levels in hyperglycemia (Mean $\pm$ SEM: 0.145 $\pm$ 0.01) (**Figure 12B**). The glutathione redox potential was elevated in hyperglycemic conditions compared to baseline in both controls ($P<0.0001$) and the EMPA treatment group (EMPA $P<0.0001$), while AIP-treatment had no significant change in redox potential between baseline and hyperglycemia. Baseline redox potential was not significantly different between

treatment groups (Mean $\pm$ SEM: Controls -303 $\pm$ 1.981mV; EMPA -302 $\pm$ 3.09mV; AIP -307 $\pm$ 3.93mV). Significant differences were observed between groups under hyperglycemic conditions. The redox potential was significantly elevated in controls compared to both EMPA (P=0.0005) and AIP (P<0.0001) treated cardiomyocytes (Mean $\pm$ SEM: Controls -255 $\pm$ 3.06mV; EMPA -271 $\pm$ 2.21mV; AIP -296 $\pm$ 3.57mV). Additionally, E_{GSH} was significantly reduced in AIP-treated myocytes compared to those treated with EMPA (P<0.0001) (**Figure 12C**).

Together, these findings indicate that CaMKII contributes to hyperglycemia-induced cytosolic ROS production, as AIP reduced ROS to near baseline levels. EMPA also attenuated ROS, though to a lesser extent, suggesting it may act upstream of or in parallel with CaMKII signaling. This same pattern was also observed in the glutathione redox potential. Collectively, these results support a role for CaMKII in mediating ROS responses during metabolic stress and implicate it in the redox effects of EMPA.

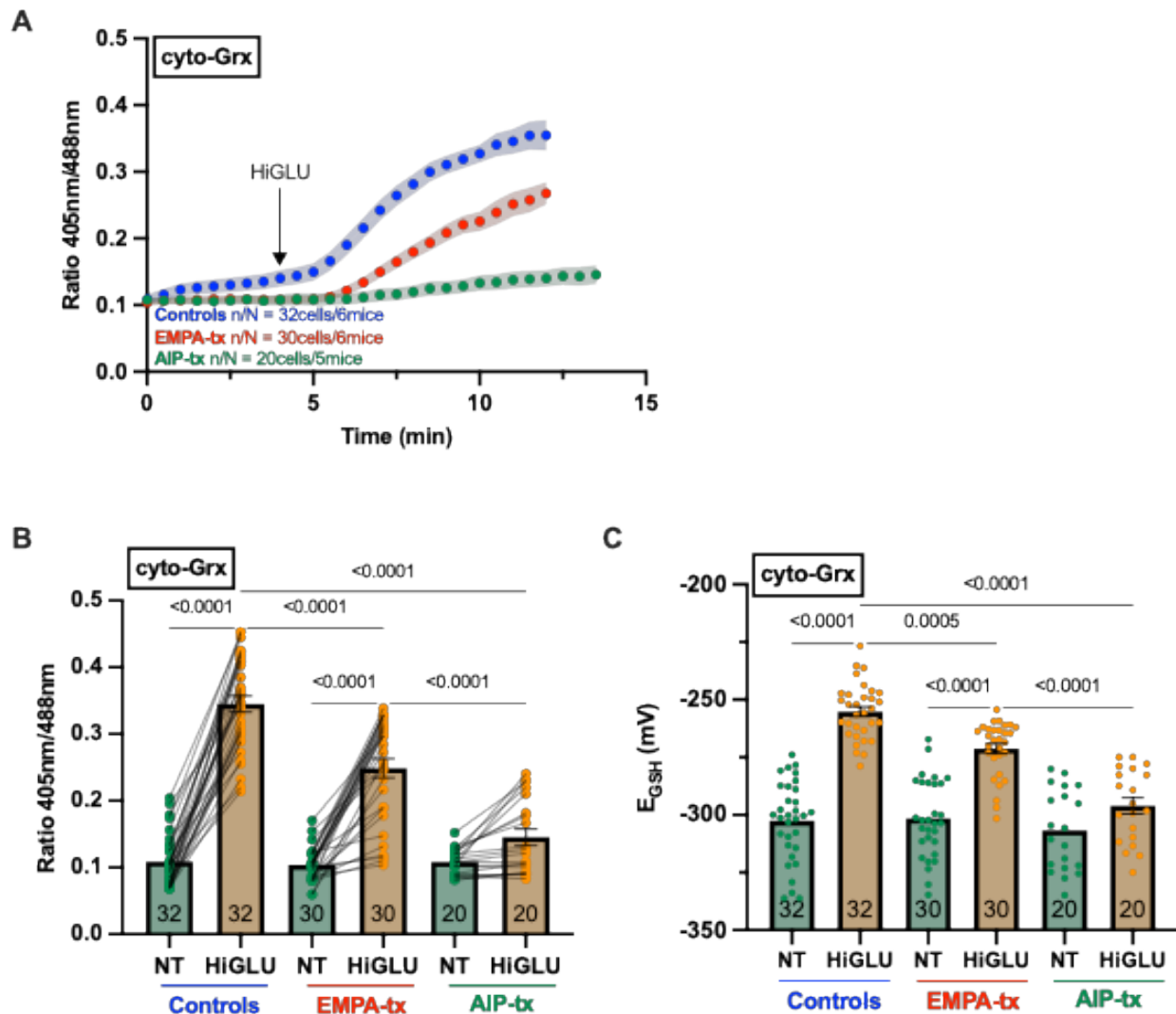


Figure 12. CaMKII effect on cytosolic ROS levels.

Comparison of EMPA-tx (1 μ M), AIP-tx (1 μ M), and control myocytes hyperglycemia-induced ROS levels. **A)** Traces of myocytes ROS levels at baseline and when exposed to hyperglycemia. **B)** One-way ANOVA comparison of ROS levels at baseline and high glucose in control vs. EMPA-treated vs. AIP-treated. **C)** Glutathione redox potential of each treatment type at baseline and hyperglycemia. One-way ANOVA followed by Šídák's multiple comparisons test. Animal numbers are shown in figure panel A and cell numbers are shown in panels B&C.

Empagliflozin Effects on NOX2 Activity

In cardiomyocytes CaMKII is known to increase cytosolic ROS production via NOX2 (Lu et al. 2020). The activity of NOX2 and EMPA's potential effects on it were tested using an NOX activity assay and a known NOX2 inhibitor, GP91, for comparison to EMPA. Utilizing a maximally inhibitory concentration of GP91 we compared its effects along with different doses of EMPA and compared the two as well as to an uninhibited control (**Figure 13**). To test whether EMPA affected other NOX isoforms, we compared maximum NOX2 inhibition (10 μ M GP91) with varying concentrations of EMPA (3, 1, 0.3, & 0.1 μ M). NOX activity is represented as units of 1 mM substrate oxidized to DCPIP per gram of protein per minute at room temperature (defined as 1 U/gpro).

No significant decrease in overall NOX activity was observed at any EMPA concentration (from 0.1 to 3 μ M). That was true whether total NOX activity was measured when NOX2 was specifically inhibited by GP91, which could have revealed effects on other NOX isoforms. GP91 (10 μ M) significantly reduced total NOX activity, by approximately 50% of the total NOX activity (from ~3.6 to 1.8 U/gpro; $P < 0.0001$).

Together, these data indicate that EMPA does not directly inhibit NOX2 activity in cardiomyocytes. While the NOX2-specific inhibitor GP91 significantly reduced NOX activity compared to the uninhibited control, EMPA at all tested concentrations (0.1–3 μ M) had no measurable effect, either alone or in combination with GP91. NOX activity in EMPA-treated cells remained comparable to the positive control, and no dose-dependent effects were observed. These findings suggest that the observed reductions in ROS with EMPA treatment are unlikely to result from direct modulation of NOX2 or

A

Dose-response curve showing NOX Activity (U/gpro) versus GP91 concentration (μM). The curve shows a sigmoidal decrease in activity as GP91 concentration increases. Key parameters are indicated:

- $\text{IC}_{50}: 4.809 \mu\text{M}$
- $R^2: 0.8279$

GP91 (μM)	NOX Activity (U/gpro)
0.1	~3.8
1	~3.5
5	~2.3
10	~1.5
100	~1.6

B

Effect of EMPA and GP91 on NOX activity. The graph shows NOX Activity (U/gpro) versus EMPA concentration (μM). Two conditions are compared: EMPA alone (red circles) and EMPA + GP91 (blue circles). Horizontal dashed lines indicate Max Activity (blue) and 10 μM GP91 (green).

EMPA (μM)	NOX Activity (U/gpro) - EMPA	NOX Activity (U/gpro) - EMPA + GP91
0.01	-	~3.5
0.1	~3.3	~1.8
0.3	~3.3	~1.9
1	~3.3	~1.8
3	~3.3	~1.8
10	-	~1.7

C

Bar graph showing NOX Activity (U/gpro) for various treatments. Individual data points are overlaid on each bar. Statistical significance is indicated by asterisks (* p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001) above the bars.

Treatment	NOX Activity (U/gpro)
0 Inhibitor	~3.5
0.1 μM EMPA	~3.3
0.3 μM EMPA	~3.3
1 μM EMPA	~3.3
3 μM EMPA	~3.3
GP + 0.1 μM EMPA	~1.8
GP + 0.3 μM EMPA	~1.8
GP + 1 μM EMPA	~1.8
GP + 3 μM EMPA	~1.7
10 μM GP	~1.6

GP91 vs. EMPA dose response (U/gpro). **A)** NOX activity (U/gpro) dose response curve of GP91 (μM) to determine maximum inhibition of NOX2. **B)** Dose response of EMPA (μM) and EMPA+GP91 (μM) groups compared to no inhibitor, or maximum NOX activity, and maximum NOX2 inhibition with 10 μM GP91. **C)** Bar graph of varying

groups. Numbers shown in figure reflect averaged triplicates from each mouse heart used for tissue lysate. One-way ANOVA was followed by Šídák's multiple comparisons test.

