GLIAL GROWTH FACTOR 2 REGULATES GLUCOSE TRANSPORT IN CARDIAC MYOCYTES VIA AN AKT-DEPENDENT PATHWAY

By

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Bachelor of Science in Medical Molecular Biology

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2012

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
July, 2016

GLIAL GROWTH FACTOR 2 REGULATES

GLUCOSE TRANSPORT IN CARDIAC MYOCYTES

VIA AN AKT-DEPENDENT PATHWAY

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ACKNOWLEDGEMENTS

Praise God from whom all blessings flow! I want acknowledge the Creator and Sustainer of life as He has walked with me through this experience and is celebrating this accomplishment with me. As my rock and my redeemer, whom shall I fear?

My parents deserve more accolades than this page can hold, but I am so appreciative for their guidance, grounding, and loving kindness they have continued to shower upon me through the entirety of my life, not excluding this time as a graduate student. Alongside my parents, my siblings, Tanner and Trevor, have contributed to my success and intelligence; they have, through there example encouraged me to conquer life and live out my dreams. Family extends to such depths, in this, I want to thank Tawna and Bob for instilling in me a love for science and the study of life. Grandma Frances, you have given me a love for writing and communication that have been my greatest asset, alongside my faith, in my successes.

I can't begin to name every teacher and professor that has had a part in my story, but I will start by thanking Dr. Lacombe who has come along beside me and developed me beyond my expectations. My committee as well as the BMB faculty deserve many thanks for your insight and wisdom. Dr. Hartson, thank you for encouraging troubleshooting and developing my "logic brain." A special thanks to my lab members Allison, Zahra, Dominic and others who have helped with experiments, data generation, and my sanity.

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Date of Degree: JULY, 2016

Title of Study: GLIAL GROWTHFACTOR 2 REGULATES GLUCOSE TRANSPORT IN CARDIAC MYOCYTES VIA AN AKT-DEPENDENT PATHWAY

Major Field: BIOCHEMISTRY AND MOLECULAR BIOLOGY

Abstract: Neuregulin, a paracrine factor in myocytes, promotes cardiac development via the ErbB receptors. Neuregulin-1β also improves cardiac function and cell survival after myocardial infarction, although the molecular mechanisms underlying these cardio-protective effects are not well elucidated. Increased glucose uptake has been shown to be cardio-protective during myocardial infarction. Therefore, we hypothesized that acute treatment with neuregulin-1β3 isoform, glial growth factor 2 (GGF2; USAN - cimaglermin alfa), will enhance glucose transport in the myocardium through insulin and calcium-dependent pathways. Isolated cardiac myocytes from healthy adult rats were incubated for 1 hour with and without Afatinib, an ErbB 2/4 receptor blocker, and/or GGF2 for an additional hour (n=4-15/group). Glucose uptake was measured using a fluorescent D-glucose analog. The translocation of the glucose transporter (GLUT) 4 to the cell surface, the rate-limiting step in glucose uptake, was measured using a photolabeled biotinylation assay. Expression and phosphorylation of key proteins of the downstream insulin signaling pathway were measured by Western blotting. Similar to insulin, acute GGF2 treatment increased glucose uptake in healthy myocytes (by 49%, $P<0.05$). GGF2 and calcium also increased GLUT4 translocation in myocytes by 117% ($P<0.02$) and 511% ($P<0.001$), respectively; while ErbB 2/4 blockage blunted these effects. GGF2 also enhanced Akt phosphorylation (at both threonine and serine sites, by 75% and 139%, respectively, $P<0.05$), which was blunted by ErbB 2/4 blockage. In addition, GGF2 treatment increased the phosphorylation of AS160 (an Akt effector) by 72% ($P<0.05$), as well as the phosphorylation of PDK-1 and PKC (by 206% and 100%, respectively, $P<0.05$). In conclusion, our data demonstrate that acute GGF2 treatment increases GLUT4 translocation and thus glucose uptake, in cardiac myocytes by activating the ErbB 2/4 receptors and subsequently key insulin downstream effectors (i.e., PDK1, Akt, AS160 and PKC). Similarly, calcium-activated GLUT4 translocation is mediated by ErbB 2/4 receptor activation. These findings highlighted some novel mechanisms of action of GGF2, which warrant further investigation in patients with heart failure.

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APPENDIX A

List of abbreviations

18F-FDG	Fludeoxyglucose (^{18}F)
2-NBDG gluocose	1-N-7-(nitrobenz-2-oxa-1,3-diazol-4-yl)amino-2-deoxy-D-
3T3-L1	Cell line derived from mouse 3T3 cells (adipocytes)
ADAM	A-disintegrin and metalloprotease
Afat	Afatinib
AICAR activator	5-Aminoimidazole-4-carboxamide ribonucleotide, an AMPK
Akt	Protein kinase B isoform 2
AMP	Adenosine monophosphate
AMPK	5'-AMP-activated protein kinase
ANP	Atrial natriuretic peptide
ARIA	Acetylcholine receptor inducing activity
AS160	Akt substrate at 160kD
ATP	Adenosine triphosphate
Ca^{2+}	Calcium
CAMKII	Ca^{2+} /calmodulin-dependent protein kinase class II
CLSQ	Calsequestrin

DCM	Diabetic cardiomyopathy
EDL	Extensor digitorum longus
EGF	Epidermal growth factor
ERK	Extracellular signal-regulated kinase
FA	Fatty acid
GDP	Guanosine diphosphate
GGF	Glial growth factor
GLUT	Glucose transporter
GTP	Guanosine triphosphate
IP3	1,4,5-inositol-triphosphate
IR	Insulin receptor
IV	Intravenous
KI	Kinase-inactive
KO	Knock out
LC	LC Laboratories
LV	Left ventricle
MI	Myocardial infarcation
microPET	Positron emission tomography
mRNA	Messenger RNA
mTOR	Mechanistic target of rapamycin
NRG(-1)	Neuregulin
PDK-1	Pyruvate dehydrogenase lipoamide kinase isozyme 1
PI3K	Phosphoinositide 3-kinase
PKCζ	Protein kinase C zeta isoform
P-PCI	Primary Percutaneous Coronary Intervention

rhNRG-1β	recombinant neuregulin-1 beta
S	Selleck Chemicals
Ser473	Akt phosphorylation at serine site
T2DM	Type II diabetes mellitus
Th308	Akt phosphorylation at threonine site

CHAPTER I

INTRODUCTION

Neuregulins (NRGs) are signaling proteins that facilitate cell-cell interactions in the nervous system, heart, breast, and other organ systems (7, 8). Neuregulin (NRG)-1β, via its ErbB2 and ErbB4 receptors, is crucial for preservation of adult heart function. Although the inhibition of ErbB2 has been shown to significantly improve the overall survival of breast cancer patients, patients also develop side effects of cardiotoxicity, including left ventricular dysfunction and heart failure. This cardiac phenotype observed in ErbB2 inhibition elucidates the essential role of ErbB2, ErbB4, and NRG-1β in cardiac maintenance of the physiological function of an adult heart (9). Observations of NRG-1β's role in cell proliferation, differentiation, and survival have established its role as a therapeutic target for heart failure. Animal studies and ongoing clinical trials have demonstrated the beneficial effects of two forms of recombinant NRG-1β on cardiac function (10-13). Recombinant NRG-1β (rhNRG-1) has been evaluated as a potential therapy in many animal models of heart injury, including myocardial infarction, ischemia/reperfusion injury, diabetic cardiomyopathy, myocarditis, and chronic rapid pacing (14). Glial growth factor 2 (GGF2) is a full-length splice variant of the neuregulin-1 gene. In addition to the C-terminal EGF-like receptor binding domain, it

contains an N-terminal Ig-like domain with one or more heparin-binding sites and the isoform-specific Kringle domain (15). While it is known that the EGF-like domain is sufficient to bind and signal via an ErbB receptor network, it has been demonstrated that a single intravenous dose of GGF2 has parallel efficacy to the EGF-domain only fragment of rhNRG-1 which was given as daily intravenous infusions over 10 days (11, 12). In addition, GGF2 administered in patients with symptomatic heart failure improved ejection fraction at day 28 and day 90 (11, 12). Yet, the underlying mechanisms described in clinical and animal studies by which NRG-1β provides a cardioprotective function *in vitro* as well as its function enhancing property *in vivo* remain incompletely understood.

As the energetic demands of the heart are extreme (to sustain constant pumping), the heart has the highest rate of oxygen consumption per gram of any tissue in the body (16). Glucose is a primary energy substrate for the heart, which contributes to 30% of the heart's overall energy through glucose oxidation (16, 17). Even with its ability to utilize other substrates such as fatty acids, lactate, ketone bodies, and amino acids, the heart must utilize more glucose than skeletal muscle, lung, or adipose tissue in order to maintain homeostasis (18, 19). Therefore, glucose uptake is crucial to healthy cardiac function. Furthermore, there is a decrease in glucose uptake reported during myocardial infarction (MI), as early as 48 hours and up to 10 weeks post-infarction (20-22).

The translocation of the glucose transporter 4 (GLUT4), the major GLUT isoform, from an intracellular pool to the cell surface (active site) is the rate limiting step in glucose uptake, and is independently stimulated by insulin- and Ca 2+/ contraction- dependent processes (23). Interestingly, it has been reported that neuregulin stimulates glucose

transport in skeletal muscle cells via a PI3K-dependent pathway, activating known downstream effectors such as PI3K, PDK1, and PKC-ζ (24). Previous studies provide evidence that neuregulin's ErbB receptors during contraction are necessary for stimulation of glucose transport and a key component of energetic metabolism during muscle contraction. Furthermore, blockage of ErbB4 abruptly impairs contraction-induced glucose uptake in both slow and fast twitch muscle fibers. Therefore, evidence suggests that contraction-induced activation of neuregulin receptors is necessary for the stimulation of glucose transport and a key element of energetics during muscle contraction (24, 25). Yet, whether neuregulin has similar metabolic effects in the heart is unknown. Though the EGF-like domain alone is adequate to activate downstream ErbB signaling, the increased efficacy of a single IV dose GGF2 is more clinically appealing compared to a 10 day IV treatment with the EGF-domain of rhNRG-1. Therefore, we hypothesize that Glial growth factor 2 (GGF2; USAN -cimaglermin alfa) will increase glucose uptake in the healthy and failing myocardium via a PI3K-dependent mechanism.

CHAPTER II

REVIEW OF LITERATURE

2.1 Neuregulin-1β (Nrg-1)

Neuregulins (NRGs) are signaling proteins that facilitate cell-cell interactions in the nervous system, heart, breast, and other organ systems (7, 8). The NRG-1 gene is located on human chromosome 8 and is approximately 1.4 megabases long, of these, only 0.3% of this gene encodes protein (Figure 1, (3)). Alternative splicing and multiple promoters yield at least 15 different NRG isoforms are produced from the single NRG1

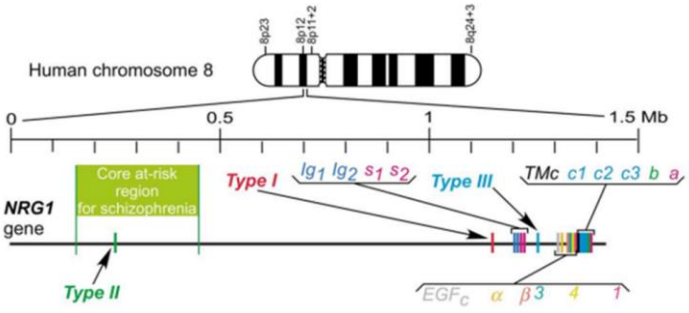

Figure 1: Human NRG1 gene structure (Genbank accession no. BK000383). The NRG1 gene is on the short arm of chromosome 8. On the expanded illustration of this region, the position of each exon included in reported NRG1 isoforms is indicated by a vertical line. Lines descending along the edge of the green box delineate the boundaries of the core at-risk haplotype for schizophrenia. Only the exon encoding the type II-specific N-terminal region lies within these bounds. Exon naming: Exons are named here for the structural region of the NRG1 protein they encode. Abbreviations used closely correspond to names of NRG protein structural regions indicated in panel B. EGFc refers to the exon encoding the portion of the EGF-like domain sequence shared by NRGs with an -type and NRGs with a -type EGF-like domain. The exon labeled TMc also includes adjacent extracellular juxtamembrane sequence and cytoplasmic tail sequence (3).

gene which begets multiple splice variants that can be divided into 3 types of isoforms (15, 26). Three structural characteristics that differentiate NRG isoforms with respect to *in vivo* functions and cell biological properties are the type of EGF-like domain (α or β), the N -terminal sequence (type I, II, or III), and whether the isoform is initially synthesized as a transmembrane or nonmembrane protein (**Figure 2, modified from (5)**). Furthermore, type I and II NRGs are characterized by an immunoglobulin-like domain and are single-pass transmembrane proteins while type III NRGs possess a cysteine-rich domain and are additionally characterized as 2-pass transmembrane proteins (7). These isoforms known as NDF (NEU differentiation factor (27)), heregulin (28), GGF (glial growth factor; (29)), ARIA (acetylcholine receptor inducing activity; (30)) or SMDF (sensory and motor neuron-derived factor; (31)) vary significantly in their size, structure, and tissue specificity. Soluble forms of the NRGs are produced by proteolytic processing in the juxtamembrane region of transmembrane precursors where proteolysis can take place either within the rough endoplasmic reticulum or at the cell membrane (32). The

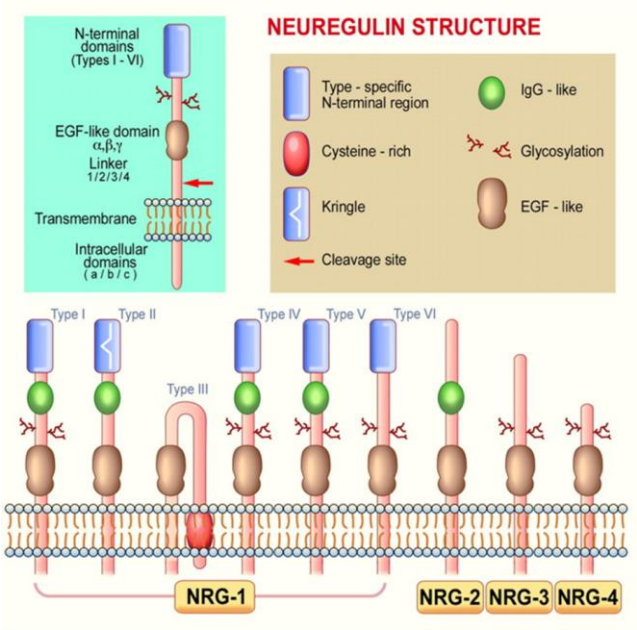

Figure 2: Illustration of NRG "coding segments." Isoforms differ in their coding segment composition due to initiation of transcription from different NRG1 gene promoters and alternative splicing. The EGF-like domain alone is sufficient for high potency activation of the cognate ErbB receptor tyrosine kinases. Not all potential combinations of coding segments have been reported (5).

production of an NRG bioactive fragment is credited to the proteolytic cleavage of type I and II neuregulin by participants of the α-disintegrin and metalloprotease (ADAM) family; including ADAM17 and ADAM19 (33, 34). Neuregulin type III cleavage creates a transmembrane N-terminal fragment (NTF) that accumulates at the cell surface. Another cleavage of the NTF in the type III NRGs near the membrane can release a fragment containing the EGF-like domain into the medium (35). Only a single domain of the NRG precursor, the EGF-like domain, has its bioactivity and importance fully appreciated. The EGF-like domain is so called due to its clear structural homology to the six-cysteine (joined in three disulfide bonds) bioactive moiety of EGF (36). All NRG isoforms that contain the EGF-like domain, including those that are transmembrane proteins, undergo alternative splicing, which yields α or β variants. Of the two variants, the β variant is considered to be the more active of the two, and it is reported that, when compared to the α, the β isoform is up to 100 times more active (7). The EGF-like domain contained in all bioactive NRG isoforms is alone sufficient for activation of ErbB receptor-tyrosine kinases (15). The stimulation of cell division or the induction of differentiation-specific genes are the known functions of the EGF-like domain alone (28, 36). As a member of the epidermal growth factor (EGF) family, NRG-1 has been reported both *in vitro* and *in vivo* of many cell types such as breast epithelial, glial, neurons, and myocytes to initiate proliferation, differentiation, and survival. (27-30). Intricately regulated by neurohormonal and biomechanical stimuli, NRG-1 is synthesized and released by endothelial cells within the myocardium (37). Upon binding to its tyrosine kinase receptors (ErbB2, ErbB3, and ErbB4), NRG-1 triggers their

phosphorylation and downstream cascades including the mTOR pathway for protein synthesis as well as JAK/STAT and RAS/ERK pathways for transcription (38).

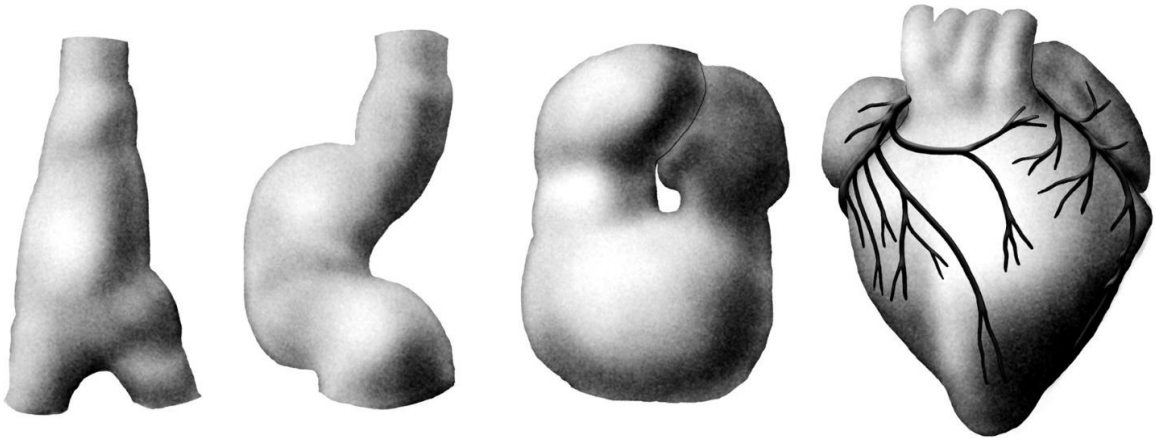

Figure 3: Four key stages in cardiac morphogenesis—tube, loop, chamber formation, completion of septation with deployment of coronary circulation. Art work by Ivan Helekal based on chick heart development studies by Manner14 and Sedmera et al. (1, 2)

2.1.1 NRG/ErbB roles in cardiac development

Function of the cardiovascular system is critical for embryonic survival and, as a consequence, the heart must beat to support circulation even before its morphogenesis is complete (39, 40). Both the function of the heart and its morphology influence the other's progress (39, 40). This intricate balance forces hearts into very similar functional designs that are conserved among widely divergent organisms. Yet, the developmental pathway between the simple cardiac tube and the mature four-chambered organ of mammals is rather convoluted **(Figure 3**, (1, 2)**).** Simply, I will briefly explain each of the four key stages in cardiac morphogenesis by developmental day and major characterizations of

each. The first cardiac contractions are recognized during the straight tube stage (**Figure 3**), approximately ED (embryonic development) 8.5 in the mouse (41) and 22 days in humans (42). These first heart beats are irregular and do not possess the force needed to propel blood through the circulation (43), yet activity advances rapidly to establish coordinated and primitive blood circulation throughout the embryo, thus driving development by decreasing diffusion distance for nutrients and oxygen. The next stage of embryonic cardiac morphogenesis transitions the straight tube to a looped heart (**Figure 3**) known as cardiac looping (44), and for some does not end with loop formation, but continues by further 'twisting' throughout the septation process (45). In homeotherm embryos, pumping function of the tubular heart is required from this stage on, ED10.5 in mice, for embryonic survival. Therefore, consequences of perturbations such as transgenic knockouts resulting in significant cardiac dysmorphogenesis cannot be analyzed in these model species (46). Morphological differentiation of myocardium along the cardiac tube becomes apparent soon after looping (47), characterized by the development of trabeculae, and, soon afterwards, pectinate muscles in the atria. These morphological developments are attended by changes in gene expression patterns (48).

