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The Physiological Glucagon Receptor in Rat Heart

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THE PHYSIOLOGICAL GLUCAGON RECEPTOR IN

RAT HEART

BY

KEVIN AGOSTINUCCI

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE

REQUIREMENTS FOR THE DEGREE OF

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MASTER OF SCIENCE IN BIOMEDICAL AND PHARMACEUTICAL SCIENCE

OF

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2017

ABSTRACT

The actions of glucagon have been extensively studied for many years. In heart, it is well established that glucagon is capable of causing inotropy. This action is mediated by glucagon binding to a G-protein coupled receptor (GPCR) which activates adenylate cyclase, increasing the intracellular concentrations of cAMP. The studies that were able to show this effect used concentrations of glucagon that were considered to be much higher than what is normally found in blood. A recent report, by Harney and Rodgers (2008), described the actions and signaling mechanisms of physiological concentrations of glucagon ($<10^{-10}$ M) on perfused rat hearts. These investigators were also able to show that a peak physiological concentration of glucagon (10^{-10} M) increased glucose uptake in the same preparation. It was also found that this action was mediated by the activation of the PI3K/AKT signaling pathway. The missing piece of this mechanism to fully show glucagon's action is the receptor responsible for glucagon binding and initiation of the reported downstream signaling effects. We hypothesize that one of two situations would occur: 1) the glucagon receptor that mediates glucose uptake is a GPCR or 2) there is a novel glucagon receptor responsible for the effects observed at low concentrations. Hearts were removed from male Sprague Dawley rats (n=1-6/grp) and isolated heart perfusion methods, either Langendorff or working mode, were used to deliver a physiological solution to the heart. Specific concentrations of hormone, one which represents peak physiological glucagon levels and another that represents a concentration of glucagon too high to be normally found in blood, were added to the physiological solution. An anti-glucagon receptor antibody was obtained which was

used to block the known glucagon receptor. Hearts were treated with no hormone or with the following treatments: 1) 10^{-10} M glucagon; 2) 4×10^{-10} M insulin; 3) 10^{-8} M glucagon; 4) 10^{-8} M glucagon plus antibody and 5) 10^{-10} M glucagon plus antibody. Immediately after perfusion, hearts were processed and probed for AKT activation. It was found that glucagon concentrations at 10^{-10} M did not induce an inotropic response during Langendorff perfusion, while glucagon concentrations at 10^{-8} M was able to. For working mode, an inotropic response was not observed when hearts were perfused with 10^{-10} M glucagon and 10^{-8} M glucagon. There was no observable activation in AKT after physiological glucagon treated hearts in either Langendorff or working mode perfusion methods. In conclusion, the receptor that mediates glucagon's actions at physiological concentrations was not able to be characterized from these experiments. Experimental strategy should be revised to optimize *ex vivo* heart perfusion method and include additional *in vitro* experiments to conclude the identity of the glucagon receptor.

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CHAPTER 1

INTRODUCTION

Glucagon is a 29-amino acid peptide that is manufactured in alpha cells of the pancreas (Jiang and Zhang, 2003). This peptide is a vital player in regulating glycemia by acting in opposition to the effects of insulin. When blood glucose and insulin are low, the tonic inhibitory effect of insulin is removed and glucagon can be released from the pancreas (Taborsky, 2010). Glucagon is transported in the blood to its primary effector organ, the liver. As the ratio of glucagon to insulin increases, glucagon's action will be more prominent than insulin's action (Unger, 1971). The major effect of glucagon is to promote glucose output by increasing glycogenolysis and gluconeogenesis. In addition, glucagon can also act on the heart and adipose tissue by increasing contractility and lipolysis, respectively (Rodgers, 2012).