Empagliflozins Effect on O-GlcNAcylation

O-GlcNAcylation of CaMKII δ at Ser280 has been implicated in the acute HiGLU-induced increase in ROS production (Lu et al. 2020). Modulation of O-GlcNAcylation and CaMKII signaling has also been proposed as a potential mechanism contributing to the cardioprotective effects of EMPA via its ability to reduce global O-GlcNAcylation and thus CaMKII activation (Hegyí & Bers 2023). One of the enzymes involved in that process is O-GlcNAc transferase (OGT), which mediates O-GlcNAcylation of proteins at serine and threonine sites using UDP-GlcNAc as the substrate. To test whether EMPA directly inhibits OGT activity, we measured OGT enzymatic activity and the ability of a known OGT inhibitor, OSMI-1. **Figure 14A** shows an OGT activity concentration-response curve for OSMI-1, revealing an IC₅₀ of 1 μ M. EMPA did not alter OGT over the entire concentration range explored (1 nM to 1 mM; **Figure 14B**).

O-GlcNAcase (OGA), is the enzyme responsible for the removal of O-GlcNAc from proteins. To measure EMPA's potential direct effects on OGA we measured enzyme activity in an assay along with the effect of a known specific OGA inhibitor, ThiametG (ThmG). **Figure 15A** shows that ThmG inhibits OGA activity with an IC₅₀ of 15.7 nM. EMPA, on the other hand has no effect at any concentration tested (**Figure 15B**) even at 1 μ M, which suppressed HiGLU-induced ROS production in **Figure 11**.

Thus, an EMPA-induced enhancement of OGA activity does not explain the inhibition of HiGLU-induced ROS production.

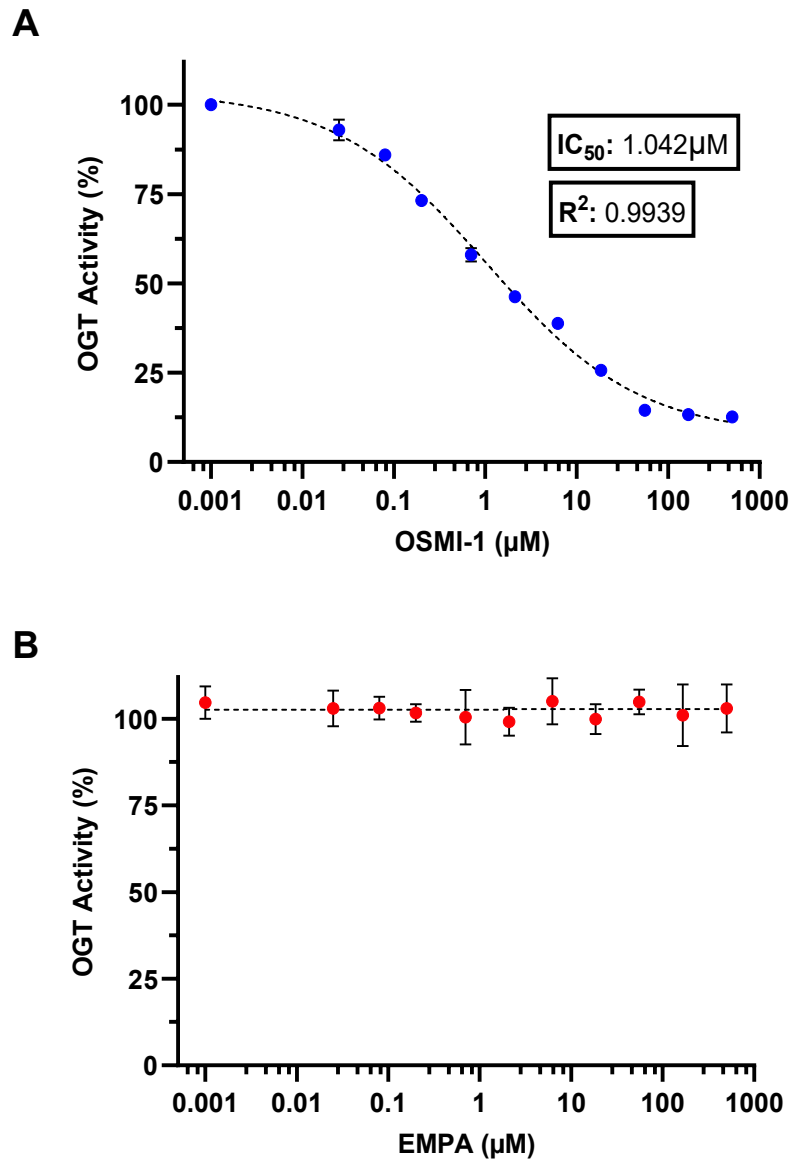


Figure 14. O-GlcNAc Transferase (OGT) activity: OSMI-1 vs. Empagliflozin (EMPA) dose response curves.

A) OGT activity (%) dose response curve of OSMI-1 (μM) **B)** and EMPA (μM).

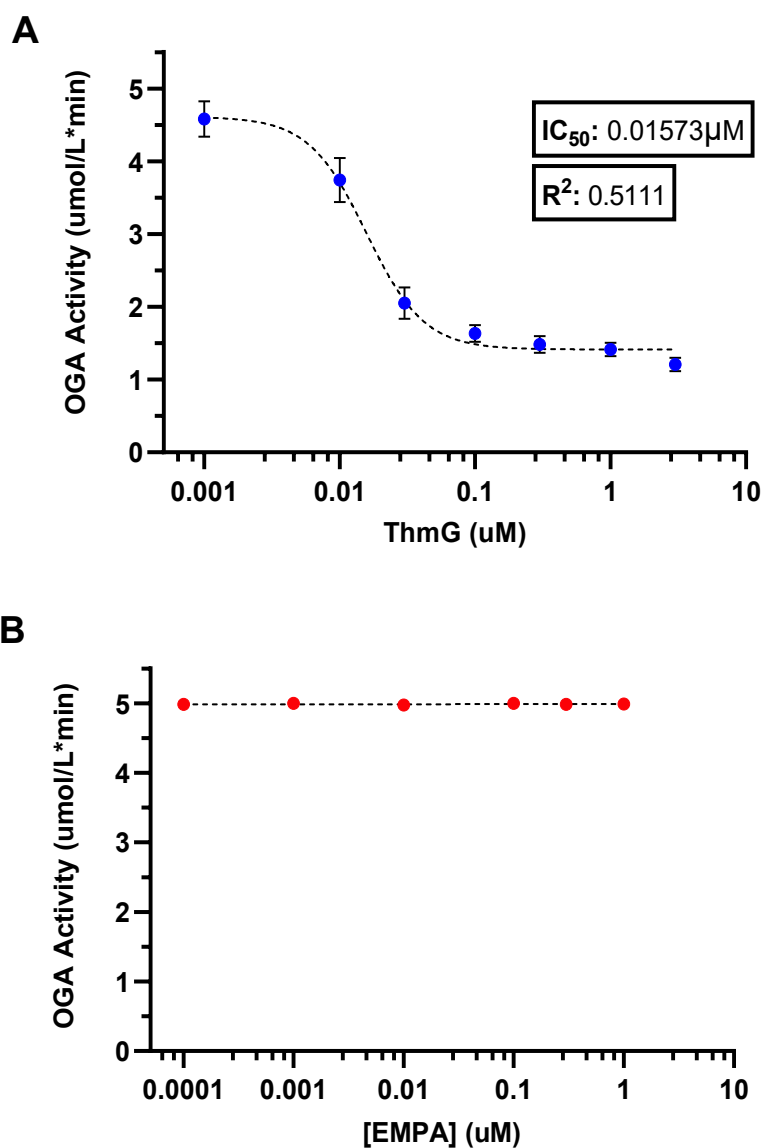


Figure 15. O-GlcNAcase (OGA) activity: ThiametG (ThmG) vs. Empagliflozin (EMPA) dose response curves.

A) OGA activity ($\mu\text{mol/L} \cdot \text{min}$) dose response curve of ThmG (μM) **B)** and EMPA (μM).

These data indicate that EMPA does not directly modulate O-GlcNAc cycling enzymes in cardiomyocytes. While the OGA inhibitor, ThiametG, and the OGT inhibitor, OSMI-1, produced clear dose-dependent inhibition of their respective enzymes, EMPA

showed no measurable effect on either OGA or OGT activity across the tested concentrations. These results suggest that EMPA's previously observed effects on O-GlcNAcylation in cardiomyocytes are unlikely to occur through direct effects on these enzymes, pointing instead to indirect regulatory mechanisms.

CHAPTER 4: DISCUSSION

HFpEF remains a growing clinical challenge where limited therapeutic options, combined with a wide array of phenotypes and comorbidities, exacerbate disease progression. SGLT2 inhibitors and EMPA, are therapeutic with overlapping efficacy for both T2DM and HF (Anker et al. 2021), and represent a unique avenue for probing disease mechanisms and potentially informing therapeutic development. Similar dysfunction is observed in diabetic and failing hearts, with hyperglycemia and oxidative stress in cardiomyocytes further promoting disease progression (Giacco and Brownlee 2010; González et al. 2023). This dissertation investigated the effects of acute hyperglycemia on cardiomyocyte ROS production and whether EMPA modulates these responses independent of systemic effects in healthy and HFpEF mouse models. Additionally, EMPA's direct effects on key enzymatic activities were evaluated.

Acute hyperglycemia induced a rapid increase in cytosolic ROS in cardiomyocytes (**Figure 8**). That is consistent with prior reports which also showed that both NOX2 and CaMKII δ and the Ser280 O-GlcNAcylation site on CaMKII δ were required for this increase in ROS levels (Liu et al. 2026; Lu et al. 2020, 2025). We found this hyperglycemia-induced ROS production was also partially attenuated by EMPA pretreatment, an effect observed in myocytes from both healthy and HFpEF mice, indicating a direct cardiomyocyte mechanism of EMPA (**Figure 11**). Notably, the hyperglycemia-induced ROS increase was exclusive to the cytosol; mitochondrial ROS was unchanged in either treatment, aligning with previous studies (Lu et al. 2020, 2025) (**Figure 7**). EMPA did not alter mitochondrial ROS production regardless of HF status (**Figure 10**), further supporting a non-mitochondrial origin of the acute ROS signal.

Baseline mitochondrial ROS levels were significantly lower in HFpEF cardiomyocytes compared with controls; however, the dynamic response to hyperglycemia and overall redox changes were otherwise similar between groups (**Figure 10**) and this was not observed in the cytosol (**Figure 11**). CaMKII inhibition produced a more pronounced suppression of hyperglycemia-induced ROS than EMPA (**Figure 12**), although not as complete as was observed in mouse cardiomyocytes lacking CaMKII δ or expressing only the O-GlcNAcylation-resistant CaMKII δ -S280A (Lu et al. 2020), suggesting additional upstream contributions to ROS amplification. EMPA did not directly inhibit NOX2, OGT, or OGA activity (**Figure 13-15**) indicating that its effects are not mediated through direct enzymatic suppression of these pathways.