Life without neuregulins is not possible. The expansive literature reveals how ubiquitous and necessary NRG signaling appears to be (**Table 1**, refer to appendix, (7)). NRGs function in vivo will be elucidated by describing the phenotypes of the NRG and ErbB knockouts. In vivo studies that blunted the expression of NRG-1, ErbB2, or ErbB4 in mice led to death in utero. This phenotype resulted from the failure of cardiac development of the endocardial cushions and trabeculae (49-51) **(Figure 4, (6)).** Both ErbB2 (50) and ErbB4 (49) KO mice exhibit analogous phenotypes of death at

embryonic day 10.5, the time at which mice embryos switch from dependence on maternal circulation to dependence on their own circulation. These phenotypes are a result of myocyte proliferation resulting in the thickening of the ventricular wall from lack of trabeculation as well as insufficient ability to maintain blood circulation due to the development of only a single-cell layer of myocytes forming the ventricle. (49, 50). Control littermates demonstrated expression of ErbB2 and ErbB4 receptors in the ventricular trabeculae determined by immunohistochemistry (49). Furthermore, ErbB2-deficient mice also exhibit failing endocardial cushions during fetal heart development (50). Similar to ErbB2 knockouts, mice deficient in NRG-1 acquire both trabeculae and endocardial cushion defects. Therefore, NRG-1 is required for both the development of trabeculae and endocardial cushion. Dying between days 10.5-11.5, NRG-1 null mice are comparable in size and overall embryonic structure to wild type, yet histological analysis revealed lack of trabeculation and an unclosed endocardial cushion yielding loss of function of the heart (51). The parallel phenotype

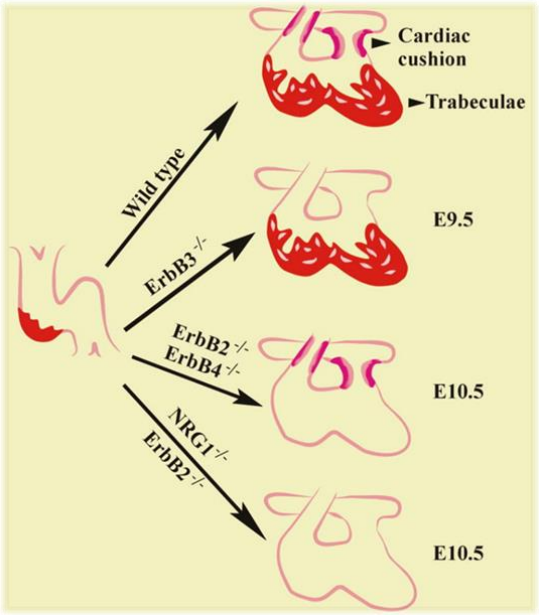

Figure 4: Role of Nrg-1/ErbB signaling during cardiac development. Wild type mouse heart develops cardiac cushion and trabeculae between day 9.5 and day 10.5 of fetal development. Lack of Nrg-1 effectively blocks the formations of such structures. Lack of ErbB2 leads to impaired formation of trabeculae and in some hearts also of cardiac cushion. ErbB4 null mice specifically display lack of trabeculation whereas ErbB3 null mice develop normal trabeculae but they do not form a normal cardiac cushion (6).

demonstrated between Nrg-1, ErbB2, and ErbB4 null mice as a requirement for ventricular trabeculation lend insight to these proteins' interactions and dependence on each other (6).

2.1.2 NRG-1/ErbB and cardiac structure and function

NRG-1 expression (52) alongside ErbB2 and ErbB4 receptors is also expressed in the adult heart, although ErbB3 ceases to be expressed (53). It has been reported that NRG-1 expression is specific to the endocardium and myocardial microvasculature, as it is not found in the coronary arteries, veins, or aorta (54). NRG-1/ErbB signaling is adaptive under a variety of circumstances through which it plays a role in regulating survival of cardiomyocytes (53, 55), angiogenesis (56), excitation-contraction (57), proliferation (53, 58), cell-to-cell contact between myocytes (59), and preventing oxidative stress induced apoptosis (60). Although ErbB2 does not bind NRG-1, it is the preferred heterodimer for both ErbB3 and ErbB4 and is necessary for ErbB signaling within the adult heart (61). Further reports confirm that not only is ErbB4 actively involved in cell protection (55, 60), but is also mediated by ErbB2 (54). These findings further validate protein interactions between NRG-1 and ErbB receptors described during embryonic cardiac development to be true in mediating NRG-1 signaling in the adult heart. Additional studies conditionally knocked out ErbB2 and ErbB4 in the post-natal heart (62, 63). I immunohistochemistry revealed not only abnormal distribution of the ErbB2 receptor but increased viniculin, a cytoskeletal protein associated with cell-cell and cell-matrix junctions, at the intercalated disks in ErbB4 knockout mice (62). ErbB2, ErbB4, and NRG-1 knockouts developed dilated cardiomyopathy, reduction of fractional shortening, and impaired contractility, alongside delayed conduction (62-64). The expression of atrial

natriuretic peptide (ANP) and skeletal a-actin genes in ErbB2 knockouts were congruous with heart failure development as well as the inability to survive aortic binding-induced pressure overload (65). Although the role of NRG-1, ErbB2, and ErbB4 has not been completely elucidated, it can be inferred that all are essential in the maintenance of ventricular structure and function in the neonatal and adult heart.

2.2 Pharmacological actions and therapeutic indications

2.2.1 Stroke and related neurological diseases

In 2012, an estimated 6.6 million Americans ≥20 years of age have had a stroke. Overall stroke prevalence during this period was an estimated 2.6% (66). Stroke is determined by poor blood flow to the brain resulting in cell death. There are two main types of stroke: ischemic, due to lack of blood flow, and hemorrhagic, due to bleeding (66). Historically, the first suggestion that NRG-1 might have some role in the heart came from studies in mice, which were genetically manipulated to remove functional NRG-1 and its receptors in order to determine the function of NRG-1 in the nervous system as it applied to stroke victims (49, 51). Mice with disrupted NRG-1, ErbB 2, 3 and 4 receptors demonstrated both abnormal neural and cardiac development (49, 51). Further studies of the neuregulin's action demonstrated its neuroprotective and neurorestorative properties in central and peripheral nervous system models of injury (67, 68). A neuregulin-1 EGF-like fragment demonstrated neuroprotection in the transient middle cerebral artery occlusion (MCAO) stroke model and drastically reduced infarct volume (69). Furthermore, a permanent MCAO rat model was used to compare two products of the neuregulin-1 gene as well as assess levels of recovery with acute versus delayed time to treatment (70). This

study demonstrated that the GGF2 isoform of NRG-1 had similar functional improvements compared to the EGF-like domain fragment at equimolar doses. GGF2 improved sensorimotor recovery with all treatment paradigms, even enhancing recovery of function with a delay of 7 days to treatment. Neurorestorative effects of this kind are of great potential clinical importance, given the difficulty of delivering neuroprotective therapies within a short time after an ischemic event in human patients (70).

2.2.2 Cardiovascular diseases

The Centers for Disease Control estimates that the number of occurrences of myocardial infarction (MI) in the US to be 715,000 in 2012, with a survival rate of 50% (71). MI, or heart attack, is characterized by the lack of blood flow to the myocardial tissue resulting in damage to or necrosis of the cardiac myocytes. According to
American Heart Association in their 2015 update, coronary artery disease is the leading cause of heart attacks (66). Myocardial infarction, which can lead to heart failure (HF), is a compounded syndrome that develops over months to years. Heart failure is described as the structural, functional, and bioenergetic derangement of the heart**,** which results in its inability to perform the hemodynamic requirements of the body. With the rate of glucose utilization being greater in the heart compared to other tissues, altered substrate utilization, resulting from decreased glucose uptake and/or oxidation, could underlie contractile dysfunction reported in HF (72, 73).

In a healthy heart, ventricular diastole, the pressure in the (left and right) ventricles, drops below the pressure in the left atrium, initiating the opening of the mitral valve allowing accumulated blood from the atrium to flow into the ventricle. The *ventricular filling*

velocity or flow into the ventricles can be described by its early and late phase; first an early (*E*) diastolic filling causes ventricular suction, and second, a late filling is created by atrial contraction (*A*). The E/A ratio can be used as a diagnostic measure, since it is reduced in diastolic dysfunction (74). Furthermore, when the smaller, upper atria chambers contract during late diastole, they force blood into the larger, lower ventricle chambers. The lower chambers when filled are accompanied by the closing of the atrial valves, initiating the isovolumetric contraction (contraction of the ventricles while all valves are closed) of the ventricles, marking the first stage of systole. The second phase of systole forces blood from the left ventricle into the aorta to be delivered to body extremities, and from the right ventricle to the lungs. Ejection fraction (EF) is, therefore, defined as the fraction of outbound blood pumped from the heart with each heartbeat. It is commonly measured by echocardiogram and serves as a measurement of a person's cardiac function (75, 76).

Similar to it neurorestorative effects, NRG-1 has been reported to correlate to a certain degree with cardiovascular health. Both exercise and injury of skeletal and cardiac muscle activates the cleavage and release of NRG-1 in rodent models and is also detectable in human circulation (14). In studies that compare the level of circulating NRG-1, it was reported that healthy subjects express levels of NRG-1 that correlate with cardiopulmonary fitness (77), yet those presenting cardiovascular disease had levels associated with cardiac stress. Even further, it has been reported that when non-ischemic heart failure was compared to ischemic cardiomyopathy, NRG-1 was significantly higher in the circulation of patients with ischemic systolic heart failure. These higher levels were also associated with increased risk of cardiac transplantation or death (78).

a. Neuregulin-1 in animal studies

It has also been established that significant changes of the cardiac NRG-1/ErbB system occur as chronic heart failure (CHF) progresses in animal models (54, 79, 80). Expression of NRG-1/ErbB increases during early stages of CHF and decreases as pump failure occurs. During the development of concentric left ventricular hypertrophy, NRG-1 mRNA levels is reported to increase, potentially as a consequence of mechanical wall strain. As eccentric ventricular hypertrophy and pump failure arise, the decline in NRG-1 expression is combined with the downregulation in mRNA levels of ErbB2 and ErbB4. This downregulation of NRG-1 mRNA during heart failure is thought to be associated with increased angiotensin II and epinephrine expression, which have been demonstrated to reduce NRG-1 mRNA synthesis in cardiac endothelium (54). How the expression of NRG-1 and ErbB lead to changes in NRG-1/ErbB signaling and further the remodeling of the ventricle is still under investigation. Dr. Lemmons speculates that fluctuations in NRG-1 and ErbB mRNA expression in early and late phases of CHF could be adaptive for tissue integrity and growth in the early phases, then in response to hemodynamic conditions and peripheral circulation during late phases (81).

Understanding that NRG-1/ErbB signaling exhibits protective effects on the myocardium, has led researchers to determine whether NRG-1 activation of ErbB downstream signaling has therapeutic benefits in HF. For instance, a short-term intravenous (IV) administration of recombinant NRG-1 demonstrated an improvement in cardiac function and survival during cardiomyopathy (13). This improvement seems to be additive to treatment with angiotensin-converting enzyme inhibitors and is observed even if NRG-1 treatment began following the onset of cardiomyopathy (13). Therefore, it can be

concluded that rhNRG-1β stimulated reverse remodeling, a contrast from simply preventing further cardiac remodeling. NRG-1β's involvement in reverse remodeling is evident by its anti-fibrotic effects in animal models of heart failure. For instance, studies in rats with diabetic cardiomyopathy demonstrated that myocardial interstitial fibrosis is attenuated by NRG-1 (82). Furthermore, reduced fibrosis was reported during cardiomyopathy in a large animal model (swine) treated with the GGF2 isoform of NRG-1β (83).

b. Neuregulin-1 treatment in human clinical trials

There are currently two forms of recombinant NRG-1 in clinical trials. As briefly mentioned above, both demonstrate promising benefits for the treatment of heart failure in animal models. Similar results have been reported in human clinical trials. Phase II clinical trials with rhNRG-1 in which patients with stable chronic heart failure received daily intravenous infusions were reported to be safe and well tolerated (10, 84). Utilizing a double-blind multicenter study, participants received either NRG-1 or placebo for 10 days, in addition to standard therapy. Patients with stable chronic heart failure receiving rhNRG-1 displayed significantly increased left ventricular ejection fraction and reduced end-systolic and -diastolic volumes (75, 76)) at day 30. These improved parameters were furthermore present at day 90 (84). From these studies, it can be concluded that NRG-1 plays a therapeutic role in reversing cardiac remodeling in humans. Fifteen patients in a second clinical trial who received daily intravenous infusions of rhNRG-1 for 10 days demonstrated improved and sustained hemodynamics (10). Currently larger Phase II trials are ongoing, as well as a Phase III trial to measure the effects of rhNRG-1 treatment on the death rate of patients presenting with chronic heart failure.

A phase I clinical trial in which 40 patients with symptomatic heart failure were given a single dose of GGF2 intravenously was generally well tolerated. The completion of this study revealed a dose-responsive increase in ejection fraction at day 28 and was consistent through day 90 (11, 12). Preliminary data illustrates that this single dose of GGF2 has parallel efficacy to the EGF-domain only fragment of rhNRG-1, which was given over 10 days. This efficacy has been suggested to be attributable to the additional Kringle domain present in GGF2, but not in the rhNRG-1 (11, 12). Exciting, NRG-1 demonstrates cardioprotective benefits and enhanced contractility in animal and human studies, respectively.

c. Neuregulin-1 in diabetic cardiomyopathy

Long et. al. reported that DCM impairs NRG-1/ErbB signaling via a rat model of DCM established using a single intraperitoneal injection of streptozotocin (STZ) and confirmed by dramatic left ventricle fibrosis and impaired left ventricle systolic function at 12 weeks STZ-induced diabetes. ErbB2 and ErbB4 mRNA expression and NRG1 protein expression in the left ventricular myocardium were significantly decreased. Furthermore, this model of DCM demonstrated a decrease in phosphorylation of the ErbB2 and ErbB4 receptors (85). Interestingly, a study in streptozotocin-induced Type 1 diabetes in rats concluded that administration of NRG-1 improved cardiac function and reversed remodeling of the heart of DCM rats by attenuating cardiac fibrosis, although the precise mechanism was unclear (86). The rhNRG-1 treatment resulted in significantly less apoptotic cells than untreated group as determined by the TUNNEL method. Furthermore, mRNA expression of bcl-2, bax, and capase-3, which are regarded as the markers of apoptosis (bcl-2 is known for anti-apoptosis, and bax and caspase-3 pro-

apoptosis) revealed that Type 1 diabetes mice expressed bcl-2 was down regulated but bax and capase-3 were up regulated, whereas, rhNRG-1 alleviated the changes. These results indicate that rhNRG-1 treatment protects cardiomyocytes against apoptosis during DCM (86).

In a complementative study, human NRG-1 (hNRG-1) lentivirus was transfected into rat cardiac microvascular endothelial cells *in vitro* by conditioned media as well as injected into the myocardium of the DCM model rats *in vivo* (87). hNRG-1 conditioned medium initiated proliferation of cardiomyocytes as well as reversed apoptosis. Furthermore, DCM rats that received the hNRG-1 gene transfer displayed improved heart function, as indicated by invasive hemodynamic measurements (cardiac catheterization to measure ventricular pressure) (87). In agreement with the Li lab (86), hNRG-1 reduced the number of apoptotic cells, decreased the expression of bax and increased the expression of bcl-2 in the myocardium of the DCM model rats (87). Additionally, mRNA levels of type I and III pro-collagen as well as myocardial fibrosis were markedly reduced in hNRG-1 transfected rats (87). The combinations of results from these studies align to conclude that NRG-1 ameliorates cardiac dysfunction in diabetes.

2.3 Glucose metabolism in striated muscle

2.3.1 Regulation of glucose metabolism

Whole-body glucose homeostasis, a delicate balance of glucose anabolism and catabolism is maintained by glucose transport in several tissue types. Glucose uptake is the rate-limiting step of glucose metabolism and is mediated by a family of specialized proteins, called glucose transporters (GLUTs) (88). GLUTs are glycoproteins,

approximately 50-60kDa in size, which facilitates glucose uptake from the blood to the cells. The major isoform expressed in myocytes, GLUT4, is translocated from an intracellular pool to the cell membrane during insulin-mediated glucose uptake in striated muscle. Under basal conditions, GLUT4 is primarily located within multiple intracellular compartments such that only 2-5% is reported at the plasma membrane (89-93). Yet, other isoforms expressed in insulin-sensitive tissues also participate in glucose uptake, including the basally expressed GLUT1 and the novel GLUT8 (94). Glucose uptake is mediated by activation of the phosphatidylinositol-3-kinase (PI3K) cascade or by PI3K-independent pathways mediated by effectors such as AMPK or calcium (95).

Although the mechanisms of insulin regulation of GLUT4 within cardiac tissue is not fully known, recent data suggests that GLUT4 may have roles in the regulation of glycolysis even beyond its ability to transport glucose (96). Cardiomyocyte-specific GLUT4 knockout mice displayed reduced levels of insulin-responsive aminopeptidase (IRAP) known to be related with impaired GLUT4 trafficking (97). Similar to skeletal muscle, cardiomyocyte GLUT4-mediated glucose uptake can be activated through a G protein, PI3K, and 1,4,5-inositol-triphosphate (IP3) signaling cascade. Interestingly, it has been reported that activation of this signaling axis increases propagation of cytoplasmic calcium signals**,** which also initiate GLUT4 translocation (98). Accordingly, these data highlight the necessary role of GLUT4 in the insulin-mediated cardiac glucose metabolism. Furthermore, heterozygous GLUT4+/- knockout mice displayed increased serum glucose and insulin, reduced muscle glucose uptake, and diabetic histopathologies in the heart consistent with observations in humans with Type II diabetes mellitus (T2DM) (99).

2.3.2 PI3K-dependent GLUT4 regulation

Absorption of glucose causes an increase in blood glucose**,** which consequently yields a rise in pancreatic beta cell release of insulin into the blood. Insulin binds to its receptor initiating an intracellular cascade**,** which results in the translocation of insulin-sensitive GLUT4 from the cytosol to the plasma membrane. The insulin-dependent or PI3K (phosphoinositide-3-kinase) pathway and has been extensively investigated **(Figure 5, artistic credit to Dominic Martin)**. Upon insulin binding to its receptor (insulin receptor, IR), autophosphorylation triggers downstream effectors to phosphorylate PI3K and further downstream targets (100-103). Studies have characterized the serine/threonine kinases downstream of the IR regulating GLUT4 translocation. One of such candidates include Akt/protein kinase B, which is activated by its binding to these membrane bound lipids via the 3'-polyphosphoinositide-dependent protein kinase PDK1 (104). It has been demonstrated in 3T3-L1 adipocytes (105), primary adipocytes (106), and L6 myotubes (107) that membrane-directed constructs of Akt/PKB stimulates GLUT4 translocation, a mechanism that is blunted by inhibitory constructs (106). In contrast, a dominant

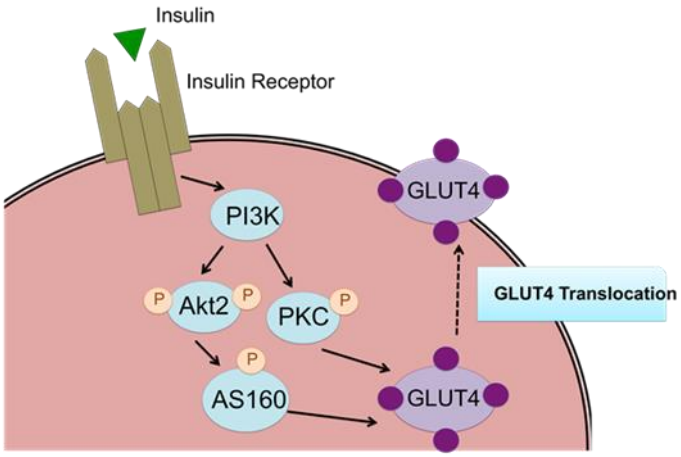

Figure 5: The downstream insulin signaling pathway. The above schematic outlines a portion of the downstream insulin-signaling pathway. Abbreviations are used as follows: P = phosphorylated, PI3K = Phosphoinositide 3-kinase, PKC = Protein kinase C, Akt2 = Protein kinase B isoform 2, AS160 = Akt substrate at 160 kD, GLUT4 = Glucose transporter isoform 4 (artistic credit to Dominic Martin).

inhibitory mutant Akt/PKB with alanine substitutions within the threonine 308 and serine 473 phosphorylation sites inhibited protein synthesis but not insulin-stimulated glucose transport, as demonstrated in both Chinese hamster ovary cells and 3T3-L1 adipocytes (108). These data suggest that Akt-initiated GLUT4 activity is dependent on location within the cell compared to activation by its phosphorylation sites. A more in depth investigation at the activation of these phosphorylation sites indicated that Akt phosphorylation at Th308 site is mediated directly by PDK-1 (109-111), while Akt phosphorylation at Ser473 is facilitated by the rictor-mammalian target of rapamycin, also known as mTOR-2 (110, 112). During experimental Type 1 diabetes, a lack of insulin production is accompanied by baseline decreases in cardiac Akt activity (113). Yet, when streptozotocin (STZ)-diabetic rats were given systemic insulin, there was a restoration of activity in nearly every protein within the PI3K/Akt signaling cascade within the myocardium, including Akt phosphorylation (114). From this, we can conclude that the lack of insulin production during insulin-deficient Type 1 diabetes induces alteration in PI3K/Akt activation. Conversely, insulin-resistant Type 2 diabetes displays diminished insulin action on the PI3K/Akt signaling cascade (115).