A G-protein coupled receptor (GPCR) is the main molecular target for glucagon (Jiang and Zhang, 2003; Rodgers, 2012). GPCR's are a type of receptor that contains a seven-transmembrane domain plus a heterotrimeric G-protein complex (Salazar et al., 2007). When glucagon binds to a GPCR, a signaling cascade is initiated. First, $G_{\alpha s}$ subunits are activated and dissociate from the G-protein complex, which causes an increase in cyclic adenosine mono-phosphate (cAMP) by increasing the activity of a membrane-bound enzyme called adenylate cyclase (AC). cAMP then activates protein kinase A (PKA) by binding to the regulatory subunit causing it to dissociate from the catalytic subunit. PKA can phosphorylate certain proteins which causes an action at the specific organ (Rababa'h et al., 2014; Towle, 2001).

The action of glucagon has been studied for many decades. The known actions of glucagon on liver, adipose tissue, and heart occur when glucagon binds to a GPCR to activate the AC/cAMP dependent pathway. The problem with these described actions is that they only occur at supra-physiological (glucagon concentration >0.1nM) levels, which do not reflect the physiologic concentrations (glucagon concentration <0.1nM) found in vivo (Rodgers, 2012). This was illustrated by constructing average dose-response curves from numerous studies. For adipose and heart, the EC_{50} value of glucagon to produce the organ's specific action, and the generation of cAMP, overlap each other and are well outside the concentration normally found in blood (Henry et al., 1975; Jean-Baptiste et al., 1982). This supports the claim that glucagon does not produce these actions *in vivo*. For liver, the EC_{50} value of glucagon to increase cAMP is well outside glucagon's physiological concentration. However, it can be shown that glucagon can cause the liver to generate similar or identical responses at much lower concentrations without activating adenylate cyclase (Clark and Jarrett, 1978; Ikezawa et al., 1998a). Since the curves for glucose output and concentration of tissue cAMP do not overlap, one can make the assertion that glucagon's action on the liver can occur through an alternative signaling mechanism *in vivo*. Collectively, these results raise the question about the "physiological relevance" of glucagon to produce these actions via the AC/cAMP dependent pathway. In order for a hormone's action to be considered "physiologically relevant", the action of the organ must occur at concentrations normally found in blood (Rodgers, 2012). It is evident from these graphs that glucagon can only generate cAMP-dependent cellular effects when concentrations are well above physiological

levels. Thus, additional research should be conducted to elucidate the physiological action of glucagon in liver, adipose tissue, and heart.

Studies have shown in liver that physiological and supra-physiological concentrations of glucagon activate two different signaling pathways but cause the same action (Livingston et al., 1985; Wakelam et al., 1986). The first piece of evidence comes from dose-response curves. Glucagon at physiological concentrations was able to increase glucose output without any increase in cAMP in perfused rat livers (Pilkis et al., 1974). Binding studies were also performed and it was found that supra-physiological glucagon concentrations were needed to activate the lower affinity glucagon receptor linked to AC. In the study performed by Bonnevie-Nielsen and Tager (1983), they determined K_d values for two different population of glucagon receptors. The K_d values of the low affinity glucagon receptor was 0.54 nM in hepatocytes and 92 nM in liver vesicles. Both these K_d values are above physiological concentrations. The high affinity receptor had K_d values of 0.19 nM and 2.6 nM in hepatocytes and liver vesicles respectively (Bonnevie-Nielsen and Tager, 1983). At physiological concentrations, glucagon binds with higher affinity to a receptor not linked to AC. It was found that the high affinity receptor participated in a calcium-dependent signaling pathway that also increased glucose output (Ikezawa et al., 1998b). A molecular cloning study was performed to identify the receptor that binds to glucagon. The experimenter expressed the glucagon receptor in kidney cell lines and measured K_d values. According the Schatchard plot they developed, they found two K_d values for the glucagon receptors that they expressed. However, the authors were not able to conclude that the receptors were anything other than the GPCR (Jelinek et

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al., 1993). Thus, one prominent hypothesis is that two glucagon receptors are GPCRs, with two different affinities to glucagon, that can either undergo the AC/cAMP or PLC/IP3 signaling pathways.