Collectively, these findings indicate that hyperglycemia-induced ROS signaling in cardiomyocytes is predominantly cytosolic and closely linked to CaMKII dependent redox amplification within this compartment. Elevated cytosolic ROS and CaMKII activity can modulate key calcium-handling proteins and kinases, such as L-type calcium channels, sodium channels, SERCA, RyR, and CaMKII itself, thereby linking acute metabolic stress to ECC dysfunction (Hegyi et al. 2021; Erickson et al. 2008; Kassmann et al. 2008; Luczak and Anderson 2014; Oda et al. 2015). In this context, EMPA may act upstream, without directly targeting NOX2 or O-GlcNAc cycling enzymes. Thus, cardioprotection by EMPA likely involves indirect modulation of non-mitochondrial cytosolic ROS-generating systems and associated redox-sensitive signaling pathway.

The compartmental restriction of ROS signaling to the cytosol suggests that acute hyperglycemia preferentially engages non-mitochondrial ROS sources (**Figures 7**

& 10). Potential contributors include NOX isoforms, uncoupled nitric oxide synthases, and other cytosolic oxidoreductase systems capable of amplifying localized redox signaling (Giacco and Brownlee 2010; Lu et al. 2020; Mazumder et al. 2004; Peoples et al. 2019). This spatial restriction of ROS to the cytosol supports early targeting of CaMKII and downstream calcium-handling machinery as primary effectors of hyperglycemia-induced myocyte dysfunction. Future studies using selective genetic or pharmacologic inhibition of candidate cytosolic ROS sources, combined with compartment-specific ROS sensors, will be necessary to define the precise target of EMPA in quieting this pathway.

ROS signals were analyzed in two complementary approaches: (1) endpoint fluorescence values at the termination of the HiGLU exposure period (**Figures 8B & 11B**), and (2) the maximal rate of ROS increase, defined as the steepest slope ($dROS/dt$) during the rising phase of the signal (not shown; reflected in time-course traces **Figure 8A & 11A**), which reflects the dynamics of ROS propagation. EMPA reduced the rate-of-rise and HiGLU endpoint fluorescence, suggesting modulation of ROS amplification kinetics that effectively lower total ROS accumulation over the acute stimulation period. This combination of effects suggests that EMPA may interrupt the potential feed-forward amplification of ROS signaling initiated by hyperglycemia. Cytosolic ROS activates CaMKII through oxidative and metabolic modifications, which can further amplify ROS production via downstream targets, contributing to a localized redox-sensitive signaling amplification, the magnitude of which may be limited in this setting (Erickson et al. 2008; Lu et al. 2020). By attenuating the rate of ROS accumulation, EMPA may dampen this component of the positive feedback loop in ROS

signaling, without directly scavenging ROS. EMPA may also modulate intracellular ion homeostasis via inhibition of NHE1, a proposed off-target effect of SGLT2 inhibitors that remains controversial. Reduced intracellular sodium could enhance calcium extrusion via NCX, thereby limiting calcium-dependent CaMKII activation and downstream ROS amplification (Baartscheer et al. 2017; Uthman et al. 2018). However, the controversial nature of the NHE1 effects, especially at neutral pH where NHE is relatively inactive (Leem et al. 1999; Vaughan-Jones et al. 1987, 2009), makes it unclear whether that mechanism is involved in the hyperglycemia-induced increase in ROS production.

Hyperglycemia-induced metabolic changes and redox remodeling may also contribute to EMPA sensitivity. Recent work suggests EMPA may shift cellular redox set points by improving metabolic efficiency or mitochondrial coupling, thereby altering thresholds for redox-sensitive signaling activation (Hoehlschen et al. 2025). Chronic metabolic stress may similarly prime cardiomyocytes for exaggerated ROS responses by altering antioxidant buffering and signaling sensitivity (Peoples et al. 2019). These observations suggest that EMPA modulates ROS signaling indirectly by altering upstream metabolic and redox conditions that govern ROS amplification, rather than functioning as a direct antioxidant. Taken together, these observations support a model in which EMPA may modulate ROS signaling kinetics rather than directly inhibiting ROS generating enzymes, and this effect may contribute to the rapid cardioprotective effects observed clinically that occur independently of glucose lowering (Packer, Butler, et al. 2021; Pellicori et al. 2020).

Unexpectedly, EMPA treatment had similar effects on cytosolic ROS in healthy, age-matched controls and HFpEF mice (**Figure 11**) where metabolic stress levels might be different.

CaMKII inhibition (AIP) strongly inhibited hyperglycemia-induced ROS, while EMPA exhibited a more limited reduction of ROS production (**Figure 12**). This underscores CaMKII as a dominant driver of the hyperglycemia-induced cytosolic ROS increase and CaMKII is also a key regulator of ECC pathways in the cardiomyocyte response to acute hyperglycemia (Bers 2002; Maier and Bers 2007; Hegyi et al. 2021). EMPA likely interrupts this pathway upstream or in parallel, without fully blocking CaMKII or NOX2 activity. Further, in relation to CaMKII posttranslational modifications, we showed that EMPA does not directly alter OGT or OGA enzymatic activity (**Figure 14 & 15**), suggesting indirect modulation of CaMKII O-GlcNAcylation via metabolic flux, or that it interferes with the ability of CaMKII to activate NOX2 (Erickson et al. 2013; Hart 2014; Zachara and Hart 2006).

Collectively, the findings of this study support a model in which acute hyperglycemia initiates a predominantly cytosolic oxidative stress response that is mediated and possibly amplified through CaMKII-dependent signaling pathways rather than through immediate mitochondrial dysfunction, similar to what is depicted in **Figure 1B**. In this framework, hyperglycemia triggers rapid activation of CaMKII mediated by O-GlcNAcylation of and activation of CaMKII that can be arrhythmogenic (Erickson et al. 2013). That activated CaMKII can also promote increased NOX2-dependent ROS production, which may subsequently promote additional activating modifications of CaMKII and establish a feed-forward cycle of oxidative stress (Hegyi et al. 2021).

The absence of a detectable increase in mitochondrial ROS during acute hyperglycemia suggests that mitochondrial involvement may occur relatively independent of this cytosolic pathway, and perhaps later in disease progression, but could also be pushed as a secondary consequence of sustained cytosolic ROS stress in hyperglycemia. Interestingly, HFpEF myocytes exhibited lower baseline mitochondrial ROS compared to controls regardless of treatment, but still demonstrated no hyperglycemic response (Figure 10B). Cytosolic basal ROS levels showed no difference between control and HFpEF myocytes (Figure 11B). Additionally, cytosolic ROS response to hyperglycemia was not different between HFpEF and age-matched controls, with significant differences observed specifically between EMPA-treated and untreated myocytes (Figure 11B). The reduced basal mitochondrial ROS in HFpEF may reflect compensatory adaptations that develop during disease progression, including upregulation of antioxidant systems, metabolic remodeling, or altered redox signaling sensitivity (Dikalov & Dikalova 2019). An alternative contributor could include shifts in substrate utilization, which are well-documented in both diabetes and HF and can have downstream effects on cellular redox balance. Together, these findings suggest that chronic HFpEF remodeling may establish a redefined redox steady state, in which mitochondrial ROS is suppressed at baseline while cytosolic ROS is maintained at levels comparable to control myocytes. This apparent normalization of cytosolic ROS, despite the presence of disease, may reflect an adaptive buffering of oxidative stress that masks expected elevations under basal conditions and during acute metabolic challenge. Within this context, the preserved cytosolic ROS response to hyperglycemia, and its selective attenuation by EMPA, supports a model in which cytosolic, rather than

mitochondrial, ROS signaling mediates the acute hyperglycemic response. Thus, although mitochondrial ROS does not appear to directly contribute to the acute effects observed here, it may still play a role in longer-term disease progression or downstream remodeling. Through modulation of cytosolic ROS signaling, EMPA may preserve calcium handling and contractile function, providing a potential cellular basis for its clinical benefits in HF.

Within this context, EMPA appears to attenuate the rate of ROS accumulation without fully suppressing the hyperglycemia-induced response, indicating that its protective effects may arise from modulation of upstream triggers rather than a simple direct inhibition of either ROS-generating enzymes or CaMKII. The near-complete suppression of ROS by CaMKII inhibition supports a central role for CaMKII as a mediator of cytosolic ROS amplification, while the lack of direct effects of EMPA on NOX2, OGT, or OGA activity suggests that the drug may act by altering the cellular environment that permits activation of this pathway. One potential interpretation is that EMPA disrupts the metabolic or ionic conditions necessary for sustained CaMKII activation or its ability to activate NOX2, thereby dampening the feed-forward cycle linking hyperglycemia to oxidative stress. Together, these findings support a model in which early cytosolic ROS-CaMKII signaling, amplified through CaMKII, represents a key mechanism linking acute metabolic stress to cardiomyocyte dysfunction and a potential pathway targeted by EMPA as an off-target cardioprotective effect of this SGLT2 inhibitor. This mechanism provides a possible explanation for the rapid cardiovascular benefits seen clinically with SGLT2 inhibitors, like EMPA, and provides an explanation for the benefits seen across disease types (i.e. T2DM, HFrEF, HFpEF)

(Anker et al. 2021; Packer, Anker, et al. 2021; Packer, Butler, et al. 2021; Pellicori et al. 2020). This also supports clinical observations that EMPA's benefits occur independent of glucose lowering (Anker et al. 2021).

However, we recognize numerous limitations to our study. Changes in acute hyperglycemia-induced oxidative stress were seen with EMPA pretreatment which occurs rapidly on increased [glucose], however we have not mimicked long-term hyperglycemia on ROS production via the pathway explored, which may be important clinically. Additionally, these studies were only performed in isolated cardiomyocytes (in part to focus on myocyte-specific mechanisms) and multicellular interactions remain to be explored. However, our experiments have the advantage that the acute EMPA effects we observe are present in myocytes that lack expression of SGLT2, and thus represent potentially beneficial off-target effects of EMPA. Our enzyme activity assays did not indicate direct effects of EMPA on OGT, OGA, or NOX2 activity, but that does not rule out indirect effects that could occur within the more physiological environment of the myocyte. Furthermore, these experiments lacked direct measurements of CaMKII modifications limiting the mechanistic certainty of conclusions on CaMKII activation state.