Akt-substrate at 160kD (AS160), is the downstream target of Akt, as its name indicates. Upon activation, AS160 actively converts Rab-GDP to Rab-GTP which initiates GLUT4 vesicles translocation to the cell membrane (116). Karlsson et. al. demonstrated in 2005 that impairment of insulin action during Type 2 diabetes results in decreased AS160 phosphorylation at its Th308 site in skeletal muscle, yet there was no change in total expression of AS160 (116). Akt impairment was further confirmed in the adipocytes of Type 2 diabetic patients as well as rodents as reported by Rodinone et. al. and Carvalho

et. al. (117, 118). Furthermore, defective phosphorylation of Akt led to defects in GLUT4 translocation (119, 120) as observed in Akt knockout mouse models during insulin resistance (121). These Akt knockout mice exhibit hyperglycemia, hyperinsulinemia, and impaired glucose tolerance. These studies elaborate on the necessary involvement of PDK-1, Akt, and AS160 during insulin-dependent GLUT4 translocation and glucose uptake.

In a similar PI3K-dependent Akt-independent mechanism, protein kinase C ζ (PKC-ζ), is activated by polyphosphoinositides via insulin in a wortmannin-sensitive manner. Insulin stimulates swift increases in PKC-ζ activity in 3T3-L1 adipocytes (122), L6 myotubes (123), rat adipocytes (124), and 32D cells (125) via PI3K-dependent stimulation (123-125). Downstream from PI3K, PDK-1 derives access to the activation of PKC-ζ through conformational regulation such that the autoinhibitory pseudosubstrate sequence of PKC-ζ must be removed in order to reveal the binding cavity for PDK-1 to phosphorylate PKC-ζ. This serves as a direct "On/Off" switch for the activity of PKC-ζ (126). It has been well established that inhibitors of PKC-ζ concurrently impede insulin effects on glucose transport as demonstrated in adipocytes (124) and L6 myotubes (123). To further confirm PKC-ζ involvement in insulin-stimulated glucose uptake, a kinase-inactive (KI) form of PKC-ζ displayed inhibition of GLUT4 translocation and glucose transport in transfected 3T3-L1 cells (122) and L6 myotubes (123). These data suggest that insulin-dependent not only initiates Akt/AS160 GLUT4 translocation, but the alternative PKC pathway is directly involved in GLUT4 translocation.

2.3.3 PI3K-independent GLUT4 regulation

Investigations of insulin-independent mechanisms lend us insight into the regulation GLUT4 translocation during skeletal muscle contraction. It has been observed that increases in glucose transport are due to the additive effects of both insulin and contraction in striated muscle, yet only insulin-dependent glucose uptake is blocked by wortmannin (127). Similar studies have reported that insulin, not contraction, stimulates PI3K and Akt activity (128). Hayashi's lab demonstrated that contraction mediates 5'-AMP-activated protein kinase's (AMPK) role in the regulation of cell stress in the rat hind limb, yet insulin did not elucidate this response (129). Interestingly, another AMPK activator, apart from contraction, mimicked the contraction-induced glucose uptake in a wortmannin-insensitive manner (129).

To further understand these concepts above, I will expand on roles of AMPK, calcium-calmodulin kinase (CAMK), and calcium-initiated glucose uptake in striated muscle, beginning with the former. AMPK, a protein complex composed of 12 molecules containing α, β and γ subunits (130, 131). The α-subunit retains catalytic activity while the β and γ subunits have important roles in substrate specificity and maintaining stability of the heterotrimer (132, 133). AMPK's role in insulin-independent glucose uptake, which is stimulated by the contraction events of skeletal muscle, is crucial in myocytes. AMPK activity is activated by the AMP:ATP ratio and further reduced by the creatine:phosphocreatine ratio (134-136). Increased AMP levels allosterically activate AMPK prompting phosphorylation of Thr172 within the catalytic α-subunit (130, 131, 137). It is therefore presumed that AMPK activation during contraction is the response to increased AMP:ADP binding and decreased ATP to γ-subunit binding (138). 5-aminoimidazole-4carboxamide-1-ß-D-ribonucleoside (AICAR) mimics the role of AMP

on AMPK as demonstrated during *in vitro* studies on isolated rat muscles exposed to AICAR, which increased glucose transport in the absence of insulin. In conjunction, healthy and Type 2 diabetic human muscles treated with AICAR responded in the same way (129, 139-141). As expected, there was an additive effect of glucose uptake when striated muscle and neuronal cells were treated with combined AICAR and insulin simultaneously (142, 143). However, when AMPK α2 or γ3 subunits are deficient, the AICAR-induced glucose uptake is blunted (144, 145). Furthermore, glucose uptake is induced by two distinct pathways when comparing AICAR and contraction. Mouse soleus and EDL muscles having α1 or α2-AMPK knockouts stimulated by muscle contraction expressed similar glucose uptake compared to wild-type mice. Interestingly, when stimulated with AICAR, only α1-AMPK KO mice displayed consistent glucose uptake. These studies conclude that AICAR-stimulated glucose uptake is dependent of the α2-AMPK subunit (144). In summary, increased levels of AMP and ADP by contraction events seem to be the primary activator of AMPK-stimulated glucose uptake.

In addition, skeletal muscle contractions can supply increased calcium concentrations within the myocytes (133, 146). This increase in calcium concentrations has been observed to stimulate glucose uptake and GLUT4 translocation (133, 147). Interestingly, it has been reported that calcium alone stimulates glucose uptake independent of contraction in skeletal muscle (148), although the mechanisms by which this occurs is not well understood. Candidates within the downstream signaling cascade of calcium-initiated glucose uptake include calmodulin-protein kinase and PKC (134, 138, 149). The calcium-calmodulin kinase (CAMK) initiates activations of AMPK via phosphorylation of Thr172, in addition to activation of GLUT4 translocation through

alternative effectors (132, 150-153). Furthermore, reports of isolated rat muscles treated with caffeine stimulates increases in intracellular calcium levels with parallel increases in glucose uptake independent of contraction (154). It has been confirmed by several labs that caffeine incubation in muscles of both mice and rats increase nucleotide turnover and AMPK activation, also independent of contraction (132, 138, 155). It has been proposed that this increased nucleotide turnover and AMPK activation is due to the pertinent demand of energy by the sarcoplasmic reticulum Ca2+-ATPase-dependent (SERCA) Ca2+ reuptake (138, 156). Concluding from these studies, metabolic stress yields increased glucose uptake, triggered by SERCA activation and muscle contraction, not the direct action of calcium (157). These studies elucidate the essential insulin-dependent and –independent activation of GLUT4 mediated glucose uptake.

2.3.4 The effect of NRG-1 on glucose metabolism in skeletal muscle

Neuregulins play a central role in muscle biology. They are synthesized by myoblast cells and initiate an autocrine signaling pathway that promotes myogenic differentiation (158). Interestingly, neuregulin has been demonstrated to increase glucose uptake in skeletal muscle, independently of insulin action (159). Sawyer's lab demonstrated that multiple isoforms of NRG and its ErbB2, -3, and -4 receptors were highly expressed in adult skeletal muscle which are activated in response to contractile activity. *In vivo* proteolytic processing of NRG with simultaneous activation of these ErbB receptors were initiated by two distinct modes of exercise. Interestingly, there was increased expression of ErbB3 in extensor digitorum longus (EDL) muscles a (predominantly fast-twitch muscle) compared to soleus muscle (a predominantly slow-twitch, oxidative muscle) (160).

Furthermore, being akin in structure slow-twitch skeletal muscle, adult cardiac myocytes do not express ErbB3 (53).

Dr. Suarez's lab reported neuregulin's rapid effects on glucose transport in muscle cells independent of changes in gene expression (159). This was elucidated by the increased translocation of GLUT1, GLUT3, and GLUT4 upon stimulation by NRG in cultured L6E9 cells, and was independent of changes in GLUT gene expression (159). Similar to insulin stimulation, NRG treatment induces the translocation of GLUT4 in skeletal muscle myocytes. Furthermore, when NRG and insulin treatments were combined, the stimulation of GLUT transport was additive. In addition, NRG's effect on glucose transport was blunted by wortmannin, a PI3K inhibitor. These findings suggest a PI3K-dependent mechanism for glucose uptake stimulated by NRG (159).

Dr. Canto and Dr. Suarez also demonstrated *in vitro* contractions stimulated ErbB4-mediated glucose uptake, but not ErbB3, in soleus muscle (25). This was secondary to their previous finding in which ErbB3 phosphorylation levels were minimal in the presence of saturating concentrations of neuregulins in soleus muscle (25). *Ex vivo* blocking of ErbB4 had more dramatic effects on calcium-mediated glucose uptake in slow-twitch soleus muscle fibers compared to fast-twitch EDL muscles (24). Furthermore, when ErbB4-specific antibodies blocked its phosphorylation *in vitro*, ErbB3 receptors were minimally phosphorylated in the presence of caffeine. Even still, caffeine did not induce glucose uptake in these circumstances. To validate that ErbB3 was not involved in the activation of glucose uptake, ErbB3-specific antibodies blunted its phosphorylation but did not impair the stimulation of glucose uptake during any conditions tested (25).

Dr. Canto further postulated that neuregulins are necessary effectors for calcium signaling to induce glucose uptake in skeletal muscle (24). To determine if calcium-mediated glucose uptake via ErbB4 receptor was dependent upon CAMKII, they inhibited CAMKII and reported no effect on ErbB4 phosphorylation, suggesting that CAMKII is not involved in calcium-mediated ErbB receptor phosphorylation (24). Similarly, to determine if AMPK-induced glucose uptake is independent of neuregulin action, they demonstrated that AICAR (5-Aminoimidazole-4-carboxamide ribonucleotide, an AMPK activator) does not increase phosphorylation of the ErbB receptors, nor does blockage of ErbB4 affect AMPK-stimulated glucose uptake (24). Therefore, calcium-mediated neuregulin stimulation increased glucose transport independent of AMPK and CAMKII.

2.3.5 Alterations of glucose metabolism during cardiovascular diseases

In order to sustain the high rate of contraction, the energetic demand of the heart is greater compared to other tissue. Therefore, the heart requires an increased rate of oxygen consumption per gram of tissue (16, 17). Glucose is a major energy substrate for the heart, which contributes to 30% of the heart's overall energy through glucose oxidation during physiological states. Even with its ability to utilize other substrates such as fatty acids, lactate, ketone bodies, and amino acids, the heart utilizes glucose more than skeletal muscle, lung, or adipose tissue in order to maintain homeostasis (18, 19). Therefore, glucose uptake is crucial to healthy cardiac function.

a. Hyperglycemia contributes to increased risk during MI and T2DM

Diabetes is the most predominant risk factor for cardiovascular events in the United States (161), with heightened risk of myocardial infarction that contributes significantly to decreased life expectancy. It has been reported that patients with diabetes have a risk for MI comparable to that of recurrent MI in a nondiabetic patient. A Finnish population-based study summarized their findings of the previous statement in that the 7-year incidence rates were 18.8% in subjects having MI without diabetes compared to 45% in subjects with MI and diabetes (162). Unfortunately, patients presenting diabetes at age 40 have an approximate 8 year decreased life expectancy compared to nondiabetics (163). This prognosis worsens in T2DM patients that have experienced a myocardial infarction and, unfortunately, the underlying mechanisms of increased mortality associated to glucose levels are not understood. Interestingly, a human study of 52 patients with acute MI, ST segment elevation (corresponds to a period of ventricular depolarization, in which, elevation can forecast abnormalities in ventricular function), and hyperglycemia displayed that basal glucose levels are a suggestive predictor of left ventricular growth following acute MI (164). Even so, it has been reported that increased mortality within 30 days of the MI was associated with increased hyperglycemia upon time of admission. Additionally, the former prognosis with the presence of diabetes was associated with poor outcomes over 3 years. From these data, it can be deduced that acute hyperglycemia observed in MI and T2DM should be considered as a risk factor in determining treatment avenues when a patient presents both MI and T2DM (165). Therefore, we will discuss the alterations in the metabolism of glucose during both diseased states.

b. myocardial infarction

Apart from T2DM, the effect of MI on insulin-mediated glucose uptake in the heart remains unclear. Mouse model investigations using cardiac insulin receptor knockout mice revealed mitochondrial dysfunction and cardiomyopathy suggesting insulin resistance as a precursor of contractile dysfunction (166). Both long term (10 weeks) and short term (2 weeks) studies have been performed to investigate changes in cardiac metabolism and insulin sensitivity after MI. In a 10-week study, investigators reported decreased insulin-stimulated glucose uptake and reduced GLUT4 expression after MI (22). Yet, at the two weeks post-MI time point, there was no change in glucose uptake compared to control. Moreover, the lack of hypertrophy as well as insulin-stimulated total and phosphorylated Akt was unchanged between groups (20). Instead of highlighting contrasts between the long and short term effect of glucose uptake post-MI, these studies provide insights into the development of insulin resistance that may accrue with time post infarct. MicroPET imaging of glucose uptake after MI indicated a moderate to severe reduction in 18F-FDG uptake in the apex, apical anterior, and apical lateral segments as early as 48 hours post infarct. Interestingly, the mid anterolateral segment displayed marked decrease in 18F-FDG uptake initially that reversed after 2 weeks post MI and remained stable throughout the remainder of the study (21). A direct measurement of insulin-stimulated glucose uptake in chronically infarcted rat hearts after MI displayed significant decreases in the MI rats compared to control. Basal rates of glucose uptake remained consistent between all groups while insulin-stimulated glucose uptake was reduced by 42% in the MI model in parallel with a 28% decrease in GLUT4 expression within the same hearts (22). Therefore, it can be concluded that MI gives rise to insulin resistance leading to decreased glucose uptake via impairment of GLUT4 translocation.

c. diabetic cardiomyopathy

The frequency of Type 2 diabetes mellitus (T2DM) has gradually increased over the last two decades. By 2030, this trend would lend close to half a billion people affected by this disease (167). Diabetic cardiomyopathy (DCM), now recognized as a complication of T2DM, is characterized by persistent hyperlipidemia, hyperglycemia, increased cardiac oxidative stress, myocardial fibrosis, inflammation, and disruption of Ca^{2+} handling along with mitochondrial dysfunction (168, 169). Clinically, DCM manifests itself in two phases; the first phase is characterized by abnormal myocardial energy metabolism, diastolic dysfunction and reduced LV strain to overt heart failure. The second phase is characterized by cardiomyocyte hypertrophy, myocardial fibrosis, and cardiomyocyte death, and whole-body insulin resistance (170-172). During DCM, cardiac dysfunction induced by pressure overload is associated with dysfunctional insulin signaling and decreased GLUT4 translocation (173). Furthermore, cardiac dysfunction is preceded by hyperinsulinemia and augmented Akt activation (173) as described in both animal (174) and human (175) patients with DCM. More specifically, hearts from obese and insulin-resistant rodents displayed lower glucose oxidation rates and decreased cardiac efficiency (176, 177). Moreover, cardiomyocytes from high-fat-diet-fed (HFD-fed) mice display decreased GLUT4 expression and translocation in response to insulin as well as increased fatty acid (FA) oxidation rates (174).

2.4 Therapies available for treatment of myocardial infarction

Fibrinolytic therapy (FT) and Primary Percutaneous Coronary Intervention (P-PCI) are the two currently available options of reperfusion therapies for the treatment of

myocardial infarction. P-PCI executed in a timely manner is more effective than FT. Unfortunately, the downfall of P-PCI is that it is not universally available (178). Another liability of P-PCI is that it must be performed within 90 minutes upon arrival to the hospital (179). Patients receiving FT are also subjected to antiplatelet therapy encompassing the benefits of Aspirin and Clopidogrel, as well as the use of adjunctive anticoagulants such as Heparin, which reinforces fibrinolytic agents used for MI (180, 181). Unfortunately, these therapies have many risks and adverse events, including recurrent ischemia, reinfarction, intracranial hemorrhage, and potential death. In addition, most patients have an increased risk to develop a thrombus after MI. Trials to increase epicardial coronary flow, prevent distal embolization, and reduce microvascular obstruction by a thrombectomy were not successful in reducing infarct size and improving left ventricular ejection fraction (182-184). Reperfusion therapy alongside adjunctive pharmacotherapy aids in the reestablishment of coronary flow, yet this restoration of coronary blood flow has been reported to cause further injury to cardiac myocytes (185). Reperfusion injury (185) is caused by oxidative stress (186, 187), calcium overload (188, 189), inflammation, and rapid restoration of pH (190). Understanding these mechanisms at a cellular level has led to renewed interest in designing treatment strategies targeting pathways**,** which mediate reperfusion injury. Research strategies focus on the cardioprotective effect mediated by multiple signaling pathways, although this is not well understood (191). Even with substantial enhancements in MI management, adverse event rates have not significantly decreased (192).

2.4.1 Metabolic therapy for treatment of myocardial infarction

In spite of the formerly stated insufficient treatments, metabolic therapy has emerged as a promising field to determine the benefits of metabolic processes in treatment of patients with MI and HF. It has been proposed that optimizing energy substrate metabolism by inhibition of fatty acid β-oxidation, while increasing glucose oxidation will enhance ATP production and utilization efficiency, and therefore restore cardiac efficiency in the ischemic/reperfused failing hearts (193). A viable strategy to decrease the delivery of FFA to the myocardium would include therapeutically reducing circulating FFA concentrations therefore decreasing cardiac fatty acid β-oxidation in the post-ischemic period as well as in heart failure. It is known in heart failure that significant reduction/depletion of plasma FFA acutely and rapidly impairs cardiac efficiency, highlighting the necessity of fatty acids as an oxidative substrate in this setting (194). Understanding this, we can infer that careful evaluation of the degree to which circulating FFA concentration is decreased will be crucial in the determination of subsequent effects on cardiac efficiency and function.

One such approach utilizes glucose–insulin–potassium (GIK) therapy which has been reported to increase the rates of glycolysis as well as decrease circulating concentrations of FFA (195, 196). Furthermore, the shift toward glucose utilization has been demonstrated to decrease infarct size (196) besides improving post-ischemic cardiac function (197). Several studies administering GIK therapy at reperfusion report its cardioprotective benefits (196, 198, 199). Yet, one must take into account that the level of circulating FFA supplied to the myocardium directly influences the effect of insulin (200). Furthermore, unbalanced stimulation of glycolysis relative to glucose oxidation is

known to uncouple the two processes, increase intracellular acidosis (201), and may diminish the cardioprotective effects of insulin (200).

Several fatty acid β-oxidation inhibitors have been developed and proven effective against ischemic heart disease and heart failure. Carnitine palmitoyltransferase I is the rate-limiting enzyme mediating mitochondrial fatty acid uptake, making an attractive target for the inhibition of myocardial fatty acid β-oxidation. A number of carnitine palmitoyltransferase I inhibitors have been developed, including etomoxir (202) and perhexiline (203), both demonstrating anti-ischemic effects as well as beneficial effects in the treatment of heart failure. Trimetazidine is a partial fatty acid β-oxidation inhibitor that competitively inhibits long chain 3-ketoacyl-CoA thiolase (204). Results from clinical studies have confirmed the effectiveness of trimetazidine as an anti-ischemic agent (205). Metabolic therapy, though currently incomplete, is a promising avenue for the treatment of myocardial infarction. We hypothesize that NRG is a novel metabolic therapy for patients with heart failure.

CHAPTER III

METHODOLOGY

3.1 Ventricular myocyte isolation

Animals were deeply anesthetized by 1mL pentobarbital sodium injection or 3-5% isoflurane, and the heart and lungs rapidly excised. The heart was perfused in a retrograde manner, using a Langendorff apparatus with Tyrode buffer (37°C, pH = 7.35 and oxygenated with 95% O_2 and 5% CO_2), which contained (in mM): NaCl (135), KCl (5.4), MgCl2 (1), NaH_2PO_4 (0.33), Hepes (10), glucose (10), and $CaCl_2$ (1). This initial perfusion was followed by a perfusion with Tyrode buffer free of $CaCl_2$. Subsequently, collagenase (type II, 1 mg/ml, Worthington Biochemical) was added to the calcium-free Tyrode buffer and recirculated for the rest of the perfusion period. When the heart was softened the ventricles were minced and the cells were subsequently washed in Tyrode solution containing $CaCl_2$ (0.125 mM). Only rod-shaped cells with sharp margins and clear striations were included in the study. All recordings were made within 5 hours of isolation (206-208). All the procedures of this study were approved by the Oklahoma State University Institutional Animal Care and Use Committee (ACUP# VM 12-3).

3.2 Glucose uptake assay

A nonradioactive assay was performed using a fluorescent D-analog, 1-N-7-(nitrobenz-2-oxa-1,3-diazol-4-yl)amino-2-deoxy-D-gluocose (2-NBDG) in isolated fresh cardiac myocytes and a glucose uptake cell-based assay kit, following manufacture's recommendation (Cayman Chemical, MI, USA). The cells were plated on a 96-well plate at a density of 5,000 cells/well in glucose-free Tyrode buffer and incubated with/without 100 ng/ml GGF2 or 0.0349 mM insulin for at least 30 minutes. 100 uM 2-NBDG was then administrated to each well. After 10 minutes incubation, cells were washed with PBS (200uL/well) twice, and fluorescence was measured using a microplate reader at excitation/emission wavelengths of 535/758 nM.