Adipose tissue also has been shown to respond to glucagon at supra-physiological concentrations via the cAMP/AC dependent pathway. However, the available evidence does not support the existence of a physiological receptor for glucagon in adipose tissue. The average dose response curves produces a monophasic curve with an EC_{50} that lies well outside physiological glucagon concentrations (Jean-Baptiste et al., 1982). Furthermore, the average EC_{50} and K_d was calculated from many studies and it was shown that these values were similar (Rodgers, 2012). The similar values show that an additional receptor is not present in adipose tissue. Thus, it is believed that glucagon does not have any action *in vivo* on adipose tissue.

It has been demonstrated multiple times, and in many experimental models, that glucagon causes the heart to generate more force at a faster rate. Because of this, the claim has been made that glucagon's acts to activate inotropic and chronotropic responses on the heart, both which are accompanied by increase in cAMP. Dr. Robert Rodgers and his colleagues believe that these effects cannot be the *in vivo* action of glucagon because concentrations above physiological levels are needed for this to occur. Dr. Rodgers constructed a concentration-effect curve that represents the findings of multiple sources to show the action of glucagon on the heart (Rodgers, 2012). From his curve, he shows that EC_{50} values for inotropy and cAMP concentration both lie well outside of the glucagon concentration found in blood. Thus, his laboratory was interested in seeking out glucagon's *in vivo* cardiac action.

They found that at physiological glucagon concentrations, glycolysis increased and palmitate oxidation decreased in isolated perfused working hearts (Harney and Rodgers, 2008). These actions are the same as insulin's actions and our experiments showed that there was no statistical difference between the actions produced by either glucagon or insulin (Harney and Rodgers, 2008; Rodgers et al., 2001a). Furthermore, a physiological glucagon concentration activated a PI3K/AKT pathway but had no effect on the cAMP/AC pathway. This is another component that is shared between glucagon and insulin (Harney and Rodgers, 2008; Saltiel and Kahn, 2001). they were able to show that glucagon mimics the action of insulin, rather than opposing it, in heart at a physiological concentration.

The glucagon receptor that mediates the insulin-like response has yet to be identified. The purpose of this investigation was to begin to explore the identity of this receptor by performing heart perfusions followed by western blot analysis. The isolated heart perfusion method was used because the physiological parameters of the heart could be measured independent of any neuro-hormonal influence, the heart is in an environment where all substrates and substrate levels are known, and without an confounding effects from other metabolically active organs (Skrzypiec-Spring et al., 2007). Hearts were treated with specific concentrations of hormone (physiological or supra-physiological) and contractility was measured to see if the hormone could activate the known GPCR. To block the known GPCR, an anti-glucagon GPCR antibody was obtained and put into a solution that was perfused through the heart. Hearts were then subjected to western blot analysis after perfusion experiments to show AKT activation. We hypothesized: 1) A GPCR linked to PI3K/AKT mediates

the insulin-like action of glucagon or 2) A receptor of a different class linked to

PI3K/akt mediates the insulin-like action of glucagon.

CHAPTER 2

METHODOLOGY

Methods:

Animal usage:

All procedures for the use of animals were approved by the University of

Rhode Island Animal Care and Use Committee. Male rats were purchased from

vendors (Taconic or Charles River). Animals were co-housed in standard cages with a

12-hour light-dark cycle. They provided water and standard chow ad libitum., and

body weights were collected routinely.

Preparation of solutions:

Hearts were perfused with Krebs-Henseleit (K-H) solution with the following

composition (mM): NaCl, 120; $CaCl_2 \cdot 2H_2O$, 1.8; $NaH_2PO_4 \cdot H_2O$ 1.2; $MgSO_4 \cdot 7H_2O$,

0.65 and KCl, 5.6, glucose, 10; and $NaHCO_3$, 25. Working and Langendorff heart

preparations were perfused with K-H solution containing 3% and 0.1% BSA,

respectively. In both preparations, the solution was gassed with 95-5% O_2-CO_2

Hearts were treated with one of the following: 1) no hormone; 2) insulin

($4 \times 10^{-10}M$) (Sigma-Aldrich); 3) and glucagon (10^{-10} or $10^{-8}M$) (Sigma-Aldrich). The

lower and higher glucagon concentrations represented physiological and supra-

physiological concentrations (Rodgers, 2012).