Future Directions

Progression toward HF often begins before clinical symptoms become apparent, initially manifesting as diastolic dysfunction and eventually as systolic impairment and eventual pump failure (Mengstie et al. 2022). Numerous pathological processes contribute to this progression, including altered calcium handling, metabolic disturbances, insulin resistance, oxidative stress, autonomic dysfunction, and

cardiomyocyte loss. These findings raise the question of how early cytosolic ROS evolves into the broader oxidative stress observed in chronic HF. One possible interpretation is that hyperglycemia may initially activate redox-sensitive mechanisms, such as CaMKII, which amplify ROS production prior to mitochondrial involvement. Over time, sustained activation of these pathways may promote mitochondrial involvement, contributing to the widespread oxidative stress observed in established HF.

The reduced baseline mitochondrial ROS observed in HF cardiomyocytes suggests adaptive remodeling, warranting investigation into how such adaptations might influence basal or long-term metabolic stress-induced ROS signaling. Such adaptations could initially serve a protective role but may also modify cellular responsiveness to acute metabolic stress. Future work should investigate the precise CaMKII-dependent pathways responsible for cytosolic ROS amplification, including whether distinct post-translational modifications contribute differentially to acute versus chronic activation. EMPA, which only partially reduced ROS production, may act upstream of CaMKII activation or disrupt the permissive cellular environment required for this feed-forward signaling rather than directly inhibiting ROS-generating enzymes.

Temporal and spatial progression of ROS signaling from early cytosolic generation to mitochondrial involvement under chronic hyperglycemic conditions warrants further investigation. Approaches capable of resolving compartment-specific redox dynamics in intact cells (as the mitochondrial and cytosolic targeted ROS sensors used here) or in vivo could provide valuable additional insight into this process. Direct assessment of CaMKII activation states and post-translational modifications in this context will be important for identifying the mechanisms by which acute signaling

transitions to sustained pathological activation. The current study utilized isolated mouse cardiomyocytes, which, while advantageous for mechanistic interrogation, does not fully recapitulate the structural and physiological complexity of the human heart. Differences in electrophysiology, metabolism, and disease progression between species highlight the importance of extending these findings to more translational models. Finally, translation to human physiology requires validation in larger animal models or whole-heart preparations to confirm whether the cytosolic ROS–CaMKII axis and EMPA modulation observed in mice similarly impact cardioprotection in vivo.

REFERENCES

- Akki, A., Zhang, M., Murdoch, C., Brewer, A., & Shah, A. M. (2009). NADPH oxidase signaling and cardiac myocyte function. *Journal of molecular and cellular cardiology*, 47(1), 15–22. doi.org/10.1016/j.yjmcc.2009.04.004
- Al-Lawati J. A. (2017). Diabetes Mellitus: A Local and Global Public Health Emergency!. *Oman medical journal*, 32(3), 177–179. doi.org/10.5001/omj.2017.34
- Anaspec. “O-Linked GlcNAc Transferase (OGT) Substrate - 1 Mg.” Anaspec. <https://www.anaspec.com/en/catalog/o-linked-glcnac-transferase-ogt-substrate-1-mg~784ae466-ac57-4bba-8553-e5e0dd59582d>.
- Anderson M. E. (2007). Multiple downstream proarrhythmic targets for calmodulin kinase II: moving beyond an ion channel-centric focus. *Cardiovascular research*, 73(4), 657–666. doi.org/10.1016/j.cardiores.2006.12.009
- Anderson, M. E., Brown, J. H., & Bers, D. M. (2011). CaMKII in myocardial hypertrophy and heart failure. *Journal of molecular and cellular cardiology*, 51(4), 468–473. doi.org/10.1016/j.yjmcc.2011.01.012
- Anker, S. D., Butler, J., Filippatos, G., Khan, M. S., Marx, N., Lam, C. S. P., Schnaidt, S., Ofstad, A. P., Brueckmann, M., Jamal, W., Bocchi, E. A., Ponikowski, P., Perrone, S. V., Januzzi, J. L., Verma, S., Böhm, M., Ferreira, J. P., Pocock, S. J., Zannad, F., & Packer, M. (2021). Effect of Empagliflozin on Cardiovascular and Renal Outcomes in Patients With Heart Failure by Baseline Diabetes Status: Results

From the EMPEROR-Reduced Trial. *Circulation*, 143(4), 337–349.

doi.org/10.1161/CIRCULATIONAHA.120.051824

Baartscheer, A., Schumacher, C. A., Wüst, R. C., Fiolet, J. W., Stienen, G. J., Coronel, R., & Zuurbier, C. J. (2017). Empagliflozin decreases myocardial cytoplasmic Na⁺ through inhibition of the cardiac Na⁺/H⁺ exchanger in rats and rabbits. *Diabetologia*, 60(3), 568–573. doi.org/10.1007/s00125-016-4134-x

Battiprolu, P. K., Gillette, T. G., Wang, Z. V., Lavandero, S., & Hill, J. A. (2010). Diabetic Cardiomyopathy: Mechanisms and Therapeutic Targets. *Drug discovery today. Disease mechanisms*, 7(2), e135–e143. doi.org/10.1016/j.ddmec.2010.08.001

Benedetti, R., Chianese, U., Papulino, C., Scisciola, L., Cortese, M., Formisano, P., Del Gaudio, N., Cabaro, S., D'Esposito, V., Pesapane, A., Conte, M., Signoriello, G., Barbieri, M., Altucci, L., & Paolisso, G. (2025). Unlocking the power of empagliflozin: Rescuing inflammation in hyperglycaemia-exposed human cardiomyocytes through comprehensive multi-level analysis. *European journal of heart failure*, 27(5), 844–856. doi.org/10.1002/ejhf.3566

Bers, D.M. (2001). *Excitation-Contraction Coupling and Cardiac Contractile Force*. Dordrecht: Springer Netherlands. doi.org/10.1007/978-94-010-0658-3.

Bers, D. M. (2002). Cardiac excitation-contraction coupling. *Nature*, 415(6868), 198–205. doi.org/10.1038/415198a

Bers, D.M., & Kranias.E.G. (2005). *Normal and Abnormal Calcium Homeostasis. Molecular Mechanisms of Cardiac Hypertrophy and Failure*, CRC Press.

- Bhatt, N. M., Aon, M. A., Tocchetti, C. G., Shen, X., Dey, S., Ramirez-Correa, G., O'Rourke, B., Gao, W. D., & Cortassa, S. (2015). Restoring redox balance enhances contractility in heart trabeculae from type 2 diabetic rats exposed to high glucose. *American journal of physiology. Heart and circulatory physiology*, 308(4), H291–H302. doi.org/10.1152/ajpheart.00378.2014
- Brown, D. I., & Griending, K. K. (2015). Regulation of signal transduction by reactive oxygen species in the cardiovascular system. *Circulation research*, 116(3), 531–549. doi.org/10.1161/CIRCRESAHA.116.303584
- Cai, W., Chong, K., Huang, Y., Huang, C., & Yin, L. (2024). Empagliflozin improves mitochondrial dysfunction in diabetic cardiomyopathy by modulating ketone body metabolism and oxidative stress. *Redox biology*, 69, 103010. doi.org/10.1016/j.redox.2023.103010
- Charlton, A., Garzarella, J., Jandeleit-Dahm, K. A. M., & Jha, J. C. (2020). Oxidative Stress and Inflammation in Renal and Cardiovascular Complications of Diabetes. *Biology*, 10(1), 18. doi.org/10.3390/biology10010018
- Chen, J., Williams, S., Ho, S., Loraine, H., Hagan, D., Whaley, J. M., & Feder, J. N. (2010). Quantitative PCR tissue expression profiling of the human SGLT2 gene and related family members. *Diabetes therapy : research, treatment and education of diabetes and related disorders*, 1(2), 57–92. doi.org/10.1007/s13300-010-0006-4

- Chen, S., Coronel, R., Hollmann, M. W., Weber, N. C., & Zuurbier, C. J. (2022). Direct cardiac effects of SGLT2 inhibitors. *Cardiovascular diabetology*, 21(1), 45. doi.org/10.1186/s12933-022-01480-1
- Cho, N. H., J. E. Shaw, S. Karuranga, Y. Huang, J. D. da Rocha Fernandes, A. W. Ohlrogge, and B. Malanda. (2018). IDF Diabetes Atlas: Global Estimates of Diabetes Prevalence for 2017 and Projections for 2045. *Diabetes Research and Clinical Practice*, 138, 271-281. doi:10.1016/j.diabres.2018.02.023.
- Chung, Y. J., K. C. Park, S. Tokar, T. R. Eykyn, W. Fuller, D. Pavlovic, P. Swietach, and M. J. Shattock. (2021). Off-Target Effects of Sodium-Glucose Co-Transporter 2 Blockers: Empagliflozin Does Not Inhibit Na⁺/H⁺ Exchanger-1 or Lower Intracellular Na⁺ in the Heart. *Cardiovascular Research*, 117(14), 2794-2806. doi.org/10.1093/cvr/cvaa323
- Cooper, L. L., Li, W., Lu, Y., Centracchio, J., Terentyeva, R., Koren, G., & Terentyev, D. (2013). Redox modification of ryanodine receptors by mitochondria-derived reactive oxygen species contributes to aberrant Ca²⁺ handling in ageing rabbit hearts. *The Journal of physiology*, 591(23), 5895–5911. doi.org/10.1113/jphysiol.2013.260521
- Corbin, K. D., Dagogo-Jack, S., Cannon, C. P., Cherney, D. Z. I., Cosentino, F., Frederich, R., Liu, J., Pong, A., Lin, J., Cater, N. B., & Pratley, R. E. (2023). Cardiorenal outcomes by indices of liver steatosis and fibrosis in individuals with type 2 diabetes and atherosclerotic cardiovascular disease: Analyses from VERTIS CV, a randomized trial of the sodium-glucose cotransporter-2 inhibitor

ertugliflozin. *Diabetes, obesity & metabolism*, 25(3), 758–766.