3.3 Western immunoblotting

Following a 1 hour incubation with or without 300 nM afatinib (Selleck Chemicals, Cat No.S1011), cells were incubated for 1 hour with 100 ng/ml GGF2 (ACORDA Therapeutics), 0.5 mM CaCl2 (Sigma-Aldrich), 100 nM AICAR (Sigma-Aldrich, A9978), or 0.0349 mM Insulin (Thermo-Fisher). Cells were then lysed with 1:500 RIPA (Thermo-Fisher) and Protease Inhibitor Cocktail (Sigma-Aldrich, P8340) and the total lysate was stored in -80˚C. The bicinchoninic acid assay (BCA assay) was the biochemical assay used to determine the total concentration of protein in solution. The total protein concentration is exhibited by a color change of the sample solution from green to purple in proportion to protein concentration, which can then be measured using colorimetric techniques. Equal amounts of protein (5–20μg) were resolved in an 8–12% SDS-polyacrylamide gel and electrophoretically transferred (Bio-Rad) to a polyvinyl-idine fluoride membrane (Bio-Rad), as previously described [22; 24–

27; 29; 30]. After blocking (1–5% non-fat dry milk or 2% goat serum albumin), membranes were incubated with optimally diluted primary antibodies overnight (polyclonal rabbit anti-human GLUT4, 1:750, AbD Serotec 4670–1704; monoclonal rabbit anti-mouse total Akt, 1:1000, Cell Signaling 4061; monoclonal rabbit anti-human phosphorylated Akt s473, 1:1000, Cell Signaling 4060; monoclonal rabbit anti-mouse phosphorylated Akt Th308, 1:1000, Cell Signaling 2965; monoclonal rabbit anti-human total AS160, 1:1000, Cell Signaling 2670 and polyclonal rabbit anti-human phosphorylated AS160, 1:1000, Cell Signaling 9611; polyclonal rabbit anti-human PDK-1, 1:1000, Cell Signaling 3062; Monoclonal rabbit anti-human phosphorylated PDK-1 S241, 1:1000, Cell Signaling 3438; Monoclonal Rabbit IgG anti-human PKC zeta Th410, 1:500, Cell Signaling 2060; polyclonal rabbit anti-human PKC zeta, 1:1000, Cell Signaling 9372); washed for 10 minutes with TPBS (twice), 5 minutes with PBS, followed by a 1 hour incubation of appropriate secondary antibodies conjugated to horseradish peroxidase (for total and phosphorylated Akt, PKC, PDK-1 and AS160, Cell Signaling 7074, 1:2000, polyclonal goat anti-rabbit; for GLUT4, GE Healthcare NA934V, polyclonal donkey anti-rabbit). Membranes were again washed for 10 minutes with TPBS (twice), and 5 minutes with PBS. Primary antibodies were chosen based on their 100% sequence homology with the protein of interest in rodents, and validated against a positive control (i.e., tissue, peptide). Antibody-bound transporter proteins were quantified by enhanced chemiluminescence reaction (KPL) and autoradiography. Band density and molecular weight were quantified using GelPro Analyzer (Media Cybernetics). The data was expressed relative to appropriate controls. Equal protein

loading was confirmed by reprobing each membrane with Calsequestrin monoclonal IgG (Thermo-Scientific PA1-903, 1:2500, polyclonal rabbit anti-rat).

3.4 Quantification of GLUT translocation to the cell surface

Following a 1 hour incubation with or without 300 nM afatinib (Selleck Chemicals, Cat No.S1011), cells were incubated for 1 hour with 100 ng/ml GGF2 (ACORDA Therapeutics), 0.5 mM $CaCl_2$ (Sigma-Aldrich), 100 nM AICAR (Sigma-Aldrich, A9978), or 0.0349 mM Insulin (Thermo-Fisher), and were photolabeled with the cell-surface-impermeant biotinylated bis-glucose photolabeling reagent (bio-LC-ATB-BGPA, 300 μM, Toronto Research Chemicals, ON, Canada), of which the hexose group interacts specifically with the extracellular binding site of GLUTs. The photolabeled reagent was cross-linked to cell surface GLUTs using a Rayonet photochemical reactor (340 nM, Southern New England UV), as previously described (209-211). Cells were then lysed with 1:500 RIPA (Thermo-Fisher) and Protease Inhibitor Cocktail (Sigma-Aldrich, P8340) and the total lysate was stored in -80˚C. Recovery of photolabeled (cell surface) GLUTs from total cardiac membranes (200μg) was achieved using streptavidin isolation (bound to 6% agarose beads) to facilitate separation of non-cell surface GLUTs ("unlabeled" or intracellular fraction that remains in the supernatant) from cell surface GLUTs ("labeled" or sarcolemmal fraction). The labeled GLUTs were then dissociated from the streptavidin by boiling in Laemmli buffer for 60 min prior to SDS-PAGE and subsequent immunoblotting with GLUT antibody. The Laemmli buffer is described as follows based upon its components. The 2-mercaptoethanol reduces the intra and inter-molecular disulfide bonds. The SDS detergent denatures the proteins and subunits and gives each an overall negative charge so that each will separate based on size. The

bromophenol blue serves as a dye front that runs ahead of the proteins and also serves to make it easier to see the sample during loading. The glycerol increases the density of the sample so that it will layer in the sample well. Proteins from the labeled fraction were quantified by densitometry relative to the positive control, as previously described (209, 210, 212).

3.5 Statistical analysis

Differences between means were assessed using Student's t-tests. Repeated measured One way ANOVA was performed with Student Newman Keuls post-hoc test for *in vitro* measurements, as appropriate. If not normally distributed, the data was analyzed with a Mann-Whitney or Friedman test. Statistical significance was defined as $P<0.05$. Data are presented as mean ± SE.

CHAPTER IV

FINDINGS

4.1 In vitro GGF2 treatment increases glucose uptake

To determine whether acute *in vitro* GGF2 treatment modulates glucose uptake in healthy cardiac myocytes, we isolated cardiac myocytes via Langendorff perfusion and subjected them to incremental doses of GGF2 or insulin for 30 minutes prior to incubation with 2-NBDG, a fluorescently labeled glucose analog, for 30 minutes. Glucose uptake was measured by fluorescent detection via microplate reader. Similar to insulin, GGF2 treatment, both 1 ng/ml and 100 ng/ml resulted in increased glucose uptake, by 39% and 49%, respectively, compared to basal ($P<0.05$ **Figure 6A**). It is worthwhile to note that incubation with 10ng/ml resulted in glucose uptake similar to insulin, however, due to the variability within the samples the changes were not significant. Furthermore, Western blotting of total GLUT4 (**Figure 6B)** demonstrated a significant increase of total GLUT4 content at 10 ng/ml GGF2 ($P=0.047$) after a 1 hour treatment.

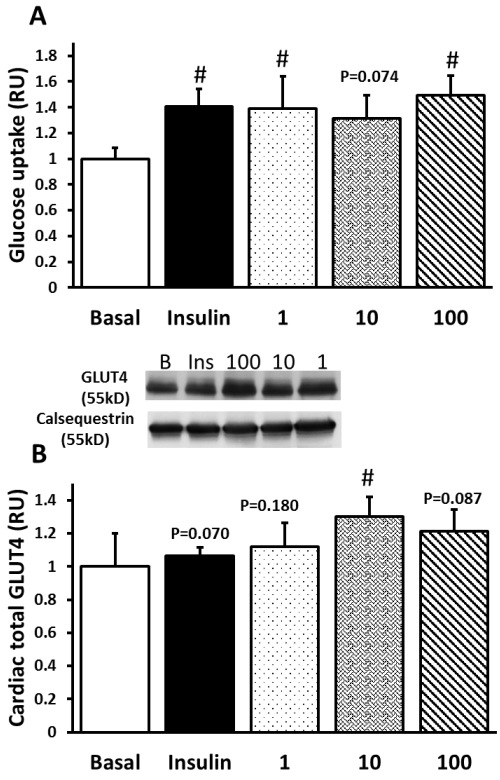

Figure 6A: Glial growth factor 2 (GGF2) stimulates glucose uptake in isolated cardiac myocytes. Mean ± SE of glucose uptake; values normalized to basal (n=6/group); # P<0.05 relative to basal. Methods: A nonradioactive assay was performed using 2-NDBG glucose uptake assay. Isolated cardiac myocytes were incubated with/without 100 ng/ml GGF2 or 0.0349 mM insulin for at least 30 minutes prior to 10 minutes incubation with 2-NDBG. Fluorescence was measured using a microplate reader at excitation/emission wavelengths of 535/758 nM. **B: GGF2 increases total GLUT4 expression.** Top panel: representative Western blot. Bottom panel: Mean ± SE of protein expression (values expressed relative to basal; n=11-15/group); # P<0.05 vs. basal (Mann-Whitney rank sum test). Methods: Total lysate of isolated rat ventricular myocytes incubated with 0.0349 mM insulin or 1 ng/ml, 10 ng/ml, or 100 ng/ml GGF2; Calsequestrin (CLSQ) was used as a loading control.

4.1.2 In vitro GGF2 treatment increases GLUT4 translocation

We have previously demonstrated in our lab that GLUT4 translocation was significantly increased in cells treated with insulin (97%, P<0.02) for one hour compared to basal treatment (212); therefore, we used insulin as a positive control. To measure GLUT4 trafficking, the rate-limiting in glucose uptake, we performed the state-of-the-art technique biotinylation photolabeled assay that specifically quantifies cell membrane GLUTs (**Figure 7A and B)**. We demonstrated significant increases of GLUT4 translocation in GGF2-treated myocytes at both 1 ng/ml and 100 ng/ml (by 99% and 184%, respectively, P<0.05) compared to basal. Afatinib is a protein kinase inhibitor that irreversibly inhibits ErbB2 and -4 by covalently binding to cysteine number 797 of the ErbB via a Michael addition. Because afatinib had not been tested in cardiac myocytes, we incubated the myocytes with ascending concentrations of 300- and 500 nM, as suggested by research in other tissue types (213). GGF2-stimulated GLUT4 translocation to the cell membrane was blunted by the addition of 300- and 500 nM afatinib, the ErbB2 and -4 inhibitor, prior to incubation with GGF2 **(Figure 7B).** These findings suggest that GGF2-modulated glucose uptake via GLUT4 translocation and that this process is dependent on the activation of its receptors ErbB2 and ErbB4.

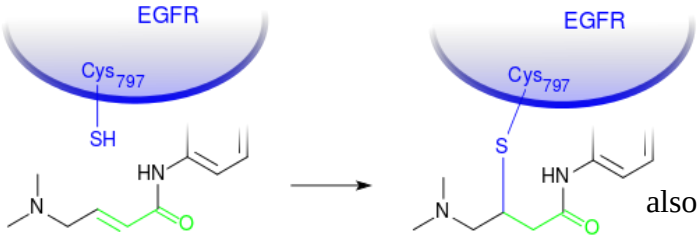

Figure 8: Afatinib covalently binds to cysteine number 797 of the epidermal growth factor receptor (EGFR) via a Michael addition (IC50 = 0.5 nM) (4).

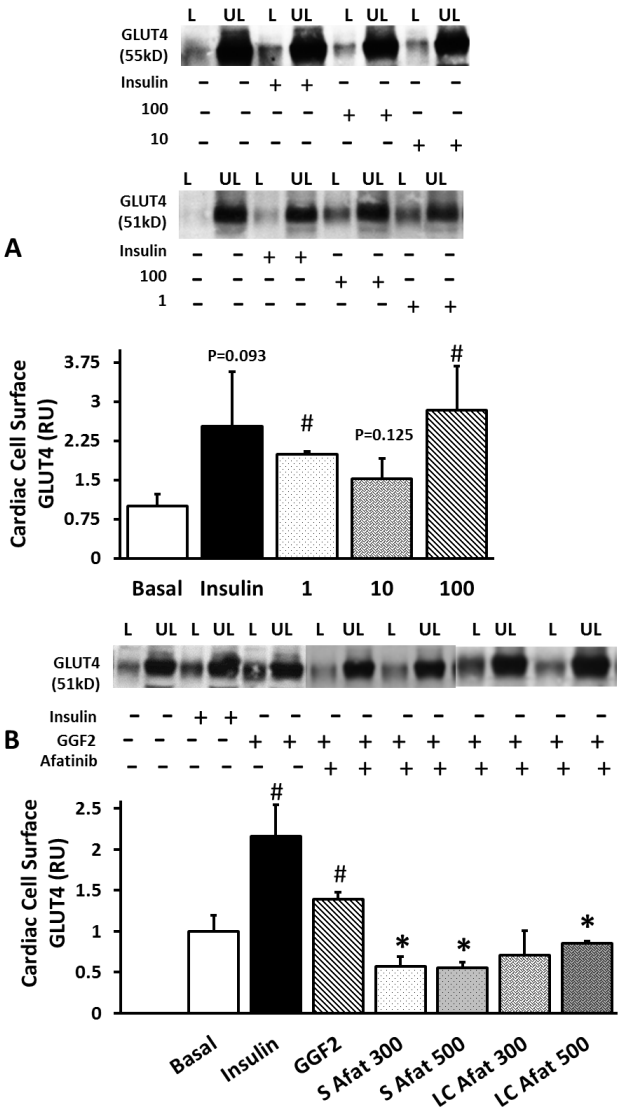

Figure 7A: Glial growth factor 2 (GGF2) stimulates GLUT4 trafficking to the cardiac cell surface. Top panels: representative Western blot. Bottom Panels: Mean ± SE of cell surface GLUT4 protein content; values normalized to basal ventricle (n=3-8/group); # P<0.05 vs. basal (t-test). Methods: Cell membrane fraction of isolated rat ventricular myocytes incubated with or without 0.0349 mM insulin, 1 ng/ml, 10 ng/ml, or 100 ng/ml GGF2 for one hour. Cell surface GLUTs were measured using biotinylated photolabeling technique in isolated rat ventricular myocytes. L: Labeled (cell surface fraction); UL: Unlabeled (intracellular fraction). **B: Afatinib attenuates GGF2 stimulated translocation of GLUT4 in ventricular myocytes.** Top panels: representative Western blot. Bottom Panels: Mean ± SE of cell surface protein expression (values expressed relative to basal); # P<0.05 vs. basal; * P<0.05 vs GGF2. S: Afatinib from Selleck Chemicals; LC: Afatinib from LC Laboratories. Methods: Cell membrane fraction of isolated rat ventricular myocytes incubated with or without ErbB blocking antibodies (Afatinib, 300 nM and 500 nM from Selleck Chemicals or LC Laboratories) for 1 hour prior to incubation with or without insulin (0.0349 mM) or GGF2 (100 ng/ml).

4.2 In vitro GGF2 treatment stimulates glucose uptake via PI3K-dependent pathway

4.2.1 GGF2 increases phosphorylation of PDK-1

It has been proposed that neuregulin-1 regulates glucose metabolism in both skeletal and cardiac muscle via activation of downstream effectors including P13K and Akt (6, 8, 159). Yet, whether GGF2 increases glucose uptake via a PI3K-dependent pathway is unknown. Therefore, we hypothesized that there will be increased phosphorylation of PI3K's downstream effector, PDK-1 in both insulin- and GGF2-treated cardiac myocytes. Insulin and GGF2 both elicited increases in phosphorylated PDK-1 compared to basal (91% and 206%, respectively, $P<0.05$**, Figure 9A**). No increase in total expression of PDK-1 after one hour treatment of GGF2 was observed, suggesting that this protein's basal abundance in the cell is not altered by GGF2 treatment (**Figure 9B**).

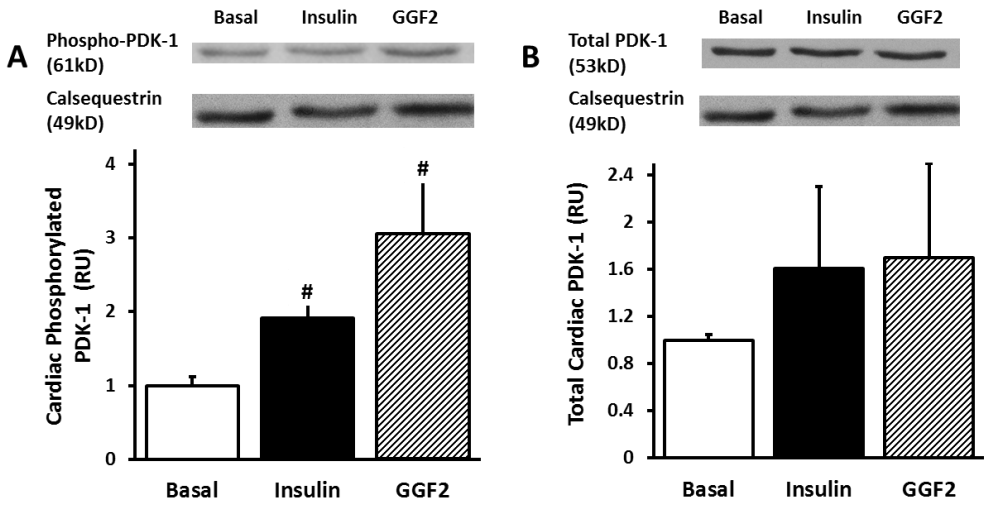

Figure 9A: Insulin and GGF2 stimulates PDK-1 phosphorylation in ventricular myocytes. Top panel: representative Western blot. Bottom panel: Mean ± SE of protein expression (values expressed relative to basal; n=4-5/group); # P<0.05 vs. basal. Methods: Total lysate of isolated rat ventricular myocytes were incubated with 0.0349 mM insulin or 100 ng/ml GGF2; Calsequestrin (CLSQ) was used as a loading control. **B: Total PDK-1 protein expression upon insulin and GGF2 treatment** of ventricular myocytes. Top panels: representative Western blot. Bottom panels: Mean ± SE of protein expression (values expressed relative to basal; n=17/group); # P<0.05 vs. basal. Methods: Total lysate of isolated rat ventricular myocytes were incubated with 0.0349 mM insulin or 100 ng/ml GGF2; Calsequestrin (CLSQ) was used as a loading control.

4.2.2 GGF2 increases Akt phosphorylation and expression

PDK-1, once activated, targets the phosphorylation of Akt, a protein well understood to play a key role in glucose uptake in insulin sensitive tissues (110). It is well documented that insulin increases Akt phosphorylation at both th-308 and ser-473 sites; therefore, our observations of phosphorylated thr-308 and ser-473 in response to insulin (57% and 524%, respectively, $P<0.05$) were consistent with previous literature (212). In cells incubated with GGF2 for 1 hour, p-thr308-Akt displayed a significant increase of 75% ($P=0.029$) compared to basal (**Figure 10A**). Furthermore, we observed a 139% ($P=0.01$) increase in p-ser473-Akt upon GGF2 treatment (**Figure 10B**). Therefore, to determine if this response was a direct response of GGF2's activation of its ErbB2 and -4 receptors, we blocked the ErbB2 and -4 receptors with afatinib. As expected, both p-thr308 and p-ser473 phosphorylation returned to their basal level upon blockage of the ErbB2 and -4 receptors (**Figure 10A &B**). Lastly, a similar response was demonstrated in total Akt expression for both insulin and GGF2 compared to basal (91% and 95%, respectively, $P<0.05$), although blocking the ErbB2 and -4 receptors with afatinib prior to treatment with GGF2 did not decrease total Akt expression (**Figure 10C**).