doi.org/10.1111/dom.14923

Cowie, M. R., & Fisher, M. (2020). SGLT2 inhibitors: mechanisms of cardiovascular benefit beyond glycaemic control. *Nature reviews. Cardiology*, 17(12), 761–772.

doi.org/10.1038/s41569-020-0406-8

Curran, J., Tang, L., Roof, S. R., Velmurugan, S., Millard, A., Shonts, S., Wang, H., Santiago, D., Ahmad, U., Perryman, M., Bers, D. M., Mohler, P. J., Ziolo, M. T., & Shannon, T. R. (2014). Nitric oxide-dependent activation of CaMKII increases diastolic sarcoplasmic reticulum calcium release in cardiac myocytes in response to adrenergic stimulation. *PloS one*, 9(2), e87495.

doi.org/10.1371/journal.pone.0087495

Curtain, J. P., Docherty, K. F., Jhund, P. S., Petrie, M. C., Inzucchi, S. E., Køber, L., Kosiborod, M. N., Martinez, F. A., Ponikowski, P., Sabatine, M. S., Bengtsson, O., Langkilde, A. M., Sjöstrand, M., Solomon, S. D., & McMurray, J. J. V. (2021). Effect of dapagliflozin on ventricular arrhythmias, resuscitated cardiac arrest, or sudden death in DAPA-HF. *European heart journal*, 42(36), 3727–3738.

doi.org/10.1093/eurheartj/ehab560

Di Franco, A., Cantini, G., Tani, A., Coppini, R., Zecchi-Orlandini, S., Raimondi, L., Luconi, M., & Mannucci, E. (2017). Sodium-dependent glucose transporters (SGLT) in human ischemic heart: A new potential pharmacological target. *International journal of cardiology*, 243, 86–90. <https://doi.org/10.1016/j.ijcard.2017.05.032>

- Dias, W. B., Cheung, W. D., & Hart, G. W. (2012). O-GlcNAcylation of kinases. *Biochemical and biophysical research communications*, 422(2), 224–228. doi.org/10.1016/j.bbrc.2012.04.124
- Dikalov, S. I., & Dikalova, A. E. (2019). Crosstalk Between Mitochondrial Hyperacetylation and Oxidative Stress in Vascular Dysfunction and Hypertension. *Antioxidants & redox signaling*, 31(10), 710–721. doi.org/10.1089/ars.2018.7632
- Dillmann W. H. (2019). Diabetic Cardiomyopathy. *Circulation research*, 124(8), 1160–1162. doi.org/10.1161/CIRCRESAHA.118.314665
- Eisner, D. A., Caldwell, J. L., Kistamás, K., & Trafford, A. W. (2017). Calcium and Excitation-Contraction Coupling in the Heart. *Circulation research*, 121(2), 181–195. doi.org/10.1161/CIRCRESAHA.117.310230
- Erickson, J. R., Joiner, M. L., Guan, X., Kutschke, W., Yang, J., Oddis, C. V., Bartlett, R. K., Lowe, J. S., O'Donnell, S. E., Aykin-Burns, N., Zimmerman, M. C., Zimmerman, K., Ham, A. J., Weiss, R. M., Spitz, D. R., Shea, M. A., Colbran, R. J., Mohler, P. J., & Anderson, M. E. (2008). A dynamic pathway for calcium-independent activation of CaMKII by methionine oxidation. *Cell*, 133(3), 462–474. doi.org/10.1016/j.cell.2008.02.048
- Erickson, J. R., Pereira, L., Wang, L., Han, G., Ferguson, A., Dao, K., Copeland, R. J., Despa, F., Hart, G. W., Ripplinger, C. M., & Bers, D. M. (2013). Diabetic

hyperglycaemia activates CaMKII and arrhythmias by O-linked glycosylation. *Nature*, 502(7471), 372–376. doi.org/10.1038/nature12537

Erickson, J. R., Nichols, C. B., Uchinoumi, H., Stein, M. L., Bossuyt, J., & Bers, D. M. (2015). S-Nitrosylation Induces Both Autonomous Activation and Inhibition of Calcium/Calmodulin-dependent Protein Kinase II δ . *The Journal of biological chemistry*, 290(42), 25646–25656. https://doi.org/10.1074/jbc.M115.650234

Fitchett, D., Inzucchi, S. E., Cannon, C. P., McGuire, D. K., Scirica, B. M., Johansen, O. E., Sambeviski, S., Kaspers, S., Pfarr, E., George, J. T., & Zinman, B. (2019). Empagliflozin Reduced Mortality and Hospitalization for Heart Failure Across the Spectrum of Cardiovascular Risk in the EMPA-REG OUTCOME Trial. *Circulation*, 139(11), 1384–1395. doi.org/10.1161/CIRCULATIONAHA.118.037778

Fitchett, D., Zinman, B., Wanner, C., Lachin, J. M., Hantel, S., Salsali, A., Johansen, O. E., Woerle, H. J., Broedl, U. C., Inzucchi, S. E., & EMPA-REG OUTCOME® trial investigators (2016). Heart failure outcomes with empagliflozin in patients with type 2 diabetes at high cardiovascular risk: results of the EMPA-REG OUTCOME® trial. *European heart journal*, 37(19), 1526–1534. doi.org/10.1093/eurheartj/ehv728

Ghodeswar, S., & Bandyopadhyay, D. (2025). ROSBASE1.0: a comprehensive database of reactive oxygen species (ROS): categorization of cell organelles, proteins, taxonomy, and diseases based on ROS-related activities. *Database : the journal of biological databases and curation*, 2025, baaf069. doi.org/10.1093/database/baaf069

- Giacco, F., & Brownlee, M. (2010). Oxidative stress and diabetic complications. *Circulation research*, 107(9), 1058–1070.
doi.org/10.1161/CIRCRESAHA.110.223545
- González, P., Lozano, P., Ros, G., & Solano, F. (2023). Hyperglycemia and Oxidative Stress: An Integral, Updated and Critical Overview of Their Metabolic Interconnections. *International journal of molecular sciences*, 24(11), 9352.
doi.org/10.3390/ijms24119352
- Gulsin, G. S., Swarbrick, D. J., Hunt, W. H., Levelt, E., Graham-Brown, M. P. M., Parke, K. S., Wormleighton, J. V., Lai, F. Y., Yates, T., Wilmot, E. G., Webb, D. R., Davies, M. J., & McCann, G. P. (2018). Relation of Aortic Stiffness to Left Ventricular Remodeling in Younger Adults With Type 2 Diabetes. *Diabetes*, 67(7), 1395–1400.
doi.org/10.2337/db18-0112
- Guo, Z., Xia, Z., Jiang, J., & McNeill, J. H. (2007). Downregulation of NADPH oxidase, antioxidant enzymes, and inflammatory markers in the heart of streptozotocin-induced diabetic rats by N-acetyl-L-cysteine. *American journal of physiology. Heart and circulatory physiology*, 292(4), H1728–H1736.
doi.org/10.1152/ajpheart.01328.2005
- Gutscher, M., Pauleau, A. L., Marty, L., Brach, T., Wabnitz, G. H., Samstag, Y., Meyer, A. J., & Dick, T. P. (2008). Real-time imaging of the intracellular glutathione redox potential. *Nature methods*, 5(6), 553–559. doi.org/10.1038/nmeth.1212

- Hadd, M. J., Bienhoff, S. E., Little, S. E., Geller, S., Ogne-Stevenson, J., Dupree, T. J., & Scott-Moncrieff, J. C. (2023). Safety and effectiveness of the sodium-glucose cotransporter inhibitor bexagliflozin in cats newly diagnosed with diabetes mellitus. *Journal of veterinary internal medicine*, 37(3), 915–924.
doi.org/10.1111/jvim.16730
- Hart G. W. (2014). Minireview series on the thirtieth anniversary of research on O-GlcNAcylation of nuclear and cytoplasmic proteins: Nutrient regulation of cellular metabolism and physiology by O-GlcNAcylation. *The Journal of biological chemistry*, 289(50), 34422–34423. doi.org/10.1074/jbc.R114.609776
- Hegyi, B. & Bers, D. M. (2022). Fixing a current problem in the cardiac Na channel. *Nature cardiovascular research*, 1(5), 408–409. doi.org/10.1038/s44161-022-00070-4
- Hegyi, B. & Bers, D. M. (2023). New cardiac targets for empagliflozin: O-GlcNAcylation, CaMKII, and calcium handling. *American journal of physiology. Heart and circulatory physiology*, 324(3), H338–H340. doi.org/10.1152/ajpheart.00003.2023
- Hegyi, B., Bers, D. M., & Bossuyt, J. (2019). CaMKII signaling in heart diseases: Emerging role in diabetic cardiomyopathy. *Journal of molecular and cellular cardiology*, 127, 246–259. doi.org/10.1016/j.yjmcc.2019.01.001
- Hegyi, B., Fasoli, A., Ko, C. Y., Van, B. W., Alim, C. C., Shen, E. Y., Ciccozzi, M. M., Tapa, S., Ripplinger, C. M., Erickson, J. R., Bossuyt, J., & Bers, D. M. (2021). CaMKII Serine 280 O-GlcNAcylation Links Diabetic Hyperglycemia to

Proarrhythmia. *Circulation research*, 129(1), 98–113.

doi.org/10.1161/CIRCRESAHA.120.318402

Hegyi, B., Ko, C. Y., Bossuyt, J., & Bers, D. M. (2021). Two-hit mechanism of cardiac arrhythmias in diabetic hyperglycaemia: reduced repolarization reserve, neurohormonal stimulation, and heart failure exacerbate susceptibility.

Cardiovascular research, 117(14), 2781–2793. doi.org/10.1093/cvr/cvab006

Hegyi, B., Mira Hernandez, J., Ko, C. Y., Hong, J., Shen, E. Y., Spencer, E. R., Smoliarchuk, D., Navedo, M. F., Bers, D. M., & Bossuyt, J. (2022). Diabetes and Excess Aldosterone Promote Heart Failure With Preserved Ejection Fraction. *Journal of the American Heart Association*, 11(23), e027164.

<https://doi.org/10.1161/JAHA.122.027164>

Hegyi, B., Mira Hernandez, J., Shen, E. Y., Habibi, N. R., Bossuyt, J., & Bers, D. M. (2022). Empagliflozin Reverses Late Na⁺ Current Enhancement and Cardiomyocyte Proarrhythmia in a Translational Murine Model of Heart Failure With Preserved Ejection Fraction. *Circulation*, 145(13), 1029–1031.

doi.org/10.1161/CIRCULATIONAHA.121.057237

Hegyi, B., Pölönen, R. P., Hellgren, K. T., Ko, C. Y., Ginsburg, K. S., Bossuyt, J., Mercola, M., & Bers, D. M. (2021). Cardiomyocyte Na⁺ and Ca²⁺ mishandling drives vicious cycle involving CaMKII, ROS, and ryanodine receptors. *Basic research in cardiology*, 116(1), 58. doi.org/10.1007/s00395-021-00900-9

- Hegyi, B., Bailey, L. R. J., Mira Hernandez, J., Ko, C. Y., Shen, E. Y., Bossuyt, J., Davis, J. M., & Bers, D. M. (2026). Excitation-contraction coupling, cardiomyocyte electrophysiology, and transcriptome profiles in two HFpEF murine models: etiology and sex-dependent differences. *American journal of physiology. Heart and circulatory physiology*, 330(2), H348–H366. doi.org/10.1152/ajpheart.00227.2025
- Heymes, C., Bendall, J. K., Ratajczak, P., Cave, A. C., Samuel, J. L., Hasenfuss, G., & Shah, A. M. (2003). Increased myocardial NADPH oxidase activity in human heart failure. *Journal of the American College of Cardiology*, 41(12), 2164–2171. [https://doi.org/10.1016/s0735-1097\(03\)00471-6](https://doi.org/10.1016/s0735-1097(03)00471-6)
- Hoehlschen, J., Gosset, É., Hofreither, D., Jahnel, S., Mendjan, S., Schittmayer, M., Tomin, T., & Birner-Gruenberger, R. (2025). Heart saver: Comprehensive investigation of (redox-) proteomic and thiol metabolite changes induced by Cana-, Dapa-, Empagliflozin treatment in 2D and 3D heart cell models reveals increased mitochondrial activity and glutathione redox defense and involvement of redox signaling. *Life sciences*, 380, 123923. doi.org/10.1016/j.lfs.2025.123923
- Hoelz, A., Nairn, A. C., & Kuriyan, J. (2003). Crystal structure of a tetradecameric assembly of the association domain of Ca²⁺/calmodulin-dependent kinase II. *Molecular cell*, 11(5), 1241–1251. doi.org/10.1016/s1097-2765(03)00171-0
- Hudmon, A., & Schulman, H. (2002). Structure-function of the multifunctional Ca²⁺/calmodulin-dependent protein kinase II. *The Biochemical journal*, 364(Pt 3), 593–611. doi.org/10.1042/BJ20020228

- Jia, G., Hill, M. A., & Sowers, J. R. (2018). Diabetic Cardiomyopathy: An Update of Mechanisms Contributing to This Clinical Entity. *Circulation research*, 122(4), 624–638. doi.org/10.1161/CIRCRESAHA.117.311586
- Jubaidi, F. F., Zainalabidin, S., Mariappan, V., & Budin, S. B. (2020). Mitochondrial Dysfunction in Diabetic Cardiomyopathy: The Possible Therapeutic Roles of Phenolic Acids. *International journal of molecular sciences*, 21(17), 6043. doi.org/10.3390/ijms21176043
- Kadosaka, T., Watanabe, M., Natsui, H., Koizumi, T., Nakao, M., Koya, T., Hagiwara, H., Kamada, R., Temma, T., Karube, F., Fujiyama, F., & Anzai, T. (2023). Empagliflozin attenuates arrhythmogenesis in diabetic cardiomyopathy by normalizing intracellular Ca^{2+} handling in ventricular cardiomyocytes. *American journal of physiology. Heart and circulatory physiology*, 324(3), H341–H354. doi.org/10.1152/ajpheart.00391.2022
- Kassmann, M., Hansel, A., Leipold, E., Birkenbeil, J., Lu, S. Q., Hoshi, T., & Heinemann, S. H. (2008). Oxidation of multiple methionine residues impairs rapid sodium channel inactivation. *Pflugers Archiv : European journal of physiology*, 456(6), 1085–1095. doi.org/10.1007/s00424-008-0477-6
- Kirchhefer, U., Schmitz, W., Scholz, H., & Neumann, J. (1999). Activity of cAMP-dependent protein kinase and Ca^{2+} /calmodulin-dependent protein kinase in failing and nonfailing human hearts. *Cardiovascular research*, 42(1), 254–261. doi.org/10.1016/s0008-6363(98)00296-x

- Köhler, A. C., Sag, C. M., & Maier, L. S. (2014). Reactive oxygen species and excitation-contraction coupling in the context of cardiac pathology. *Journal of molecular and cellular cardiology*, 73, 92–102. doi.org/10.1016/j.yjmcc.2014.03.001
- Kolwicz, S. C., Jr, Purohit, S., & Tian, R. (2013). Cardiac metabolism and its interactions with contraction, growth, and survival of cardiomyocytes. *Circulation research*, 113(5), 603–616. doi.org/10.1161/CIRCRESAHA.113.302095
- Kushnir, A., Shan, J., Betzenhauser, M. J., Reiken, S., & Marks, A. R. (2010). Role of CaMKII δ phosphorylation of the cardiac ryanodine receptor in the force frequency relationship and heart failure. *Proceedings of the National Academy of Sciences of the United States of America*, 107(22), 10274–10279. doi.org/10.1073/pnas.1005843107
- Laddha, A. P., & Kulkarni, Y. A. (2020). NADPH oxidase: A membrane-bound enzyme and its inhibitors in diabetic complications. *European journal of pharmacology*, 881, 173206. doi.org/10.1016/j.ejphar.2020.173206
- Lee, Techung Lee. O-GlcNAcase Assay Kit. SUNY Buffalo. www.bmrservice.com/ogaassay.html
- Leem, C. H., Lagadic-Gossman, D., & Vaughan-Jones, R. D. (1999). Characterization of intracellular pH regulation in the guinea-pig ventricular myocyte. *The Journal of physiology*, 517 (Pt 1)(Pt 1), 159–180. doi.org/10.1111/j.1469-7793.1999.0159z.x
- Li, Q., Su, D., O'Rourke, B., Pogwizd, S. M., & Zhou, L. (2015). Mitochondria-derived ROS bursts disturb Ca²⁺ cycling and induce abnormal automaticity in guinea pig

cardiomyocytes: a theoretical study. *American journal of physiology. Heart and circulatory physiology*, 308(6), H623–H636. doi.org/10.1152/ajpheart.00493.2014

Li, X., Lu, Q., Qiu, Y., do Carmo, J. M., Wang, Z., da Silva, A. A., Mouton, A., Omoto, A. C. M., Hall, M. E., Li, J., & Hall, J. E. (2021). Direct Cardiac Actions of the Sodium Glucose Co-Transporter 2 Inhibitor Empagliflozin Improve Myocardial Oxidative Phosphorylation and Attenuate Pressure-Overload Heart Failure. *Journal of the American Heart Association*, 10(6), e018298. doi.org/10.1161/JAHA.120.018298

Liang, J., Wu, M., Chen, C., Mai, M., Huang, J., & Zhu, P. (2020). Roles of Reactive Oxygen Species in Cardiac Differentiation, Reprogramming, and Regenerative Therapies. *Oxidative medicine and cellular longevity*, 2020, 2102841. doi.org/10.1155/2020/2102841

Lim, G., Venetucci, L., Eisner, D. A., & Casadei, B. (2008). Does nitric oxide modulate cardiac ryanodine receptor function? Implications for excitation-contraction coupling. *Cardiovascular research*, 77(2), 256–264. doi.org/10.1093/cvr/cvm012

Lima, J.E.B.F., Moreira, N.C.S., & Sakamoto-Hojo, E.T. (2022). Mechanisms Underlying the Pathophysiology of Type 2 Diabetes: From Risk Factors to Oxidative Stress, Metabolic Dysfunction, and Hyperglycemia. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 874–875: 503437. doi.org/10.1016/j.mrgentox.2021.503437

Liu, M. B., de Lange, E., Garfinkel, A., Weiss, J. N., & Qu, Z. (2015). Delayed afterdepolarizations generate both triggers and a vulnerable substrate promoting

reentry in cardiac tissue. *Heart rhythm*, 12(10), 2115–2124.

doi.org/10.1016/j.hrthm.2015.06.019

Liu, R., Cao, X., Fan, Z., Tian, Y., Li, G., Shen, Y., Guo, L., Zhang, J., & Qi, Z. (2026).

Hyperglycemia Aggravates Cardiomyocyte Oxidative Stress via Caveolin-

1/Nrf2/Keap1 Signaling Axis Activation. *Endocrine and Metabolic Science* 20:

100286. doi.org/10.1016/j.endmts.2025.100286

Lopaschuk, G. D., & Verma, S. (2020). Mechanisms of Cardiovascular Benefits of

Sodium Glucose Co-Transporter 2 (SGLT2) Inhibitors: A State-of-the-Art

Review. *JACC. Basic to translational science*, 5(6), 632–644.

doi.org/10.1016/j.jacbts.2020.02.004

Lu, S., Baier, M. J., Polonen, R. P., Liao, Z., Martin, J. L., Ginsburg, K. S., Mustroph, J.,

& Bers, D. M. (2025). Hyperglycaemia-induced reactive oxygen species production

in cardiac ventricular myocytes differs among mammals. *The Journal of physiology*.

doi.org/10.1113/JP287886

Lu, S., Liao, Z., Lu, X., Katschinski, D. M., Mercola, M., Chen, J., Heller Brown, J.,

Molkentin, J. D., Bossuyt, J., & Bers, D. M. (2020). Hyperglycemia Acutely Increases

Cytosolic Reactive Oxygen Species via O-linked GlcNAcylation and CaMKII

Activation in Mouse Ventricular Myocytes. *Circulation research*, 126(10), e80–e96.