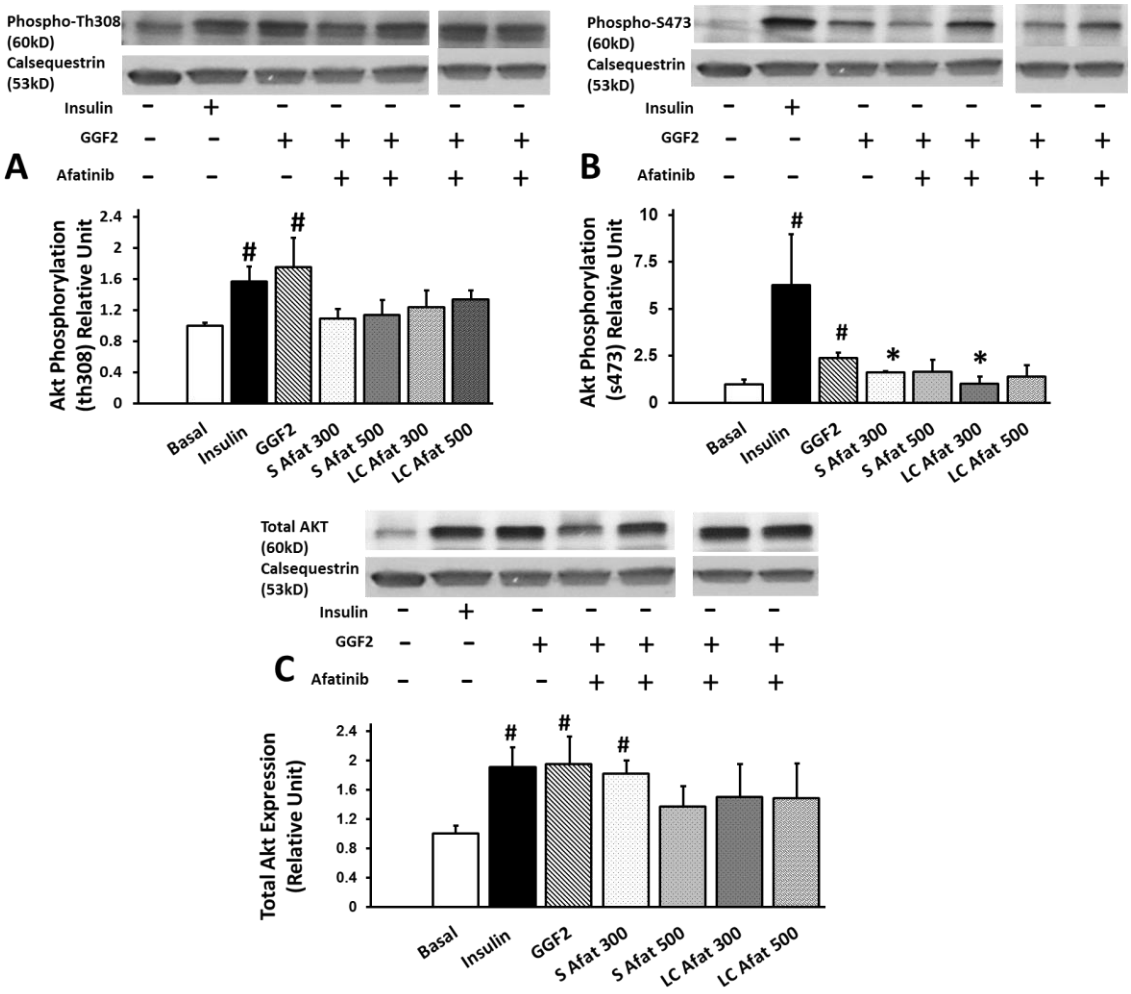

Figure 10: Analysis of the downstream insulin signaling pathways in the healthy myocardium. Afatinib attenuates GGF2 stimulation of Akt phosphorylation at A) Th308 and B) s473 sites in ventricular myocytes. Top panels: representative Western blot. Bottom panels: Mean ± SE of protein expression (values expressed relative to basal; n=3-4/group); # P<0.05 vs. basal; * P<0.05 vs GGF2 Methods: Total lysate of isolated rat ventricular myocytes incubated with insulin (0.0349 mM), ErbB blocking antibodies (Afatinib, 300 nM and 500 nM) or GGF2 (100 ng/ml); Calsequestrin (CLSQ) was used as a loading control. Bottom panels: Mean ± SE of protein expression (values expressed relative to basal; n=3-4/group); # P<0.05 vs. basal; * P<0.05 vs GGF2. **C) Total Akt protein expression upon insulin, GGF2 and GGF2+Afatinib treatment** of ventricular myocytes. Top panels: representative Western blot. Bottom panels: Mean ± SE of protein expression (values expressed relative to basal; n=4-5/group); # P<0.05 vs. basal. Methods: Total lysate of isolated rat ventricular myocytes were incubated with 0.0349 mM insulin or 100 ng/ml GGF2; Calsequestrin (CLSQ) was used as a loading control.

4.2.3 GGF2 stimulates AS160 phosphorylation and expression

As its name suggests, Akt substrate at 160kD, commonly known as AS160, is a direct target of Akt (116). Upon phosphorylation of AS160, GLUT4 vesicles are released to the cell membrane. Therefore, to confirm that GGF2 does, in fact, activate GLUT4 via an AS160-dependent mechanisms, we quantified total and phosphorylated AS160 after cells were incubated with GGF2 or insulin for 1 hour (**Figure 11A**). Increased AS160 phosphorylation was observed after insulin and GGF2 stimulation (69% and 72%, respectively, $P<0.05$). Furthermore, insulin and GGF2 treatment in adult cardiac myocytes induced a 96% and 99% increase, respectively, in total AS160 compared to basal ($P<0.05\%$) (**Figure 11B**). These data suggest that similar to insulin, acute *in vitro* GGF2 treatment activates the downstream effectors of the PI3K pathway, including AS160, which in turn stimulates GLUT4 translocation in healthy adult cardiomyocytes.

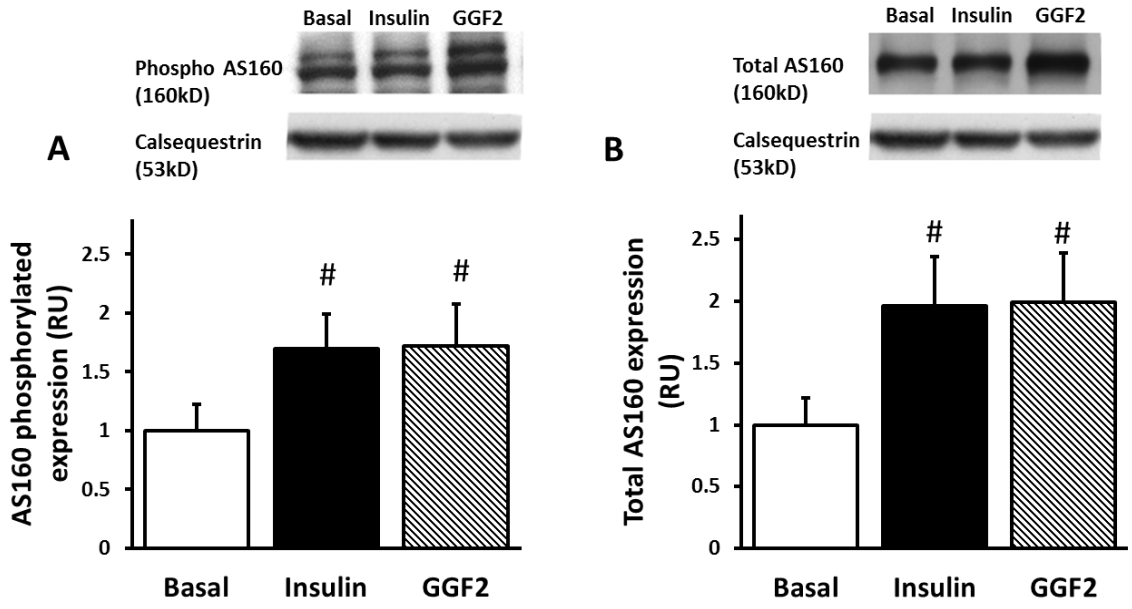

Figure 11: Analysis of the downstream insulin signaling pathways in the healthy myocardium. A) Insulin and GGF2 stimulates AS160 phosphorylation in ventricular myocytes. Top panel: representative Western blot. Bottom panel: Mean ± SE of protein expression (values expressed relative to basal; n=14-15/group); # $P<0.05$ vs. basal. Methods: Total lysate of isolated rat ventricular myocytes were incubated with 0.0349 mM insulin or 100 ng/ml GGF2; Calsequestrin (CLSQ) was used as a loading control. **B) Total AS160 protein expression upon insulin and GGF2 treatment** of ventricular myocytes. Top panels: representative Western blot. Bottom panels: Mean ± SE of protein expression (values expressed relative to basal; n=17/group); # $P<0.05$ vs. basal. Methods: Total lysate of isolated rat ventricular myocytes were incubated with 0.0349 mM insulin or 100 ng/ml GGF2; Calsequestrin (CLSQ) was used as a loading control.

4.2.4 GGF2 increases phosphorylation of PKCζ in adult cardiac myocytes

To determine if GGF2 activates glucose uptake independently of Akt, we explored an alternative target of PDK-1. In the presence of insulin, the downstream target PDK-1 is known to activate PKCζ-induced GLUT4 translocation, independently of Akt and AS160 (123). When compared to basal conditions, our data indicated increases in p-PKCζ expression in the presence of insulin and, as well as, GGF2 (158% and 100%, respectively, $P<0.05$, **Figure 12A**). Total expression of PKCζ was unchanged compared to basal (**Figure 12B**).

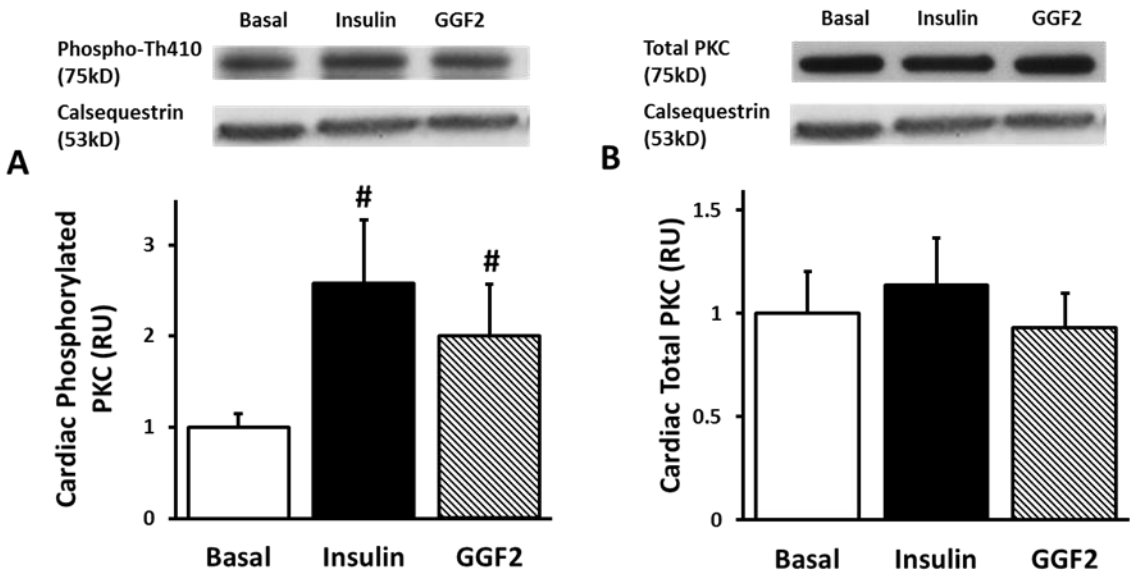

Figure 12: Analysis of the downstream insulin signaling pathways in the healthy myocardium. A) Insulin and GGF2 stimulates phosphorylation of PKC zeta at Th410 site in ventricular myocytes. Top panels: representative Western blot. Bottom panels: Mean ± SE of protein expression (values expressed relative to basal; n=9/group); # P<0.05 vs. basal. Methods: Total lysate of isolated rat ventricular myocytes were incubated with 0.0349 mM insulin or 100 ng/ml GGF2; Calsequestrin (CLSQ) was used as a loading control. **B) Total PKC protein expression upon insulin and GGF2 treatment** of ventricular myocytes. Top panels: representative Western blot. Bottom panels: Mean ± SE of protein expression (values expressed relative to basal; n=7/group). Methods: Total lysate of isolated rat ventricular myocytes were incubated with 0.0349 mM insulin or 100 ng/ml GGF2; Calsequestrin (CLSQ) was used as a loading control.

4.3 Calcium activated GLUT4 translocation is dependent on ErbB2 and -4

It has been reported that the inhibition of ErbB2 receptors use for the treatment of lung cancer resulted in severe cardiac complications (214). It is also known that calcium not only plays a major role in cardiac contraction but also glucose uptake (148), although the mechanisms are not fully understood. Therefore, we hypothesized that calcium activates GLUT4 translocation through an ErbB2 and -4 signaling pathway. We incubated cardiac ventricular myocytes with/or without ErbB2 and -4 inhibitor, afatinib, for one hour prior to incubation with calcium. Calcium stimulation yielded a 511% ($P<0.001$) increase in GLUT4 translocation compared to basal levels. Upon ErbB2 and -4 blockage, we observed a 408% ($P<0.05$) decrease in cell surface GLUT4 in contrast to calcium alone (**Figure 13**). Blocking ErbB2 and -4 did not completely eradicate calcium-stimulated glucose uptake. This data suggested that calcium-stimulated glucose uptake is mediated by other insulin-independent pathways, including AMPK and CAMK (129, 151).

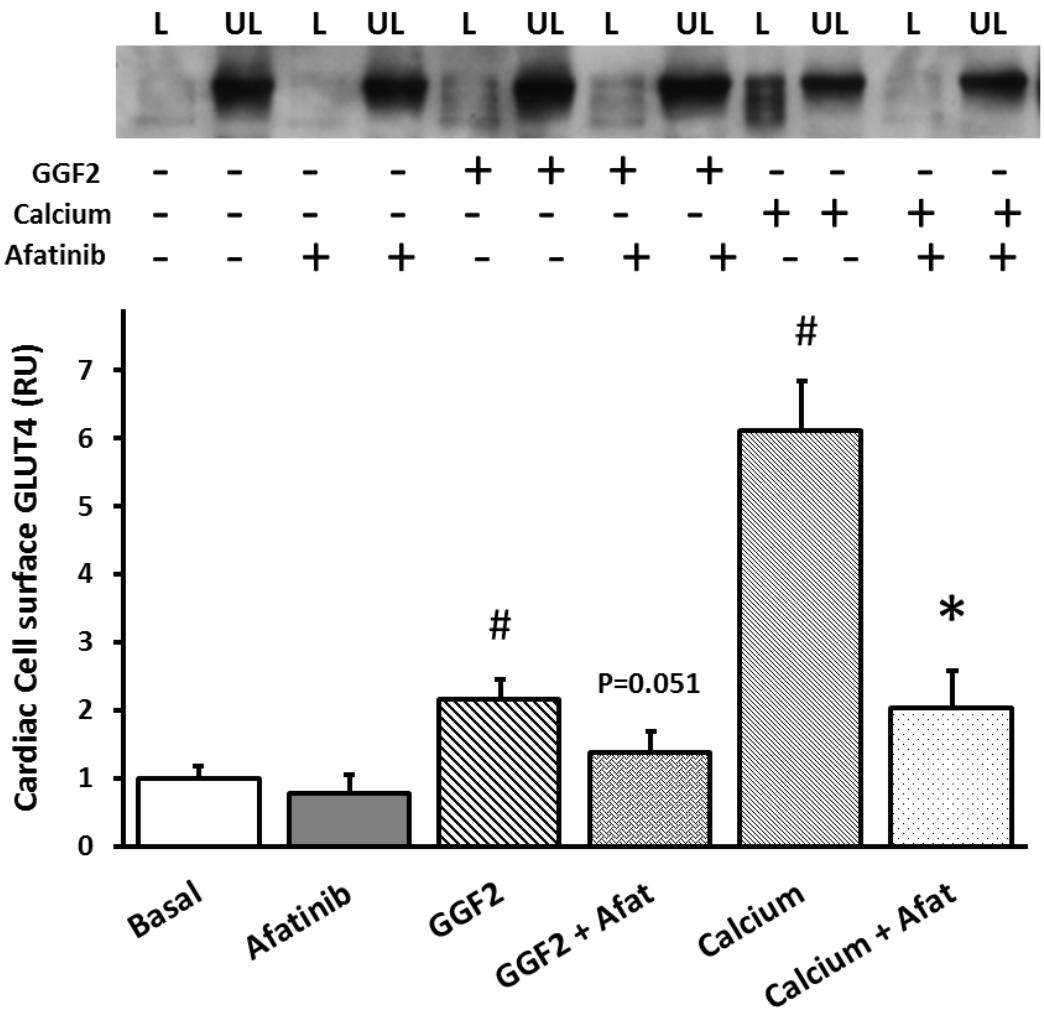

Figure 13: Analysis of the downstream calcium signaling pathway in the healthy myocardium. Afatinib attenuates calcium stimulated translocation of GLUT4 in ventricular myocytes. Top panels: representative Western blot. Mean ± SE of protein expression (values expressed relative to basal; n=4-6/group); # $P<0.05$ vs. basal; * $P<0.05$ vs. calcium. Methods: Cell surface GLUTs were measured using biotinylated photolabeling technique on isolated rat ventricular myocytes incubated with and without 300 nM Afatinib prior to incubation with 100 ng/ml GGF2 or 0.5 mM $CaCl_2$. L: Labeled (cell surface fraction); UL: Unlabeled (intracellular fraction).

CHAPTER V

DISSCUSSION

Although neuregulin 1-β and its ErbB2 and -4 receptors are essential in the development and maintenance of the heart, little is known about the mechanisms by which it promotes cell survival and differentiation. In this study, we demonstrated that NRG1β isoform, glial growth factor 2 (GGF2), stimulates GLUT4 translocation, and thus increases glucose uptake in healthy adult rat cardiac myocytes via PDK-1-, Akt-, AS160-, and PKCζ-dependent mechanisms. Furthermore, we reported that calcium-mediated glucose uptake in isolated cardiomyocytes is dependent on ErbB2 and -4 receptors. These findings highlighted some novel mechanisms of action of GGF2, which warrant further investigation in patients with heart failure.

5.1 GGF2 increases glucose uptake in adult cardiac myocytes

Neuregulin-1β and its ErbB 2/4 receptor have vital roles in the development and maintenance of the heart (27). Being within the epidermal growth factor family, NRG-1 action on cells promotes proliferation, differentiation, and survival (27-30). In this study, we demonstrated that GGF2 treatment modulates glucose uptake in healthy adult cardiac myocytes via a PDK-1, Akt, AS160, and PKCζ-dependent mechanism. Studies done in

both L6E9 myotubes (25) and soleus muscle (159) support the implication of a neuregulin-stimulated PI3K-dependent mechanism facilitating glucose uptake. In a study using L6E9 myotubes treated with neuregulin, Dr. Canto's group indicated an increased activation of the PDK-1 pathway via the ErbB3 receptor (25). Although, ErbB3 is not reported to be expressed in adult cardiac myocytes, we demonstrated similar responses in the PDK-1 pathway activation when treated with neuregulin. Furthermore, Canto's lab reported neuregulin-stimulated glucose transport was additive to insulin, suggesting this as an alternative mechanism to insulin-stimulated glucose uptake (25). Recently Dr. Pentassuglia and colleagues demonstrated that NRG1-β enhanced glucose uptake in neonatal cardiomyocytes as efficiently as insulin-like growth factor-1 and insulin (215). The metabolism of neonatal cardiomyocytes is different than adult myocytes and do not reflect the physiological process in the heart. For instance, shortly after birth, cardiac myocytes switch from predominant expression of glucose transporter GLUT1 to the insulin-sensitive isoform GLUT4. This switch coincides with the development of insulin sensitivity and shift from a straight carbohydrate to a mixed fat/carbohydrate diet (216). Furthermore, our study utilized GGF2 over rhNRG-1 as studies have shown that single intravenous dose of GGF2 has parallel efficacy to the EGF-domain only fragment of rhNRG-1 which was given as daily intravenous infusions over 10 days (11, 12).

5.1.1 GGF2 increases GLUT4 translocation in adult cardiac myocytes

Glucose uptake, the rate-limiting step in whole-body glucose homeostasis, is regulated by a family of specialized proteins, called the GLUTs. GLUT4 is the major isoform expressed in myocytes and is translocated from an intracellular pool to the cell membrane during insulin-mediated glucose uptake. Under basal conditions, GLUT4 is primarily

located within multiple intracellular compartments such that only 2-5% is observed at the plasma membrane (89-93). Many techniques have been developed to study metabolism in the myocardium, yet, the quantification of cell surface GLUTs, which is necessary to reveal the molecular mechanisms of glucose transport regulation, has proved very challenging in murine models. As an example, it is difficult to apply conventional membrane fractionation techniques to rodents due to the size limitation of their hearts. Furthermore, immunohistochemistry and immunofluorescent techniques cannot differentiate the inactive GLUTs from the active GLUTs. Additionally, neonatal cardiac myocyte studies, for which GLUT4 is only a minor isoform, is not physiologically relevant to intact adult myocytes. Therefore, we measured GLUT4 trafficking by the state-of-the-art technique biotinylation photolabeled assay that quantifies cell membrane GLUTs. In this way, we are able to overcome a substantial obstacle in metabolism research, in that we directly quantified the activation and translocation of the GLUT4. Our lab has further validated this technique in quantifying GLUTs in the insulin stimulated GLUT4, -8 translocation as well as insulin-independent GLUT12 in the rat ventricular myocytes as well as intact mouse ventricle and atria (23, 212, 217). Our results suggest that short-term GGF2 treatment stimulates GLUT4 translocation in cardiomyocytes to the same extent as insulin. Similarly, Dr. Suarez and colleagues demonstrated a 43% increase in GLUT4 abundance within plasma membrane fractions of skeletal myocytes. Furthermore, his research demonstrated that GLUT1 and GLUT3 translocate to the same extent in response to the treatment of skeletal muscle with neuregulin (159). However, to our knowledge, this is the first report on the effect of acute in vitro GGF2 treatment on GLUT4 translocation in cardiomyocytes.

These cardioprotective mechanisms are initiated upon NRG-1 binding its tyrosine kinase receptors (ErbB2, ErbB3, and ErbB4), triggering phosphorylation and the downstream cascade of effectors (38). In alignment with previous studies on incubated soleus muscle (24) and neonatal cardiomyocytes (215), we demonstrated that inhibition of ErbB2 and ErbB4 impaired neuregulin-induced glucose transport. We further confirmed that GGF2 action on GLUT4 translocation in cardiac myocytes was significantly blunted by the irreversible ErbB 2/4 inhibitor, afatinib. By isolating the cardiac myocytes from whole tissue, we eliminated the possibility of endogenous receptor activation by endothelial cells producing NRG to bind the ErbB receptors in the cardiac myocytes. Therefore, activation of the ErbB receptors were due to the addition of exogenous GGF2.