doi.org/10.1161/CIRCRESAHA.119.316288

- Luczak, E. D., & Anderson, M. E. (2014). CaMKII oxidative activation and the pathogenesis of cardiac disease. *Journal of molecular and cellular cardiology*, 73, 112–116. doi.org/10.1016/j.yjmcc.2014.02.004
- Luo, M., Guan, X., Luczak, E. D., Lang, D., Kutschke, W., Gao, Z., Yang, J., Glynn, P., Sossalla, S., Swaminathan, P. D., Weiss, R. M., Yang, B., Rokita, A. G., Maier, L. S., Efimov, I. R., Hund, T. J., & Anderson, M. E. (2013). Diabetes increases mortality after myocardial infarction by oxidizing CaMKII. *The Journal of clinical investigation*, 123(3), 1262–1274. doi.org/10.1172/JCI65268
- MacDonald, M. R., Petrie, M. C., Varyani, F., Ostergren, J., Michelson, E. L., Young, J. B., Solomon, S. D., Granger, C. B., Swedberg, K., Yusuf, S., Pfeffer, M. A., McMurray, J. J., & CHARM Investigators (2008). Impact of diabetes on outcomes in patients with low and preserved ejection fraction heart failure: an analysis of the Candesartan in Heart failure: Assessment of Reduction in Mortality and morbidity (CHARM) programme. *European heart journal*, 29(11), 1377–1385. doi.org/10.1093/eurheartj/ehn153
- Maier, L.S., & Bers, D.M. (2007). Role of Ca²⁺/Calmodulin-Dependent Protein Kinase (CaMK) in Excitation–Contraction Coupling in the Heart. *Cardiovascular Research*, 73(4), 631–40. doi.org/10.1016/j.cardiores.2006.11.005
- Makielski, J.C. (2016). Late Sodium Current: A Mechanism for Angina, Heart Failure, and Arrhythmia. *Trends in Cardiovascular Medicine*, 26(2), 115–22. doi.org/10.1016/j.tcm.2015.05.006

- Mazumder, P. K., O'Neill, B. T., Roberts, M. W., Buchanan, J., Yun, U. J., Cooksey, R. C., Boudina, S., & Abel, E. D. (2004). Impaired cardiac efficiency and increased fatty acid oxidation in insulin-resistant ob/ob mouse hearts. *Diabetes*, 53(9), 2366–2374. doi.org/10.2337/diabetes.53.9.2366
- Mengstie, M. A., Abebe, E. C., Teklemariam, A. B., Mulu, A. T., Teshome, A. A., Zewde, E. A., Muche, Z. T., & Azezew, M. T. (2022). Molecular and cellular mechanisms in diabetic heart failure: Potential therapeutic targets. *Frontiers in endocrinology*, 13, 947294. doi.org/10.3389/fendo.2022.947294
- Moellmann, J., Klinkhammer, B. M., Droste, P., Kappel, B., Haj-Yehia, E., Maxeiner, S., Artati, A., Adamski, J., Boor, P., Schütt, K., Lopaschuk, G. D., Verma, S., Marx, N., & Lehrke, M. (2020). Empagliflozin improves left ventricular diastolic function of db/db mice. *Biochimica et biophysica acta. Molecular basis of disease*, 1866(8), 165807. doi.org/10.1016/j.bbadis.2020.165807
- Murphy, E., & Eisner, D.A. (2021). Does the Cardioprotective Effect of Empagliflozin Involve Inhibition of the Sodium–Proton Exchanger?. *Cardiovascular Research*, 117(14), 2709–2711. doi.org/10.1093/cvr/cvab137
- Mustroph, J., Wagemann, O., Lücht, C. M., Trum, M., Hammer, K. P., Sag, C. M., Lebek, S., Tarnowski, D., Reinders, J., Perbellini, F., Terracciano, C., Schmid, C., Schopka, S., Hilker, M., Zausig, Y., Pabel, S., Sossalla, S. T., Schweda, F., Maier, L. S., & Wagner, S. (2018). Empagliflozin reduces Ca/calmodulin-dependent kinase II activity in isolated ventricular cardiomyocytes. *ESC heart failure*, 5(4), 642–648. doi.org/10.1002/ehf2.12336

“NADH Oxidase (NOX) Activity Assay Kit - Elabscience®.”

www.elabscience.com/p/nadh-oxidase-nox-activity-assay-kit--e-bc-k806-m

Neal, B., Perkovic, V., Mahaffey, K.W., de Zeeuw, D., Fulcher, G., Erond, N., Shaw, W., Law, G., Desai, M., Matthews, D.R. (2017). Canagliflozin and Cardiovascular and Renal Events in Type 2 Diabetes. *The New England Journal of Medicine*, 377(7), 644–57. doi.org/10.1056/NEJMoa1611925

Nichols, G. A., Gullion, C. M., Koro, C. E., Ephross, S. A., & Brown, J. B. (2004). The incidence of congestive heart failure in type 2 diabetes: an update. *Diabetes care*, 27(8), 1879–1884. doi.org/10.2337/diacare.27.8.1879

Njegic, A., Wilson, C., & Cartwright, E. J. (2020). Targeting Ca^{2+} Handling Proteins for the Treatment of Heart Failure and Arrhythmias. *Frontiers in physiology*, 11, 1068. doi.org/10.3389/fphys.2020.01068

Oda, T., Yang, Y., Uchinoumi, H., Thomas, D. D., Chen-Izu, Y., Kato, T., Yamamoto, T., Yano, M., Cornea, R. L., & Bers, D. M. (2015). Oxidation of ryanodine receptor (RyR) and calmodulin enhance Ca release and pathologically alter RyR structure and calmodulin affinity. *Journal of molecular and cellular cardiology*, 85, 240–248. doi.org/10.1016/j.yjmcc.2015.06.009

Ohkuma, T., Komorita, Y., Peters, S. A. E., & Woodward, M. (2019). Diabetes as a risk factor for heart failure in women and men: a systematic review and meta-analysis of 47 cohorts including 12 million individuals. *Diabetologia*, 62(9), 1550–1560. <https://doi.org/10.1007/s00125-019-4926-x>

“OSMI-1 | O-GlcNAc Transferase Inhibitor | MedChemExpress.”

www.medchemexpress.com/osmi-1.

Packer, M., Anker, S. D., Butler, J., Filippatos, G., Ferreira, J. P., Pocock, S. J., Carson, P., Anand, I., Doehner, W., Haass, M., Komajda, M., Miller, A., Pehrson, S., Teerlink, J. R., Brueckmann, M., Jamal, W., Zeller, C., Schnaidt, S., & Zannad, F. (2021). Effect of Empagliflozin on the Clinical Stability of Patients With Heart Failure and a Reduced Ejection Fraction: The EMPEROR-Reduced Trial. *Circulation*, 143(4), 326–336.

doi.org/10.1161/CIRCULATIONAHA.120.051783

Packer, M., Butler, J., Zannad, F., Filippatos, G., Ferreira, J. P., Pocock, S. J., Carson, P., Anand, I., Doehner, W., Haass, M., Komajda, M., Miller, A., Pehrson, S., Teerlink, J. R., Schnaidt, S., Zeller, C., Schnee, J. M., & Anker, S. D. (2021). Effect of Empagliflozin on Worsening Heart Failure Events in Patients With Heart Failure and Preserved Ejection Fraction: EMPEROR-Preserved Trial. *Circulation*, 144(16), 1284–1294. doi.org/10.1161/CIRCULATIONAHA.121.056824

Padda, I.S., Mahtani, A.U., & Parmar, M. (2025). Sodium-Glucose Transport 2 (SGLT2) Inhibitors. StatPearls [Internet], Treasure Island (FL): StatPearls Publishing. www.ncbi.nlm.nih.gov/books/NBK576405/

Pellicori, P., Ofstad, A. P., Fitchett, D., Zeller, C., Wanner, C., George, J., Zinman, B., Brueckmann, M., & Lindenfeld, J. (2020). Early benefits of empagliflozin in patients with or without heart failure: findings from EMPA-REG OUTCOME. *ESC heart failure*, 7(6), 3401–3407. doi.org/10.1002/ehf2.12891

- Peoples, J.N., Saraf, A., Ghazal, N., Pham, T.T., & Kwong, J.Q. (2019). Mitochondrial Dysfunction and Oxidative Stress in Heart Disease. *Experimental & Molecular Medicine*, 51(12), 1–13. doi.org/10.1038/s12276-019-0355-7
- Pereira, L., Cheng, H., Lao, D. H., Na, L., van Oort, R. J., Brown, J. H., Wehrens, X. H., Chen, J., & Bers, D. M. (2013). Epac2 mediates cardiac β 1-adrenergic-dependent sarcoplasmic reticulum Ca^{2+} leak and arrhythmia. *Circulation*, 127(8), 913–922. doi.org/10.1161/CIRCULATIONAHA.12.148619
- Phaniendra, A., Jestadi, D. B., & Periyasamy, L. (2015). Free radicals: properties, sources, targets, and their implication in various diseases. *Indian journal of clinical biochemistry : IJCB*, 30(1), 11–26. doi.org/10.1007/s12291-014-0446-0
- Philippaert, K., Kalyaanamoorthy, S., Fatehi, M., Long, W., Soni, S., Byrne, N. J., Barr, A., Singh, J., Wong, J., Palechuk, T., Schneider, C., Darwesh, A. M., Maayah, Z. H., Seubert, J. M., Barakat, K., Dyck, J. R. B., & Light, P. E. (2021). Cardiac Late Sodium Channel Current Is a Molecular Target for the Sodium/Glucose Cotransporter 2 Inhibitor Empagliflozin. *Circulation*, 143(22), 2188–2204. doi.org/10.1161/CIRCULATIONAHA.121.053350
- “Pierce™ BCA Protein Assay Kits 1 Kit (1 L) | Buy Online | Thermo Scientific™.” www.thermofisher.com/order/catalog/product/23225 (May 5, 2025).
- Rastogi, R., Geng, X., Li, F., & Ding, Y. (2017). NOX Activation by Subunit Interaction and Underlying Mechanisms in Disease. *Frontiers in cellular neuroscience*, 10, 301. doi.org/10.3389/fncel.2016.00301.

- “Recombinant Human O-GlcNAcTransferase/OGT Protein, CF 8446-GT: R&D Systems.” www.rndsystems.com/products/recombinant-human-o-glcnactransferase-ogt-protein-cf_8446-gt
- Rellos, P., Pike, A. C., Niesen, F. H., Salah, E., Lee, W. H., von Delft, F., & Knapp, S. (2010). Structure of the CaMKII δ /calmodulin complex reveals the molecular mechanism of CaMKII kinase activation. *PLoS biology*, 8(7), e1000426. doi.org/10.1371/journal.pbio.1000426
- Roh, J., Hill, J. A., Singh, A., Valero-Muñoz, M., & Sam, F. (2022). Heart Failure With Preserved Ejection Fraction: Heterogeneous Syndrome, Diverse Preclinical Models. *Circulation research*, 130(12), 1906–1925. doi.org/10.1161/CIRCRESAHA.122.320257
- Rosano, G. M., Vitale, C., & Seferovic, P. (2017). Heart Failure in Patients with Diabetes Mellitus. *Cardiac failure review*, 3(1), 52–55. doi.org/10.15420/cfr.2016:20:2
- Schiattarella, G. G., Altamirano, F., Tong, D., French, K. M., Villalobos, E., Kim, S. Y., Luo, X., Jiang, N., May, H. I., Wang, Z. V., Hill, T. M., Mammen, P. P. A., Huang, J., Lee, D. I., Hahn, V. S., Sharma, K., Kass, D. A., Lavandro, S., Gillette, T. G., & Hill, J. A. (2019). Nitrosative stress drives heart failure with preserved ejection fraction. *Nature*, 568(7752), 351–356. doi.org/10.1038/s41586-019-1100-z
- Schneider, A., Hage, A., Stein, I. C. A. P., Kriedemann, N., Zweigerdt, R., & Leffler, A. (2024). A Possible Role of Tetrodotoxin-Sensitive Na⁺ Channels for Oxidation-

Induced Late Na⁺ Currents in Cardiomyocytes. *International journal of molecular sciences*, 25(12), 6596. doi.org/10.3390/ijms25126596

Schulman, H., & Anderson, M. E. (2010). Ca/Calmodulin-dependent Protein Kinase II in Heart Failure. *Drug discovery today. Disease mechanisms*, 7(2), e117–e122. doi.org/10.1016/j.ddmec.2010.07.005

Shindler, D. M., Kostis, J. B., Yusuf, S., Quinones, M. A., Pitt, B., Stewart, D., Pinkett, T., Ghali, J. K., & Wilson, A. C. (1996). Diabetes mellitus, a predictor of morbidity and mortality in the Studies of Left Ventricular Dysfunction (SOLVD) Trials and Registry. *The American journal of cardiology*, 77(11), 1017–1020. doi.org/10.1016/s0002-9149(97)89163-1

Skulachev V. P. (1999). Anion carriers in fatty acid-mediated physiological uncoupling. *Journal of bioenergetics and biomembranes*, 31(5), 431–445. doi.org/10.1023/a:1005492205984

Solaro R. J. (2010). Sarcomere control mechanisms and the dynamics of the cardiac cycle. *Journal of biomedicine & biotechnology*, 2010, 105648. doi.org/10.1155/2010/105648

Steinhorn, B., Sorrentino, A., Badole, S., Bogdanova, Y., Belousov, V., & Michel, T. (2018). Chemogenetic generation of hydrogen peroxide in the heart induces severe cardiac dysfunction. *Nature communications*, 9(1), 4044. doi.org/10.1038/s41467-018-06533-2

- Swain, L., Kesemeyer, A., Meyer-Roxlau, S., Vettel, C., Zieseniss, A., Güntsch, A., Jatho, A., Becker, A., Nanadikar, M. S., Morgan, B., Dennerlein, S., Shah, A. M., El-Armouche, A., Nikolaev, V. O., & Katschinski, D. M. (2016). Redox Imaging Using Cardiac Myocyte-Specific Transgenic Biosensor Mice. *Circulation research*, 119(9), 1004–1016. doi.org/10.1161/CIRCRESAHA.116.309551
- Thrainsdottir, I. S., Aspelund, T., Thorgeirsson, G., Gudnason, V., Hardarson, T., Malmberg, K., Sigurdsson, G., & Rydén, L. (2005). The association between glucose abnormalities and heart failure in the population-based Reykjavik study. *Diabetes care*, 28(3), 612–616. doi.org/10.2337/diacare.28.3.612
- Tian, C. J., Zhang, J. H., Liu, J., Ma, Z., & Zhen, Z. (2021). Ryanodine receptor and immune-related molecules in diabetic cardiomyopathy. *ESC heart failure*, 8(4), 2637–2646. doi.org/10.1002/ehf2.13431
- Tsao, C. W., Aday, A. W., Almarzooq, Z. I., Anderson, C. A. M., Arora, P., Avery, C. L., Baker-Smith, C. M., Beaton, A. Z., Boehme, A. K., Buxton, A. E., Commodore-Mensah, Y., Elkind, M. S. V., Evenson, K. R., Eze-Nliam, C., Fugar, S., Generoso, G., Heard, D. G., Hiremath, S., Ho, J. E., Kalani, R., ... American Heart Association Council on Epidemiology and Prevention Statistics Committee and Stroke Statistics Subcommittee (2023). Heart Disease and Stroke Statistics-2023 Update: A Report From the American Heart Association. *Circulation*, 147(8), e93–e621. doi.org/10.1161/CIR.0000000000001123
- Uthman, L., Baartscheer, A., Bleijlevens, B., Schumacher, C. A., Fiolet, J. W. T., Koeman, A., Jancev, M., Hollmann, M. W., Weber, N. C., Coronel, R., & Zuurbier, C.

- J. (2018). Class effects of SGLT2 inhibitors in mouse cardiomyocytes and hearts: inhibition of Na^+/H^+ exchanger, lowering of cytosolic Na^+ and vasodilation. *Diabetologia*, 61(3), 722–726. doi.org/10.1007/s00125-017-4509-7
- Vasquez-Vivar, J., Kalyanaraman, B., & Kennedy, M. C. (2000). Mitochondrial aconitase is a source of hydroxyl radical. An electron spin resonance investigation. *The Journal of biological chemistry*, 275(19), 14064–14069. doi.org/10.1074/jbc.275.19.14064
- Vaughan-Jones, R. D., Eisner, D. A., & Lederer, W. J. (1987). Effects of changes of intracellular pH on contraction in sheep cardiac Purkinje fibers. *The Journal of general physiology*, 89(6), 1015–1032. doi.org/10.1085/jgp.89.6.1015
- Vaughan-Jones, R. D., Spitzer, K. W., & Swietach, P. (2009). Intracellular pH regulation in heart. *Journal of molecular and cellular cardiology*, 46(3), 318–331. doi.org/10.1016/j.yjmcc.2008.10.024
- Viatchenko-Karpinski, S., Korniyeyev, D., El-Bizri, N., Budas, G., Fan, P., Jiang, Z., Yang, J., Anderson, M. E., Shryock, J. C., Chang, C. P., Belardinelli, L., & Yao, L. (2014). Intracellular Na^+ overload causes oxidation of CaMKII and leads to Ca^{2+} mishandling in isolated ventricular myocytes. *Journal of molecular and cellular cardiology*, 76, 247–256. doi.org/10.1016/j.yjmcc.2014.09.009
- Wagner, S., Dybkova, N., Rasenack, E. C., Jacobshagen, C., Fabritz, L., Kirchhof, P., Maier, S. K., Zhang, T., Hasenfuss, G., Brown, J. H., Bers, D. M., & Maier, L. S. (2006). Ca^{2+} /calmodulin-dependent protein kinase II regulates cardiac Na^+

channels. *The Journal of clinical investigation*, 116(12), 3127–3138.

doi.org/10.1172/JCI26620

Wagner, S., Hacker, E., Grandi, E., Weber, S. L., Dybkova, N., Sossalla, S., Sowa, T., Fabritz, L., Kirchhof, P., Bers, D. M., & Maier, L. S. (2009). Ca/calmodulin kinase II differentially modulates potassium currents. *Circulation. Arrhythmia and electrophysiology*, 2(3), 285–294. doi.org/10.1161/CIRCEP.108.842799

Wan, B., LaNoue, K. F., Cheung, J. Y., & Scaduto, R. C., Jr (1989). Regulation of citric acid cycle by calcium. *The Journal of biological chemistry*, 264(23), 13430–13439.

Wang, P., Lloyd, S. G., Zeng, H., Bonen, A., & Chatham, J. C. (2005). Impact of altered substrate utilization on cardiac function in isolated hearts from Zucker diabetic fatty rats. *American journal of physiology. Heart and circulatory physiology*, 288(5), H2102–H2110. doi.org/10.1152/ajpheart.00935.2004

Yano, M., Okuda, S., Oda, T., Tokuhisa, T., Tateishi, H., Mochizuki, M., Noma, T., Doi, M., Kobayashi, S., Yamamoto, T., Ikeda, Y., Ohkusa, T., Ikemoto, N., & Matsuzaki, M. (2005). Correction of defective interdomain interaction within ryanodine receptor by antioxidant is a new therapeutic strategy against heart failure. *Circulation*, 112(23), 3633–3643. doi.org/10.1161/CIRCULATIONAHA.105.555623

Ye, Y., Li, J., & Yuan, Z. (2013). Effect of antioxidant vitamin supplementation on cardiovascular outcomes: a meta-analysis of randomized controlled trials. *PloS one*, 8(2), e56803. doi.org/10.1371/journal.pone.0056803

Zachara, N. E., & Hart, G. W. (2006). Cell signaling, the essential role of O-GlcNAc. *Biochimica et biophysica acta*, 1761(5-6), 599–617.

doi.org/10.1016/j.bbailip.2006.04.007

Zima, A. V., & Blatter, L. A. (2006). Redox regulation of cardiac calcium channels and transporters. *Cardiovascular research*, 71(2), 310–321.

doi.org/10.1016/j.cardiores.2006.02.019

Zinman, B., Wanner, C., Lachin, J. M., Fitchett, D., Bluhmki, E., Hantel, S., Mattheus, M., Devins, T., Johansen, O. E., Woerle, H. J., Broedl, U. C., Inzucchi, S. E., & EMPA-REG OUTCOME Investigators (2015). Empagliflozin, Cardiovascular Outcomes, and Mortality in Type 2 Diabetes. *The New England journal of medicine*, 373(22), 2117–2128. doi.org/10.1056/NEJMoa1504720

Ziolo, M. T., Kohr, M. J., & Wang, H. (2008). Nitric oxide signaling and the regulation of myocardial function. *Journal of molecular and cellular cardiology*, 45(5), 625–632.

doi.org/10.1016/j.yjmcc.2008.07.015