5.2 GGF2 stimulates glucose uptake via an Akt-dependent pathway in adult cardiac myocytes

It has been well elucidated that ErbB2 and ErbB4 activation initiates PI3K signaling cascades in different tissue types (36, 218). Similarly, our results indicated an increase in PDK-1 phosphorylation, a downstream target of PI3K, in response to GGF2

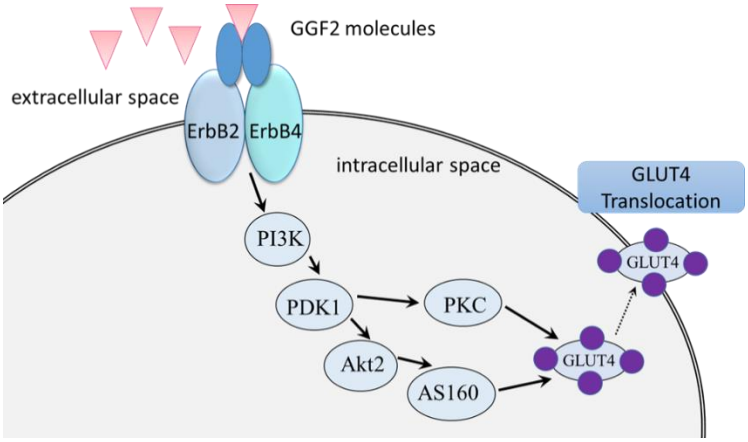

Figure 14: The downstream GGF2 signaling pathway. The above schematic outlines a portion of the downstream GGF2-signaling pathway. Abbreviations are used as follows: PI3K = Phosphoinositide 3-kinase, PDK1= Phosphoinositide-dependent kinase-1, PKC = Protein kinase C, Akt2 = Protein kinase B isoform 2, AS160 = Akt substrate at 160 kD, GLUT4 = Glucose transporter isoform 4 (artistic credit to Allison Campolo).

treatment in adult cardiomyocytes. In L6E9 myotubes, PDK-1 is also demonstrated to be an essential downstream target of PI3K, in the induction of glucose transport by neuregulin stimulation (25). The marked increase in p-PDK-1 leads us to infer that it is highly sensitive to GGF2 similar to the traditionally accepted activation by insulin (104).

Furthermore, our results showed increase in phosphorylation of Akt, a downstream target of PDK1 (104), following GGF2 treatment. It is well documented that insulin stimulation increases Akt phosphorylation at both th-308 and ser-473 sites in cardiomyocytes; therefore, our observations of phosphorylated th-308 and ser-473 in response to insulin were in agreement with previous literature (212). Similarly, NRG-1 is known to activate Akt phosphorylation in neonatal myocytes, L6E9 myotubes, and soleus muscle (25) (215). In the current study, inhibition of the ErbB 2/4 receptors by afatinib diminished neuregulin-stimulated Akt phosphorylation at both the serine and threonine sites. These findings suggest that Akt is an essential downstream target of ErbB2/4 phosphorylation in the stimulation of glucose uptake. In addition, upon GGF2 stimulation, we observed a significant increase in the activation AS160, the downstream effector of Akt. Taken together, these data indicated that GGF2 treatment increases glucose uptake in cardiomyocytes independent of insulin via the PI3K/AS160 pathway (**Figure 13, artistic credit to Allison Campolo**).

A lesser known pathway, but just as necessary, is the PDK1 activation of PKCζ in the stimulation of glucose uptake. An insulin stimulus is known to rapidly increase PKC-ζ activity in adipocytes (122), L6 myotubes (123), and 32D cells (125) via PI3K-dependent stimulation (123-125). In the current study, we identified an alternative pathway for glucose uptake in which acute GGF2 treatment significantly increases activation of PKCζ

in cardiomyocytes, to the same extent as insulin stimulation. This is in contrast with previous reports in which neuregulin had a stronger maximal effect on PKC than insulin in L6E9 myotubes.(25). From these data we conclude that GGF2 increases glucose uptake in a PKCζ-dependent mechanism in cardiac myocytes, but to a lesser extent than what is observed in skeletal muscle.

5.3 Calcium activated GLUT4 translocation is dependent on ErbB2 and -4 in adult cardiac myocytes

In striated muscle, GLUT4 translocation is activated through the signaling pathways of both contraction and insulin, which act independently of each other to induce glucose uptake. Notably, during insulin resistance, there is no alterations on the effects of contraction-mediated glucose uptake, suggesting that contraction and insulin utilize independent mechanisms to stimulate glucose uptake (219, 220). Although it is previously described that increases in intracellular Ca^{2+}, even at minimal concentrations that do not excite muscle contraction, stimulates GLUT4 translocation in skeletal muscle (221) and adipose (222), less is known about Ca^{2+}-mediated glucose transport in the heart. Previously our lab provided insights into this mechanism using a transgenic mouse model with cardiac-specific upregulation the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump. We demonstrated that the SERCA pump, the only mechanism to maintain SR/ER Ca^{2+} stores within cardiac myocytes, regulated cardiac glucose homeostasis via an insulin-independent pathway (23).

Ex vivo electrically-stimulated muscle contractions in both slow- and fast-twitch muscles activated both ErbB2 and ErbB4 receptors via a ligand-dependent manner. Both slow- and fast-twitch muscles upon electrical stimulation released NRG molecules into the

media (24). Furthermore, blockage of ErbB4 receptor impaired the contraction-induced glucose uptake observed in these tissues. In a separate experiment by Dr. Canto, caffeine-induced calcium release from the sarcoplasmic reticulum increased ErbB 2/4 phosphorylation (24). This receptor activation was, again, blocked upon treatment with ryanodine receptor blocker, dantrolene, which inhibits calcium recycling within the cell (24). In the present study, we incubated isolated cardiac myocytes with physiological concentrations of calcium. As our results indicated, upon ErbB 2/4 blockage by afatinib, a marked reduction in calcium-initiated GLUT4 translocation was observed. However, blockage of ErbB 2/4 did not completely inhibit calcium-activated GLUT4. Therefore, one could assume that calcium activates alternative pathways, including AMPK or CaMK. In accordance with previous literature reporting a marked increase in cardiac glucose uptake compared to insulin-stimulated (24). Thus, to our knowledge, this is the first report that calcium-stimulated GLUT4 translocation is primarily mediated by ErbB 2/4 receptors in cardiac myocytes. With our findings we conclude that calcium-stimulated glucose uptake is not exclusively, but predominantly, regulated via Erb2 and -4 receptors.

CHAPTER VI

CONCLUSIONS

Using a cell surface biotinylation assay, we demonstrated that the major GLUT isoform, GLUT4, is a GGF2-sensitive glucose transporter. Our data further suggested that GLUT translocation to the cell surface, which regulated glucose uptake, is modulated by the downstream PI3K-dependent pathway via Akt and AS160 phosphorylation as well as PKCζ. We demonstrated that inhibiting the ErbB2 and -4 receptors impairs the GGF2-stimulated trafficking of GLUT-4. Furthermore, we determined that inhibition of the ErbB2 and -4 receptors pointedly diminishes calcium-induced glucose transport.

In spite of the currently insufficient treatments for MI, metabolic therapy is emerging as a promising field utilizing the benefits of metabolic processes for treatment of patients with MI and HF. Indeed, increased glucose uptake has been shown to be cardio-protective during myocardial infarction (193). Therefore, the enhancement of glucose transport reported by this study give further insights into the cardioprotective role of GGF2, alongside its ErbB receptors, in patients with myocardial infarction. Therefore, better understanding of the regulation of GGF2-stimulated glucose transport may lead to its use as a novel therapeutic target for the treatment of patients who have suffered a myocardial infarction.

Source of funding: Research support was provided by Acorda Therapeutics.

REFERENCES

1. Männer J. Cardiac looping in the chick embryo: A morphological review with special reference to terminological and biomechanical aspects of the looping process. The Anatomical Record. 2000;259(3):248-62.
2. Sedmera D, Pexieder T, Hu N, Clark EB. Developmental changes in the myocardial architecture of the chick. Anat Rec. 1997;248(3):421-32.
3. Stefansson H, Sigurdsson E, Steinthorsdottir V, Bjornsdottir S, Sigmundsson T, Ghosh S, et al. Neuregulin 1 and Susceptibility to Schizophrenia. American Journal of Human Genetics. 2002;71(4):877-92.
4. Schubert-Zsilavecz M, Wurglics, M. Neue Arzneimittel Frühjahr. 2013.
5. Gumà A, Martínez-Redondo V, López-Soldado I, Cantó C, Zorzano A. Emerging role of neuregulin as a modulator of muscle metabolism. American Journal of Physiology - Endocrinology and Metabolism. 2010;298(4):E742-E50.
6. Pentassuglia L, Sawyer DB. The role of Neuregulin-1beta/ErbB signaling in the heart. Experimental cell research. 2009;315(4):627-37.
7. Falls DL. Neuregulins: functions, forms, and signaling strategies. Experimental cell research. 2003;284(1):14-30.
8. Wadugu B, Kühn B. The role of neuregulin/ErbB2/ErbB4 signaling in the heart with special focus on effects on cardiomyocyte proliferation. American Journal of Physiology - Heart and Circulatory Physiology. 2012;302(11):H2139-H47.
9. Tania B, Christopher B, Eileen H, Xinhua Y. Anti-HER2 Cancer Therapy and Cardiotoxicity. Current Pharmaceutical Design. 2014;20(30):4911-9.
10. Jabbour A, Hayward CS, Keogh AM, Kotlyar E, McCrohon JA, England JF, et al. Parenteral administration of recombinant human neuregulin-1 to patients with stable chronic heart failure produces favourable acute and chronic haemodynamic responses. European journal of heart failure. 2011;13(1):83-92.
11. Brittain E MJ, Geisberg C, Caggiano A, Eisen A, Anderson S, Sawyer D, Mendes L, Lenihan D. Evaluation of cardiac function in symptomatic heart failure patients in a single infusion, Phase 1, dose escalation study of glial growth factor 2. Journal of the American College of Cardiology. 2013;61.
12. Lenihan DJ AS, Geisberg C, Caggiano A, Eisen A, Brittain E, Muldowney JAS, III, Mendes L, Sawyer D. SAFETY AND TOLERABILITY OF GLIAL GROWTH FACTOR 2 IN PATIENTS WITH CHRONIC HEART FAILURE: A PHASE I SINGLE DOSE ESCALATION STUDY. Journal of the American College of Cardiology. 2013;61.
13. Liu X, Gu X, Li Z, Li X, Li H, Chang J, et al. Neuregulin-1/erbB-activation improves cardiac function and survival in models of ischemic, dilated, and viral cardiomyopathy. Journal of the American College of Cardiology. 2006;48(7):1438-47.
14. Galindo CL, Ryzhov S, Sawyer DB. Neuregulin as a heart failure therapy and mediator of reverse remodeling. Current heart failure reports. 2014;11(1):40-9.
15. Buonanno A, Fischbach GD. Neuregulin and ErbB receptor signaling pathways in the nervous system. Current opinion in neurobiology. 2001;11(3):287-96.

16. Messer JV, Neill WA. Symposium on the Coronary CirculationThe oxygen supply of the human heart∗. The American journal of cardiology. 1962;9(3):384-94.
17. Messer JV, Wagman RJ, Levine HJ, Neill WA, Krasnow N, Gorlin R. PATTERNS OF HUMAN MYOCARDIAL OXYGEN EXTRACTION DURING REST AND EXERCISE*. The Journal of Clinical Investigation.41(4):725-42.
18. Stanley WC, Recchia FA, Lopaschuk GD. Myocardial substrate metabolism in the normal and failing heart. Physiological reviews. 2005;85(3):1093-129.
19. Aerni-Flessner L, Abi-Jaoude M, Koenig A, Payne M, Hruz PW. GLUT4, GLUT1, and GLUT8 are the dominant GLUT transcripts expressed in the murine left ventricle. Cardiovascular diabetology. 2012;11:63.
20. Amorim PA, Nguyen TD, Shingu Y, Schwarzer M, Mohr FW, Schrepper A, et al. Myocardial infarction in rats causes partial impairment in insulin response associated with reduced fatty acid oxidation and mitochondrial gene expression. J Thorac Cardiovasc Surg. 2010;140(5):1160-7.
21. Penuelas I, Abizanda G, Garcia-Velloso MJ, Gavira JJ, Marti-Climent JM, Ecay M, et al. 18F-FDG metabolism in a rat model of chronic infarction: a 17-sector semiquantitative analysis. Nuklearmedizin. 2007;46(4):149-54.
22. Murray AJ, Lygate CA, Cole MA, Carr CA, Radda GK, Neubauer S, et al. Insulin resistance, abnormal energy metabolism and increased ischemic damage in the chronically infarcted rat heart. Cardiovascular research. 2006;71(1):149-57.
23. Waller AP, Kalyanasundaram A, Hayes S, Periasamy M, Lacombe VA. Sarcoplasmic reticulum Ca2 + ATPase pump is a major regulator of glucose transport in the healthy and diabetic heart. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease. 2015;1852(5):873-81.
24. Cantó C, Chibalin AV, Barnes BR, Glund S, Suárez E, Ryder JW, et al. Neuregulins Mediate Calcium-induced Glucose Transport during Muscle Contraction. Journal of Biological Chemistry. 2006;281(31):21690-7.
25. Cantó C, Suárez E, Lizcano JM, Griñó E, Shepherd PR, Fryer LGD, et al. Neuregulin Signaling on Glucose Transport in Muscle Cells. Journal of Biological Chemistry. 2004;279(13):12260-8.
26. and GDF, Rosen KM. ARIA:A Neuromuscular Junction Neuregulin. Annual Review of Neuroscience. 1997;20(1):429-58.
27. Wen D, Peles E, Cupples R, Suggs SV, Bacus SS, Luo Y, et al. Neu differentiation factor: A transmembrane glycoprotein containing an EGF domain and an immunoglobulin homology unit. Cell. 1992;69(3):559-72.
28. Holmes WE, Sliwkowski MX, Akita RW, Henzel WJ, Lee J, Park JW, et al. Identification of Heregulin, a Specific Activator of p185erbB2. Science. 1992;256(5060):1205-10.
29. Marchionni MA, Goodearl ADJ, Su Chen M, Bermingham-McDonogh O, Kirk C, Hendricks M, et al. Glial growth factors are alternatively spliced erbB2 ligands expressed in the nervous system. Nature. 1993;362(6418):312-8.
30. Falls DL, Rosen KM, Corfas G, Lane WS, Fischbach GD. ARIA, a protein that stimulates acetylcholine receptor synthesis, is a member of the neu ligand family. Cell. 1993;72(5):801-13.
31. Ho W-H, Armanini MP, Nuijens A, Phillips HS, Osheroff PL. Sensory and Motor Neuron-derived Factor. A NOVEL HEREGULIN VARIANT HIGHLY EXPRESSED IN

SENSORY AND MOTOR NEURONS. Journal of Biological Chemistry. 1995;270(24):14523-32.
32. Burgess TL, Ross SL, Qian Y-x, Brankow D, Hu S. Biosynthetic Processing of neu Differentiation Factor: GLYCOSYLATION, TRAFFICKING, AND REGULATED CLEAVAGE FROM THE CELL SURFACE. Journal of Biological Chemistry. 1995;270(32):19188-96.
33. Shirakabe K, Wakatsuki S, Kurisaki T, Fujisawa-Sehara A. Roles of Meltrin beta /ADAM19 in the processing of neuregulin. The Journal of biological chemistry. 2001;276(12):9352-8.
34. Montero JC, Yuste L, Diaz-Rodriguez E, Esparis-Ogando A, Pandiella A. Differential shedding of transmembrane neuregulin isoforms by the tumor necrosis factor-alpha-converting enzyme. Molecular and cellular neurosciences. 2000;16(5):631-48.
35. Wang JY, Miller SJ, Falls DL. The N-terminal region of neuregulin isoforms determines the accumulation of cell surface and released neuregulin ectodomain. The Journal of biological chemistry. 2001;276(4):2841-51.
36. Carraway KL, 3rd, Burden SJ. Neuregulins and their receptors. Current opinion in neurobiology. 1995;5(5):606-12.
37. Lemmens K, Segers VFM, Demolder M, De Keulenaer GW. Role of Neuregulin-1/ErbB2 Signaling in Endothelium-Cardiomyocyte Cross-talk. Journal of Biological Chemistry. 2006;281(28):19469-77.
38. Holbro T, Hynes NE. ErbB receptors: directing key signaling networks throughout life. Annual review of pharmacology and toxicology. 2004;44:195-217.
39. Sedmera D. Form follows function: developmental and physiological view on ventricular myocardial architecture. European Journal of Cardio-Thoracic Surgery. 2005;28(4):526-8.
40. Sedmera D. Function and form in the developing cardiovascular system. Cardiovascular research. 2011;91(2):252-9.
41. Chen F, De Diego C, Chang MG, McHarg JL, John S, Klitzner TS, et al. Atrioventricular conduction and arrhythmias at the initiation of beating in embryonic mouse hearts. Developmental Dynamics. 2010;239(7):1941-9.
42. Sadler TW. Selective serotonin reuptake inhibitors (SSRIs) and heart defects: potential mechanisms for the observed associations. Reprod Toxicol. 2011;32(4):484-9.
43. Hirota A, Kamino K, Komuro H, Sakai T, Yada T. Early events in development of electrical activity and contraction in embryonic rat heart assessed by optical recording. The Journal of Physiology. 1985;369:209-27.
44. Patten BM. The formation of the cardiac loop in the chick. American Journal of Anatomy. 1922;30(3):373-97.
45. Bartram U, Molin DGM, Wisse LJ, Mohamad A, Sanford LP, Doetschman T, et al. Double-Outlet Right Ventricle and Overriding Tricuspid Valve Reflect Disturbances of Looping, Myocardialization, Endocardial Cushion Differentiation, and Apoptosis in TGF-β2–Knockout Mice. Circulation. 2001;103(22):2745-52.
46. Hutchins GM, Bulkley BH, Moore GW, Piasio MA, Lohr FT. Shape of the human cardiac ventricles. American Journal of Cardiology.41(4):646-54.

47. Knaapen MWM, Vrolijk BCM, Wenink ACG. Nuclear and cellular size of myocytes in different segments of the developing rat heart. The Anatomical Record. 1996;244(1):118-25.
48. MOORMAN AFM, CHRISTOFFELS VM. Cardiac Chamber Formation: Development, Genes, and Evolution. Physiological reviews. 2003;83(4):1223-67.
49. Gassmann M, Casagranda F, Orioli D, Simon H, Lai C, Klein R, et al. Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. Nature. 1995;378(6555):390-4.
50. Lee KF, Simon H, Chen H, Bates B, Hung MC, Hauser C. Requirement for neuregulin receptor erbB2 in neural and cardiac development. Nature. 1995;378(6555):394-8.
51. Meyer D, Birchmeier C. Multiple essential functions of neuregulin in development. Nature. 1995;378(6555):386-90.
52. Cote GM, Miller TA, Lebrasseur NK, Kuramochi Y, Sawyer DB. Neuregulin-1alpha and beta isoform expression in cardiac microvascular endothelial cells and function in cardiac myocytes in vitro. Experimental cell research. 2005;311(1):135-46.
53. Zhao YY, Sawyer DR, Baliga RR, Opel DJ, Han X, Marchionni MA, et al. Neuregulins promote survival and growth of cardiac myocytes. Persistence of ErbB2 and ErbB4 expression in neonatal and adult ventricular myocytes. The Journal of biological chemistry. 1998;273(17):10261-9.
54. Lemmens K, Segers VF, Demolder M, De Keulenaer GW. Role of neuregulin-1/ErbB2 signaling in endothelium-cardiomyocyte cross-talk. The Journal of biological chemistry. 2006;281(28):19469-77.
55. Fukazawa R, Miller TA, Kuramochi Y, Frantz S, Kim YD, Marchionni MA, et al. Neuregulin-1 protects ventricular myocytes from anthracycline-induced apoptosis via erbB4-dependent activation of PI3-kinase/Akt. Journal of molecular and cellular cardiology. 2003;35(12):1473-9.
56. Russell KS, Stern DF, Polverini PJ, Bender JR. Neuregulin activation of ErbB receptors in vascular endothelium leads to angiogenesis. The American journal of physiology. 1999;277(6 Pt 2):H2205-11.
57. Timolati F, Ott D, Pentassuglia L, Giraud MN, Perriard JC, Suter TM, et al. Neuregulin-1 beta attenuates doxorubicin-induced alterations of excitation-contraction coupling and reduces oxidative stress in adult rat cardiomyocytes. Journal of molecular and cellular cardiology. 2006;41(5):845-54.
58. Baliga RR, Pimental DR, Zhao YY, Simmons WW, Marchionni MA, Sawyer DB, et al. NRG-1-induced cardiomyocyte hypertrophy. Role of PI-3-kinase, p70(S6K), and MEK-MAPK-RSK. The American journal of physiology. 1999;277(5 Pt 2):H2026-37.
59. Kuramochi Y, Guo X, Sawyer DB. Neuregulin activates erbB2-dependent src/FAK signaling and cytoskeletal remodeling in isolated adult rat cardiac myocytes. Journal of molecular and cellular cardiology. 2006;41(2):228-35.
60. Kuramochi Y, Cote GM, Guo X, Lebrasseur NK, Cui L, Liao R, et al. Cardiac endothelial cells regulate reactive oxygen species-induced cardiomyocyte apoptosis through neuregulin-1beta/erbB4 signaling. The Journal of biological chemistry. 2004;279(49):51141-7.

61. Graus-Porta D, Beerli RR, Daly JM, Hynes NE. ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. The EMBO journal. 1997;16(7):1647-55.
62. Crone SA, Zhao YY, Fan L, Gu Y, Minamisawa S, Liu Y, et al. ErbB2 is essential in the prevention of dilated cardiomyopathy. Nature medicine. 2002;8(5):459-65.
63. Garcia-Rivello H, Taranda J, Said M, Cabeza-Meckert P, Vila-Petroff M, Scaglione J, et al. Dilated cardiomyopathy in Erb-b4-deficient ventricular muscle. American journal of physiology Heart and circulatory physiology. 2005;289(3):H1153-60.
64. Liu FF, Stone JR, Schuldt AJ, Okoshi K, Okoshi MP, Nakayama M, et al. Heterozygous knockout of neuregulin-1 gene in mice exacerbates doxorubicin-induced heart failure. American journal of physiology Heart and circulatory physiology. 2005;289(2):H660-6.
65. Ozcelik C, Erdmann B, Pilz B, Wettschureck N, Britsch S, Hubner N, et al. Conditional mutation of the ErbB2 (HER2) receptor in cardiomyocytes leads to dilated cardiomyopathy. Proceedings of the National Academy of Sciences of the United States of America. 2002;99(13):8880-5.
66. Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, et al. Heart disease and stroke statistics--2015 update: a report from the American Heart Association. Circulation. 2015;131(4):e29-322.
67. ter Laak MP, Hamers FPT, Kirk CJ, Gispen WH. rhGGF2 protects against cisplatin-induced neuropathy in the rat. Journal of Neuroscience Research. 2000;60(2):237-44.
68. Zhang L, Fletcher-Turner A, Marchionni MA, Apparsundaram S, Lundgren KH, Yurek DM, et al. Neurotrophic and neuroprotective effects of the neuregulin glial growth factor-2 on dopaminergic neurons in rat primary midbrain cultures. Journal of Neurochemistry. 2004;91(6):1358-68.
69. Xu Z, Jiang J, Ford G, Ford BD. Neuregulin-1 is neuroprotective and attenuates inflammatory responses induced by ischemic stroke. Biochemical and Biophysical Research Communications. 2004;322(2):440-6.
70. Iaci JF, Ganguly A, Finklestein SP, Parry TJ, Ren J, Saha S, et al. Glial growth factor 2 promotes functional recovery with treatment initiated up to 7 days after permanent focal ischemic stroke. Neuropharmacology. 2010;59(7-8):640-9.
71. Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, et al. Heart disease and stroke statistics--2015 update: a report from the American Heart Association. Circulation. 2015;131(4):e29-322.
72. Ware B, Bevier M, Nishijima Y, Rogers S, Carnes CA, Lacombe VA. Chronic heart failure selectively induces regional heterogeneity of insulin-responsive glucose transporters. American journal of physiology Regulatory, integrative and comparative physiology. 2011;301(5):R1300-6.
73. Lacombe VA. Expression and regulation of facilitative glucose transporters in equine insulin-sensitive tissue: from physiology to pathology. ISRN veterinary science. 2014;2014:409547.
74. Mohamed AL, Yong J, Masiyati J, Lim L, Tee SC. The Prevalence of Diastolic Dysfunction in Patients with Hypertension Referred for Echocardiographic Assessment

of Left Ventricular Function. The Malaysian Journal of Medical Sciences : MJMS. 2004;11(1):66-74.
75. Quiñones MA, Greenberg BH, Kopelen HA, Koilpillai C, Limacher MC, Shindler DM, et al. Echocardiographic predictors of clinical outcome in patients with left ventricular dysfunction enrolled in the SOLVD registry and trials: significance of left ventricular hypertrophy∗∗. Journal of the American College of Cardiology. 2000;35(5):1237-44.
76. Thune JJ, Køber L, Pfeffer MA, Skali H, Anavekar NS, Bourgoun M, et al. Comparison of Regional Versus Global Assessment of Left Ventricular Function in Patients with Left Ventricular Dysfunction, Heart Failure, or Both After Myocardial Infarction: The Valsartan in Acute Myocardial Infarction Echocardiographic Study. Journal of the American Society of Echocardiography.19(12):1462-5.
77. Moondra V, Sarma S, Buxton T, Safa R, Cote G, Storer T, et al. Serum Neuregulin-1beta as a Biomarker of Cardiovascular Fitness. The open biomarkers journal. 2009;2:1-5.
78. Ky B, Kimmel SE, Safa RN, Putt ME, Sweitzer NK, Fang JC, et al. Neuregulin-1 beta is associated with disease severity and adverse outcomes in chronic heart failure. Circulation. 2009;120(4):310-7.
79. Rohrbach S, Yan X, Weinberg EO, Hasan F, Bartunek J, Marchionni MA, et al. Neuregulin in cardiac hypertrophy in rats with aortic stenosis. Differential expression of erbB2 and erbB4 receptors. Circulation. 1999;100(4):407-12.
80. Rohrbach S, Niemann B, Silber RE, Holtz J. Neuregulin receptors erbB2 and erbB4 in failing human myocardium -- depressed expression and attenuated activation. Basic research in cardiology. 2005;100(3):240-9.
81. Katrien Lemmens M, PhD; Kris Doggen, MSc; Gilles W. De Keulenaer, MD, PhD. Role of Neuregulin-1/ErbB Signaling in Cardiovascular Physiology and Disease: Implications for Therapy of Heart Failure. Circulation. 2007(116):954-60

82. Li B, Zheng Z, Wei Y, Wang M, Peng J, Kang T, et al. Therapeutic effects of neuregulin-1 in diabetic cardiomyopathy rats. Cardiovascular diabetology. 2011;10:69.
83. Kasasbeh E MA, Ahmad FA, Tu E, Williams P, Nunnally A, Adcock J, Caggiano AO, Parry TJ, Ganguly A, et al. Neuregulin-1β Improves Cardiac Remodeling After Myocardial Infarction In Swine. Circulation. 2011;124.
84. Gao R, Zhang J, Cheng L, Wu X, Dong W, Yang X, et al. A Phase II, randomized, double-blind, multicenter, based on standard therapy, placebo-controlled study of the efficacy and safety of recombinant human neuregulin-1 in patients with chronic heart failure. Journal of the American College of Cardiology. 2010;55(18):1907-14.
85. Gui C, Zhu L, Hu M, Lei L, Long Q. Neuregulin-1/ErbB signaling is impaired in the rat model of diabetic cardiomyopathy. Cardiovascular Pathology.21(5):414-20.
86. Li B, Zheng Z, Wei Y, Wang M, Peng J, Kang T, et al. Therapeutic effects of neuregulin-1 in diabetic cardiomyopathy rats. Cardiovascular diabetology. 2011;10:69-.
87. Li B, Xiao J, Li Y, Zhang J, Zeng M. Gene transfer of human neuregulin-1 attenuates ventricular remodeling in diabetic cardiomyopathy rats. Experimental and Therapeutic Medicine. 2013;6(5):1105-12.

88. Mueckler M. Facilitative glucose transporters. Eur J Biochem. 1994;219(3):713-25.
89. Kandror KV, Pilch PF. Compartmentalization of protein traffic in insulin-sensitive cells. The American journal of physiology. 1996;271(1 Pt 1):E1-14.
90. Rea S, James DE. Moving GLUT4: the biogenesis and trafficking of GLUT4 storage vesicles. Diabetes. 1997;46(11):1667-77.
91. Simpson F, Whitehead JP, James DE. GLUT4--at the cross roads between membrane trafficking and signal transduction. Traffic. 2001;2(1):2-11.
92. Holman GD, Sandoval IV. Moving the insulin-regulated glucose transporter GLUT4 into and out of storage. Trends Cell Biol. 2001;11(4):173-9.
93. Pessin JE, Thurmond DC, Elmendorf JS, Coker KJ, Okada S. Molecular basis of insulin-stimulated GLUT4 vesicle trafficking. Location! Location! Location! The Journal of biological chemistry. 1999;274(5):2593-6.
94. Scheepers A, Joost HG, Schurmann A. The glucose transporter families SGLT and GLUT: molecular basis of normal and aberrant function. JPEN Journal of parenteral and enteral nutrition. 2004;28(5):364-71.
95. Koistinen HA, Zierath JR. Regulation of glucose transport in human skeletal muscle. Annals of medicine. 2002;34(6):410-8.
96. Tian R, Abel ED. Responses of GLUT4-deficient hearts to ischemia underscore the importance of glycolysis. Circulation. 2001;103(24):2961-6.
97. Abel ED, Graveleau C, Betuing S, Pham M, Reay PA, Kandror V, et al. Regulation of insulin-responsive aminopeptidase expression and targeting in the insulin-responsive vesicle compartment of glucose transporter isoform 4-deficient cardiomyocytes. Mol Endocrinol. 2004;18(10):2491-501.
98. Contreras-Ferrat AE, Toro B, Bravo R, Parra V, Vasquez C, Ibarra C, et al. An inositol 1,4,5-triphosphate (IP3)-IP3 receptor pathway is required for insulin-stimulated glucose transporter 4 translocation and glucose uptake in cardiomyocytes. Endocrinology. 2010;151(10):4665-77.
99. Stenbit AE, Tsao T-S, Li J, Burcelin R, Geenen DL, Factor SM, et al. GLUT4 heterozygous knockout mice develop muscle insulin resistance and diabetes. Nature medicine. 1997;3(10):1096-101.
100. Goodyear LJ, Kahn BB. Exercise, glucose transport, and insulin sensitivity. Annu Rev Med. 1998;49:235-61.
101. Czech MP, Corvera S. Signaling mechanisms that regulate glucose transport. The Journal of biological chemistry. 1999;274(4):1865-8.
102. Ryder JW, Chibalin AV, Zierath JR. Intracellular mechanisms underlying increases in glucose uptake in response to insulin or exercise in skeletal muscle. Acta Physiol Scand. 2001;171(3):249-57.
103. Watson RT, Pessin JE. Intracellular organization of insulin signaling and GLUT4 translocation. Recent Prog Horm Res. 2001;56:175-93.
104. Coffer PJ, Jin J, Woodgett JR. Protein kinase B (c-Akt): a multifunctional mediator of phosphatidylinositol 3-kinase activation. Biochem J. 1998;335 (Pt 1):1-13.
105. Kohn AD, Summers SA, Birnbaum MJ, Roth RA. Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. The Journal of biological chemistry. 1996;271(49):31372-8.

106. Cong LN, Chen H, Li Y, Zhou L, McGibbon MA, Taylor SI, et al. Physiological role of Akt in insulin-stimulated translocation of GLUT4 in transfected rat adipose cells. Mol Endocrinol. 1997;11(13):1881-90.
107. Hajduch E, Alessi DR, Hemmings BA, Hundal HS. Constitutive activation of protein kinase B alpha by membrane targeting promotes glucose and system A amino acid transport, protein synthesis, and inactivation of glycogen synthase kinase 3 in L6 muscle cells. Diabetes. 1998;47(7):1006-13.
108. Kitamura T, Ogawa W, Sakaue H, Hino Y, Kuroda S, Takata M, et al. Requirement for activation of the serine-threonine kinase Akt (protein kinase B) in insulin stimulation of protein synthesis but not of glucose transport. Mol Cell Biol. 1998;18(7):3708-17.
109. Manning BD, Cantley LC. AKT/PKB signaling: navigating downstream. Cell. 2007;129(7):1261-74.
110. Stokoe D, Stephens LR, Copeland T, Gaffney PR, Reese CB, Painter GF, et al. Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B. Science. 1997;277(5325):567-70.
111. Alessi DR, James SR, Downes CP, Holmes AB, Gaffney PR, Reese CB, et al. Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. Curr Biol. 1997;7(4):261-9.
112. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science. 2005;307(5712):1098-101.
113. Dobrzynski E, Montanari D, Agata J, Zhu J, Chao J, Chao L. Adrenomedullin improves cardiac function and prevents renal damage in streptozotocin-induced diabetic rats. Am J Physiol Endocrinol Metab. 2002;283(6):E1291-8.
114. Laviola L, Belsanti G, Davalli AM, Napoli R, Perrini S, Weir GC, et al. Effects of Streptozocin Diabetes and Diabetes Treatment by Islet Transplantation on In Vivo Insulin Signaling in Rat Heart. Diabetes. 2001;50(12):2709-20.
115. Kobayashi T, Taguchi K, Nemoto S, Nogami T, Matsumoto T, Kamata K. Activation of the PDK-1/Akt/eNOS pathway involved in aortic endothelial function differs between hyperinsulinemic and insulin-deficient diabetic rats. American Journal of Physiology - Heart and Circulatory Physiology. 2009;297(5):H1767-H75.
116. Karlsson HK, Zierath JR, Kane S, Krook A, Lienhard GE, Wallberg-Henriksson H. Insulin-stimulated phosphorylation of the Akt substrate AS160 is impaired in skeletal muscle of type 2 diabetic subjects. Diabetes. 2005;54(6):1692-7.
117. Carvalho E, Eliasson B, Wesslau C, Smith U. Impaired phosphorylation and insulin-stimulated translocation to the plasma membrane of protein kinase B/Akt in adipocytes from Type II diabetic subjects. Diabetologia. 2000;43(9):1107-15.
118. Rondinone CM, Carvalho E, Wesslau C, Smith UP. Impaired glucose transport and protein kinase B activation by insulin, but not okadaic acid, in adipocytes from subjects with Type II diabetes mellitus. Diabetologia. 1999;42(7):819-25.
119. Carvalho E, Rondinone C, Smith U. Insulin resistance in fat cells from obese Zucker rats--evidence for an impaired activation and translocation of protein kinase B and glucose transporter 4. Mol Cell Biochem. 2000;206(1-2):7-16.
120. Tremblay F, Lavigne C, Jacques H, Marette A. Defective insulin-induced GLUT4 translocation in skeletal muscle of high fat-fed rats is associated with alterations in both

Akt/protein kinase B and atypical protein kinase C (zeta/lambda) activities. Diabetes. 2001;50(8):1901-10.
121. Cho H, Mu J, Kim JK, Thorvaldsen JL, Chu Q, Crenshaw EB, 3rd, et al. Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta). Science. 2001;292(5522):1728-31.
122. Bandyopadhyay G, Standaert ML, Zhao L, Yu B, Avignon A, Galloway L, et al. Activation of protein kinase C (alpha, beta, and zeta) by insulin in 3T3/L1 cells. Transfection studies suggest a role for PKC-zeta in glucose transport. The Journal of biological chemistry. 1997;272(4):2551-8.
123. Bandyopadhyay G, Standaert ML, Galloway L, Moscat J, Farese RV. Evidence for involvement of protein kinase C (PKC)-zeta and noninvolvement of diacylglycerol-sensitive PKCs in insulin-stimulated glucose transport in L6 myotubes. Endocrinology. 1997;138(11):4721-31.
124. Standaert ML, Galloway L, Karnam P, Bandyopadhyay G, Moscat J, Farese RV. Protein kinase C-zeta as a downstream effector of phosphatidylinositol 3-kinase during insulin stimulation in rat adipocytes. Potential role in glucose transport. The Journal of biological chemistry. 1997;272(48):30075-82.
125. Mendez R, Kollmorgen G, White MF, Rhoads RE. Requirement of protein kinase C zeta for stimulation of protein synthesis by insulin. Mol Cell Biol. 1997;17(9):5184-92.
126. Toker A, Newton AC. Cellular Signaling: Pivoting around PDK-1. Cell. 2000;103(2):185-8.
127. Cortright RN, Dohm GL. Mechanisms by which insulin and muscle contraction stimulate glucose transport. Can J Appl Physiol. 1997;22(6):519-30.
128. Brozinick JT, Jr., Birnbaum MJ. Insulin, but not contraction, activates Akt/PKB in isolated rat skeletal muscle. The Journal of biological chemistry. 1998;273(24):14679-82.
129. Hayashi T, Hirshman MF, Kurth EJ, Winder WW, Goodyear LJ. Evidence for 5' AMP-activated protein kinase mediation of the effect of muscle contraction on glucose transport. Diabetes. 1998;47(8):1369-73.
130. Hardie DG. Minireview: the AMP-activated protein kinase cascade: the key sensor of cellular energy status. Endocrinology. 2003;144(12):5179-83.
131. Goodyear LJ, Hirshman MF, Valyou PM, Horton ES. Glucose transporter number, function, and subcellular distribution in rat skeletal muscle after exercise training. Diabetes. 1992;41(9):1091-9.
132. Jensen TE, Rose AJ, Hellsten Y, Wojtaszewski JF, Richter EA. Caffeine-induced Ca(2+) release increases AMPK-dependent glucose uptake in rodent soleus muscle. Am J Physiol Endocrinol Metab. 2007;293(1):E286-92.
133. Jessen N, Goodyear LJ. Contraction signaling to glucose transport in skeletal muscle. J Appl Physiol (1985). 2005;99(1):330-7.
134. Chen ZP, McConell GK, Michell BJ, Snow RJ, Canny BJ, Kemp BE. AMPK signaling in contracting human skeletal muscle: acetyl-CoA carboxylase and NO synthase phosphorylation. Am J Physiol Endocrinol Metab. 2000;279(5):E1202-6.
135. Fujii N, Hayashi T, Hirshman MF, Smith JT, Habinowski SA, Kaijser L, et al. Exercise induces isoform-specific increase in 5'AMP-activated protein kinase activity in human skeletal muscle. Biochem Biophys Res Commun. 2000;273(3):1150-5.

136. Wojtaszewski JF, MacDonald C, Nielsen JN, Hellsten Y, Hardie DG, Kemp BE, et al. Regulation of 5'AMP-activated protein kinase activity and substrate utilization in exercising human skeletal muscle. Am J Physiol Endocrinol Metab. 2003;284(4):E813-22.
137. Kemp BE, Stapleton D, Campbell DJ, Chen ZP, Murthy S, Walter M, et al. AMP-activated protein kinase, super metabolic regulator. Biochem Soc Trans. 2003;31(Pt 1):162-8.
138. Richter EA, Hargreaves M. Exercise, GLUT4, and skeletal muscle glucose uptake. Physiological reviews. 2013;93(3):993-1017.
139. Bergeron R, Russell RR, 3rd, Young LH, Ren JM, Marcucci M, Lee A, et al. Effect of AMPK activation on muscle glucose metabolism in conscious rats. The American journal of physiology. 1999;276(5 Pt 1):E938-44.
140. Koistinen HA, Galuska D, Chibalin AV, Yang J, Zierath JR, Holman GD, et al. 5-amino-imidazole carboxamide riboside increases glucose transport and cell-surface GLUT4 content in skeletal muscle from subjects with type 2 diabetes. Diabetes. 2003;52(5):1066-72.
141. Miyamoto L, Toyoda T, Hayashi T, Yonemitsu S, Nakano M, Tanaka S, et al. Effect of acute activation of 5'-AMP-activated protein kinase on glycogen regulation in isolated rat skeletal muscle. J Appl Physiol (1985). 2007;102(3):1007-13.
142. Shah AK, Gupta A, Dey CS. AICAR induced AMPK activation potentiates neuronal insulin signaling and glucose uptake. Arch Biochem Biophys. 2011;509(2):142-6.
143. Fediuc S, Gaidhu MP, Ceddia RB. Inhibition of insulin-stimulated glycogen synthesis by 5-aminoimidasole-4-carboxamide-1-beta-d-ribofuranoside-induced adenosine 5'-monophosphate-activated protein kinase activation: interactions with Akt, glycogen synthase kinase 3-3alpha/beta, and glycogen synthase in isolated rat soleus muscle. Endocrinology. 2006;147(11):5170-7.
144. Jorgensen SB, Viollet B, Andreelli F, Frosig C, Birk JB, Schjerling P, et al. Knockout of the alpha2 but not alpha1 5'-AMP-activated protein kinase isoform abolishes 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranosidebut not contraction-induced glucose uptake in skeletal muscle. The Journal of biological chemistry. 2004;279(2):1070-9.
145. Mu J, Brozinick JT, Jr., Valladares O, Bucan M, Birnbaum MJ. A role for AMP-activated protein kinase in contraction- and hypoxia-regulated glucose transport in skeletal muscle. Mol Cell. 2001;7(5):1085-94.
146. Chin ER. Role of Ca2+/calmodulin-dependent kinases in skeletal muscle plasticity. J Appl Physiol (1985). 2005;99(2):414-23.
147. Kurth-Kraczek EJ, Hirshman MF, Goodyear LJ, Winder WW. 5' AMP-activated protein kinase activation causes GLUT4 translocation in skeletal muscle. Diabetes. 1999;48(8):1667-71.
148. Youn JH, Gulve EA, Holloszy JO. Calcium stimulates glucose transport in skeletal muscle by a pathway independent of contraction. The American journal of physiology. 1991;260(3 Pt 1):C555-61.
149. Wheatley CM, Rattigan S, Richards SM, Barrett EJ, Clark MG. Skeletal muscle contraction stimulates capillary recruitment and glucose uptake in insulin-resistant obese Zucker rats. Am J Physiol Endocrinol Metab. 2004;287(4):E804-9.

150. Ojuka EO. Role of calcium and AMP kinase in the regulation of mitochondrial biogenesis and GLUT4 levels in muscle. Proc Nutr Soc. 2004;63(2):275-8.
151. Ojuka EO, Jones TE, Nolte LA, Chen M, Wamhoff BR, Sturek M, et al. Regulation of GLUT4 biogenesis in muscle: evidence for involvement of AMPK and Ca(2+). Am J Physiol Endocrinol Metab. 2002;282(5):E1008-13.
152. Wijesekara N, Tung A, Thong F, Klip A. Muscle cell depolarization induces a gain in surface GLUT4 via reduced endocytosis independently of AMPK. Am J Physiol Endocrinol Metab. 2006;290(6):E1276-86.
153. Wright DC, Hucker KA, Holloszy JO, Han DH. Ca2+ and AMPK Both Mediate Stimulation of Glucose Transport by Muscle Contractions. Diabetes. 2004;53(2):330-5.
154. Terada S, Muraoka I, Tabata I. Changes in [Ca2+]i induced by several glucose transport-enhancing stimuli in rat epitrochlearis muscle. J Appl Physiol (1985). 2003;94(5):1813-20.
155. Egawa T, Hamada T, Ma X, Karaike K, Kameda N, Masuda S, et al. Caffeine activates preferentially alpha1-isoform of 5'AMP-activated protein kinase in rat skeletal muscle. Acta Physiol (Oxf). 2011;201(2):227-38.
156. Norris SM, Bombardier E, Smith IC, Vigna C, Tupling AR. ATP consumption by sarcoplasmic reticulum Ca2+ pumps accounts for 50% of resting metabolic rate in mouse fast and slow twitch skeletal muscle. Am J Physiol Cell Physiol. 2010;298(3):C521-9.
157. ALVIM RO, CHEUHEN MR, MACHADO SR, SOUSA AGP, SANTOS PCJL. General aspects of muscle glucose uptake. Anais da Academia Brasileira de Ciências. 2015;87:351-68.
158. Kim D, Chi S, Lee KH, Rhee S, Kwon YK, Chung CH, et al. Neuregulin Stimulates Myogenic Differentiation in an Autocrine Manner. Journal of Biological Chemistry. 1999;274(22):15395-400.
159. Suárez E, Bach D, Cadefau J, Palacín M, Zorzano A, Gumà A. A Novel Role of Neuregulin in Skeletal Muscle: NEUREGULIN STIMULATES GLUCOSE UPTAKE, GLUCOSE TRANSPORTER TRANSLOCATION, AND TRANSPORTER EXPRESSION IN MUSCLE CELLS. Journal of Biological Chemistry. 2001;276(21):18257-64.
160. Lebrasseur NK, Coté GM, Miller TA, Fielding RA, Sawyer DB. Regulation of neuregulin/ErbB signaling by contractile activity in skeletal muscle. American Journal of Physiology - Cell Physiology. 2003;284(5):C1149-C55.
161. Grundy SM, Benjamin IJ, Burke GL, Chait A, Eckel RH, Howard BV, et al. Diabetes and Cardiovascular Disease: A Statement for Healthcare Professionals From the American Heart Association. Circulation. 1999;100(10):1134-46.
162. Haffner SM, Lehto S, Ronnemaa T, Pyorala K, Laakso M. Mortality from coronary heart disease in subjects with type 2 diabetes and in nondiabetic subjects with and without prior myocardial infarction. N Engl J Med. 1998;339(4):229-34.
163. Roper NA, Bilous RW, Kelly WF, Unwin NC, Connolly VM. Excess mortality in a population with diabetes and the impact of material deprivation: longitudinal, population based study. BMJ. 2001;322(7299):1389-93.
164. Nicolau JC, Maia LN, Vitola JV, Mahaffey KW, Machado MN, Ramires JAF. Baseline glucose and left ventricular remodeling after acute myocardial infarction. Journal of Diabetes and Its Complications.21(5):294-9.

165. Ishihara M, Kagawa E, Inoue I, Kawagoe T, Shimatani Y, Kurisu S, et al. Impact of admission hyperglycemia and diabetes mellitus on short- and long-term mortality after acute myocardial infarction in the coronary intervention era. The American journal of cardiology. 2007;99(12):1674-9.
166. Boudina S, Bugger H, Sena S, O'Neill BT, Zaha VG, Ilkun O, et al. Contribution of impaired myocardial insulin signaling to mitochondrial dysfunction and oxidative stress in the heart. Circulation. 2009;119(9):1272-83.
167. Shaw JE, Sicree RA, Zimmet PZ. Global estimates of the prevalence of diabetes for 2010 and 2030. Diabetes Res Clin Pract. 2010;87(1):4-14.
168. Bugger H, Abel ED. Molecular mechanisms of diabetic cardiomyopathy. Diabetologia. 2014;57(4):660-71.
169. Jia G, DeMarco VG, Sowers JR. Insulin resistance and hyperinsulinaemia in diabetic cardiomyopathy. Nat Rev Endocrinol. 2016;12(3):144-53.
170. Voulgari C, Papadogiannis D, Tentolouris N. Diabetic cardiomyopathy: from the pathophysiology of the cardiac myocytes to current diagnosis and management strategies. Vasc Health Risk Manag. 2010;6:883-903.
171. Falcao-Pires I, Leite-Moreira AF. Diabetic cardiomyopathy: understanding the molecular and cellular basis to progress in diagnosis and treatment. Heart failure reviews. 2012;17(3):325-44.
172. Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Blaha MJ, et al. Heart disease and stroke statistics--2014 update: a report from the American Heart Association. Circulation. 2014;129(3):e28-e292.
173. Shimizu I, Minamino T, Toko H, Okada S, Ikeda H, Yasuda N, et al. Excessive cardiac insulin signaling exacerbates systolic dysfunction induced by pressure overload in rodents. J Clin Invest. 2010;120(5):1506-14.
174. Wright JJ, Kim J, Buchanan J, Boudina S, Sena S, Bakirtzi K, et al. Mechanisms for increased myocardial fatty acid utilization following short-term high-fat feeding. Cardiovascular research. 2009;82(2):351-60.
175. Cook SA, Varela-Carver A, Mongillo M, Kleinert C, Khan MT, Leccisotti L, et al. Abnormal myocardial insulin signalling in type 2 diabetes and left-ventricular dysfunction. European heart journal. 2010;31(1):100-11.
176. Mazumder PK, O'Neill BT, Roberts MW, Buchanan J, Yun UJ, Cooksey RC, et al. Impaired cardiac efficiency and increased fatty acid oxidation in insulin-resistant ob/ob mouse hearts. Diabetes. 2004;53(9):2366-74.
177. Buchanan J, Mazumder PK, Hu P, Chakrabarti G, Roberts MW, Yun UJ, et al. Reduced cardiac efficiency and altered substrate metabolism precedes the onset of hyperglycemia and contractile dysfunction in two mouse models of insulin resistance and obesity. Endocrinology. 2005;146(12):5341-9.
178. Antman EM. Time is muscle: translation into practice. Journal of the American College of Cardiology. 2008;52(15):1216-21.
179. Bonnefoy E, Steg PG, Boutitie F, Dubien PY, Lapostolle F, Roncalli J, et al. Comparison of primary angioplasty and pre-hospital fibrinolysis in acute myocardial infarction (CAPTIM) trial: a 5-year follow-up. European heart journal. 2009;30(13):1598-606.

180. Chen ZM, Jiang LX, Chen YP, Xie JX, Pan HC, Peto R, et al. Addition of clopidogrel to aspirin in 45,852 patients with acute myocardial infarction: randomised placebo-controlled trial. Lancet (London, England). 2005;366(9497):1607-21.
181. Eisenberg PR. Role of heparin in coronary thrombolysis. Chest. 1992;101(4 Suppl):131s-9s.
182. Burzotta F, De Vita M, Gu YL, Isshiki T, Lefevre T, Kaltoft A, et al. Clinical impact of thrombectomy in acute ST-elevation myocardial infarction: an individual patient-data pooled analysis of 11 trials. European heart journal. 2009;30(18):2193-203.
183. Ali A, Cox D, Dib N, Brodie B, Berman D, Gupta N, et al. Rheolytic thrombectomy with percutaneous coronary intervention for infarct size reduction in acute myocardial infarction: 30-day results from a multicenter randomized study. Journal of the American College of Cardiology. 2006;48(2):244-52.
184. Antoniucci D, Valenti R, Migliorini A, Parodi G, Memisha G, Santoro GM, et al. Comparison of rheolytic thrombectomy before direct infarct artery stenting versus direct stenting alone in patients undergoing percutaneous coronary intervention for acute myocardial infarction. The American journal of cardiology. 2004;93(8):1033-5.
185. Bolli R, Becker L, Gross G, Mentzer R, Jr., Balshaw D, Lathrop DA. Myocardial protection at a crossroads: the need for translation into clinical therapy. Circulation research. 2004;95(2):125-34.
186. Zweier JL. Measurement of superoxide-derived free radicals in the reperfused heart. Evidence for a free radical mechanism of reperfusion injury. The Journal of biological chemistry. 1988;263(3):1353-7.
187. Downey JM. Free radicals and their involvement during long-term myocardial ischemia and reperfusion. Annual review of physiology. 1990;52:487-504.
188. Piper HM, Garcia-Dorado D, Ovize M. A fresh look at reperfusion injury. Cardiovascular research. 1998;38(2):291-300.
189. Vinten-Johansen J. Involvement of neutrophils in the pathogenesis of lethal myocardial reperfusion injury. Cardiovascular research. 2004;61(3):481-97.
190. Kim JS, Jin Y, Lemasters JJ. Reactive oxygen species, but not Ca2+ overloading, trigger pH- and mitochondrial permeability transition-dependent death of adult rat myocytes after ischemia-reperfusion. American journal of physiology Heart and circulatory physiology. 2006;290(5):H2024-34.
191. Downey JM, Davis AM, Cohen MV. Signaling pathways in ischemic preconditioning. Heart failure reviews. 2007;12(3-4):181-8.
192. Reddy K, Khaliq A, Henning RJ. Recent advances in the diagnosis and treatment of acute myocardial infarction. World journal of cardiology. 2015;7(5):243-76.
193. Jaswal JS, Keung W, Wang W, Ussher JR, Lopaschuk GD. Targeting fatty acid and carbohydrate oxidation — A novel therapeutic intervention in the ischemic and failing heart. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research. 2011;1813(7):1333-50.
194. Tuunanen H, Engblom E, Naum A, Nagren K, Hesse B, Airaksinen KE, et al. Free fatty acid depletion acutely decreases cardiac work and efficiency in cardiomyopathic heart failure. Circulation. 2006;114(20):2130-7.
195. Jonassen AK, Aasum E, Riemersma RA, Mjos OD, Larsen TS. Glucose-insulin-potassium reduces infarct size when administered during reperfusion. Cardiovascular

drugs and therapy / sponsored by the International Society of Cardiovascular Pharmacotherapy. 2000;14(6):615-23.
196. Sodi-Pallares D, Testelli MR, Fishleder BL, Bisteni A, Medrano GA, Friedland C, et al. Effects of an intravenous infusion of a potassium-glucose-insulin solution on the electrocardiographic signs of myocardial infarction. A preliminary clinical report. The American journal of cardiology. 1962;9:166-81.
197. Zhang HX, Zang YM, Huo JH, Liang SJ, Zhang HF, Wang YM, et al. Physiologically tolerable insulin reduces myocardial injury and improves cardiac functional recovery in myocardial ischemic/reperfused dogs. J Cardiovasc Pharmacol. 2006;48(6):306-13.
198. Diaz R, Paolasso EA, Piegas LS, Tajer CD, Moreno MG, Corvalan R, et al. Metabolic modulation of acute myocardial infarction. The ECLA (Estudios Cardiologicos Latinoamerica) Collaborative Group. Circulation. 1998;98(21):2227-34.
199. van der Horst IC, Zijlstra F, van 't Hof AW, Doggen CJ, de Boer MJ, Suryapranata H, et al. Glucose-insulin-potassium infusion inpatients treated with primary angioplasty for acute myocardial infarction: the glucose-insulin-potassium study: a randomized trial. Journal of the American College of Cardiology. 2003;42(5):784-91.
200. Folmes CD, Clanachan AS, Lopaschuk GD. Fatty acids attenuate insulin regulation of 5'-AMP-activated protein kinase and insulin cardioprotection after ischemia. Circulation research. 2006;99(1):61-8.
201. Liu Q, Docherty JC, Rendell JC, Clanachan AS, Lopaschuk GD. High levels of fatty acids delay the recovery of intracellular pH and cardiac efficiency in post-ischemic hearts by inhibiting glucose oxidation. Journal of the American College of Cardiology. 2002;39(4):718-25.
202. Reaven GM, Chang H, Hoffman BB. Additive Hypoglycemic Effects of Drugs That Modify Free-Fatty Acid Metabolism by Different Mechanisms in Rats With Streptozocin-Induced Diabetes. Diabetes. 1988;37(1):28-32.
203. Lee L, Campbell R, Scheuermann-Freestone M, Taylor R, Gunaruwan P, Williams L, et al. Metabolic Modulation With Perhexiline in Chronic Heart Failure: A Randomized, Controlled Trial of Short-Term Use of a Novel Treatment. Circulation. 2005;112(21):3280-8.
204. Lopaschuk GD, Barr R, Thomas PD, Dyck JRB. Beneficial Effects of Trimetazidine in Ex Vivo Working Ischemic Hearts Are Due to a Stimulation of Glucose Oxidation Secondary to Inhibition of Long-Chain 3-Ketoacyl Coenzyme A Thiolase. Circulation research. 2003;93(3):e33-e7.
205. Saeedi R, Grist M, Wambolt RB, Bescond-Jacquet A, Lucien A, Allard MF. Trimetazidine Normalizes Postischemic Function of Hypertrophied Rat Hearts. Journal of Pharmacology and Experimental Therapeutics. 2005;314(1):446-54.
206. Dirksen WP, Lacombe VA, Chi M, Kalyanasundaram A, Viatchenko-Karpinski S, Terentyev D, et al. A mutation in calsequestrin, CASQ2D307H, impairs Sarcoplasmic Reticulum Ca2+ handling and causes complex ventricular arrhythmias in mice. Cardiovascular research. 2007;75(1):69-78.
207. Lacombe VA, Viatchenko-Karpinski S, Terentyev D, Sridhar A, Emani S, Bonagura JD, et al. Mechanisms of impaired calcium handling underlying subclinical diastolic dysfunction in diabetes. American journal of physiology Regulatory, integrative and comparative physiology. 2007;293(5):R1787-97.

208. Lacombe VA, Sogaro-Robinson C, Reed SM. Diagnostic utility of computed tomography imaging in equine intracranial conditions. Equine Vet J. 2010;42(5):393-9.
209. Waller AP, George M, Kalyanasundaram A, Kang C, Periasamy M, Hu K, et al. GLUT12 functions as a basal and insulin-independent glucose transporter in the heart. Biochim Biophys Acta. 2013;1832(1):121-7.
210. Waller AP, Kalyanasundaram A, Hayes S, Periasamy M, Lacombe VA. Sarcoplasmic reticulum Ca2+ ATPase pump is a major regulator of glucose transport in the healthy and diabetic heart. Biochim Biophys Acta. 2015;1852(5):873-81.
211. Miller EJ, Li J, Sinusas KM, Holman GD, Young LH. Infusion of a biotinylated bis-glucose photolabel: a new method to quantify cell surface GLUT4 in the intact mouse heart. Am J Physiol Endocrinol Metab. 2007;292(6):E1922-8.
212. Maria Z, Campolo AR, Lacombe VA. Diabetes Alters the Expression and Translocation of the Insulin-Sensitive Glucose Transporters 4 and 8 in the Atria. PLoS One. 2015;10(12):e0146033.
213. Li D, Ambrogio L, Shimamura T, Kubo S, Takahashi M, Chirieac LR, et al. BIBW2992, an irreversible EGFR/HER2 inhibitor highly effective in preclinical lung cancer models. Oncogene. 2008;27(34):4702-11.
214. Hervent A-S, De Keulenaer GW. Molecular Mechanisms of Cardiotoxicity Induced by ErbB Receptor Inhibitor Cancer Therapeutics. International Journal of Molecular Sciences. 2012;13(10):12268-86.
215. Pentassuglia L, Heim P, Lebboukh S, Morandi C, Xu L, Brink M. Neuregulin-1β promotes glucose uptake via PI3K/Akt in neonatal rat cardiomyocytes. American Journal of Physiology - Endocrinology and Metabolism. 2016;310(9):E782-E94.
216. Postic C, Leturque A, Printz RL, Maulard P, Loizeau M, Granner DK, et al. Development and regulation of glucose transporter and hexokinase expression in rat. The American journal of physiology. 1994;266(4 Pt 1):E548-59.
217. Waller AP, George M, Kalyanasundaram A, Kang C, Periasamy M, Hu K, et al. GLUT12 functions as a basal and insulin-independent glucose transporter in the heart. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease. 2013;1832(1):121-7.
218. Canfield K, Li J, Wilkins OM, Morrison MM, Ung M, Wells W, et al. Receptor tyrosine kinase ERBB4 mediates acquired resistance to ERBB2 inhibitors in breast cancer cells. Cell Cycle. 2015;14(4):648-55.
219. Holloszy JO. A forty-year memoir of research on the regulation of glucose transport into muscle. Am J Physiol Endocrinol Metab. 2003;284(3):E453-67.
220. Rose AJ, Richter EA. Skeletal muscle glucose uptake during exercise: how is it regulated? Physiology (Bethesda). 2005;20:260-70.
221. Wright DC, Geiger PC, Holloszy JO, Han DH. Contraction- and hypoxia-stimulated glucose transport is mediated by a Ca2+-dependent mechanism in slow-twitch rat soleus muscle. Am J Physiol Endocrinol Metab. 2005;288(6):E1062-6.
222. Holloszy JO, Narahara HT. Enhanced permeability to sugar associated with muscle contraction. Studies of the role of Ca++. J Gen Physiol. 1967;50(3):551-62.

APPENDICES

APPENDIX B

Table: Selected proposed functions of NRGs

Organ/cell type/ structure	Effect	Reference(s)
Nervous system		
Schwann cells	Survival, proliferation, migration, differentiation, myelination	[24,29,31–33]
Oligodendrocytes	Proliferation, survival, differentiation, myelination	[48,129–134,153]
Neuromuscular synapse	Nerve–muscle interaction controlling initial formation, acetylcholine receptor synthesis during development and in the adult, nerve terminal interactions with "terminal Schwann cells"	[17,20,26–28,146]
Muscle spindle	Muscle spindle development (Muscle spindles are muscle length/stretch sensors.)	[172]
Cranial sensory neurons	Initial population of cranial sensory ganglia (ganglia of cranial nerves) with neural-crest derived sensory neurons (However, the initial population of cranial sensory ganglia with placode-derived sensory neurons appears to be unaffected by *NRG1* mutations.)	[36–39,44]
Motor and sensory neurons	Survival (spinal and probably also cranial)	[42]
Peripheral and cranial nerves	Fasciculation (bundling) of axons and/or integrity of nerves	[36–39,42,44]
Sympathetic neurons/adrenal medulla	Migration of sympathetic neuron/adrenal chromaffin precursors to the anlage of sympathetic ganglia/adrenal medullas	[40]
Cerebellum	Production of cerebellar neuron precursors	[38]
Cortical neuron precursors/ cerebellar granule cells	Migration of CNS neuronal precursors along radial glia	[71,72]
Hypothalamus	Hypothalamic control of mammalian female sexual maturation	[135,173]
Parasympathetic	Enteric ganglia development	[136,174,175]
Hippocampus	Inhibition of long-term potentiation (LTP) induction (LTP is a model for studying the neurophysiological basis of learning and memory.)	[97]
Various neurons of CNS and PNS	Regulation of neuronal neurotransmitter receptors (NMDA, GABA, neuronal nicotinic acetylcholine receptors) and other neuronal ion channels	[64,74–76,176]
Heart	Development of ventricular wall trabeculae, AV-septum, and cardiac valves	[36–38,44,137]
Heart	Development of cardiac conduction system	[138]
Heart	Growth, repair, survival of adult cardiomyocytes; response to increased work load	[89–91,139]
Blood vessels	Angiogenesis	[144]
Breast	Breast development during pregnancy and lactation	[45]; Review in this issue by D. Stern
Lung	Development of pulmonary epithelium (autocrine effect?)	[142]
Muscle	Myogenesis (autocrine effect?)	[140]

Table 1: Selected proposed functions of NRGs. This list is not intended to be comprehensive, and further investigation will be required to confirm the physiological significance of many of the proposed roles. Some proposed functions have been inferred principally from the effects of exogenously supplied recombinant NRG1, ErbB blockade, or ErbB knock-out; in these cases the physiological signal may actually be NRG2, 3, or 4 or another ErbB ligand. Caveat emptor. Functions proposed solely on the basis of mRNA/protein expression data are not included. Reviews have been cited for proposed functions of NRG1s in Schwann cell and neuromuscular synapse development due to the large body of relevant literature (7).

VITA

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