Isolated Heart Perfusion:

The isolated heart perfusion technique was used to measure the hearts contractility when administering different treatments. The two types of perfusion techniques used were the Langendorff (Skrzypiec-Spring et al., 2007) and the working mode preparation (Harney and Rodgers, 2008; Rodgers et al., 2001b), both which have been described previously. Both preparation allows for the delivery of K-H solution to the coronary arteries of the heart so it can contract without being inside the animal. The pressure produced during perfusion was measured using a Millar catheter (SPR-524) inside a K-H solution filled column which was connected to the left ventricle. Pressure data was collected using Data Translation software (Colorado Springs, CO) and the data was analyzed using ADinstruments Lab Chart software. At the end of the perfusion, hearts were snapped frozen in liquid nitrogen and store in -80ºC freezer.

Western Blot and AKT probing:

The procedure used to isolate proteins from the heart tissue was established by Harney and Rodgers (2008). Briefly, left ventricles of the heart were isolated then added to a lysis buffer containing the following solutes (mM) NaCl – 150; Tris-HCl – 50; $NaVO_4$ – 1; EDTA – 5; PMSF – 1; and protease tablet. Tissues were homogenized and centrifuged at the following speeds (times g): 1,000; 100,000; and 15,000. The final solution contained only soluble proteins. Proteins in solution were quantified using BCA assay kit.

Protein in solution were separated via SDS-PAGE. Equal amount of protein was added to each lane of a 10% gel and were electrophoresed at 100V. At the

completion of the electrophoresis, electroblotting was performed to transfer proteins from the polyacrylamide gel to a PVDF membrane. Electroblotting proceeded at 30V for 30 minutes.

Anti-AKT and anti-phospho-AKT (S473) were purchased from Cell Signaling Technologies and were used according to manufacturer's protocol. Proteins were visualized using Biorad ChemiDoc imaging. ImageJ software was used to quantify the signal that was developed by the imaging software.

CHAPTER 3

FINDINGS

It is well established that high concentrations of glucagon are capable of inducing an inotropic response on the heart (Gonzalez-Muñoz et al., 2008; MacLeod et al., 1981; Rodgers et al., 1981). This response is mediated by the known glucagon GPCR linked to AC/cAMP. Physiological glucagon concentrations and insulin should not be able to induce this response (Fu et al., 2014; Harney and Rodgers, 2008). Therefore, we tested this theory by administering either 0.4 nM of insulin, 0.1 nM (physiological concentration) or 10nM (supra-physiological) of glucagon to isolated perfused hearts and measure the pressure produced by the left ventricle.

Figure 1 is a representative pressure tracing of one heart perfused via the Langendorff preparation. From this figure, the left ventricular developed pressure (LVDP) can be determined by taking the difference between the peak systolic and diastolic pressures. The developed pressures were calculated for each heart and the average pressures were determined for each hormone group. The average LVDP values are represented in figure 2. Hearts that were perfused with no hormone had an average pulse pressure of 65.6 +/- 7.06 mmHg (mean +/- SEM). When 0.4nM insulin or 0.1nM of glucagon the pressure produced were 68.02 +/- 13.11 mmHg and 80.67 +/- 19.78 mmHg, respectively. This is in line with what we were expecting because peak physiological concentration of either insulin or glucagon is not capable of inducing inotropy (Fu et al., 2014; Harney and Rodgers, 2008). Hearts produced the

largest inotropic response after administration of glucagon at supra-physiological

concentrations, as expected. The pressure that was developed for supra-physiological

glucagon administration was 133.11 +/- 6.67 mmHg.

Hearts that were perfused via working mode produced higher pressures, with

all treatments, compare to hearts perfused in the Langendorff mode. Figure 3 is a

representative pressure tracing of a heart perfused in the working mode. The same

calculations were performed, as for figure 1, and average pressures were determined

for each group and are represented in figure 4. Hearts produced pressures of 110.80

+/- 10.7 mmHg, 100.85 mmHg, 90.95 +/- 24.6 mmHg and 92.96 +/- 4.18 mmHg when

administered with no hormone, 0.4nM insulin, 0.1nM glucagon, and 10nM glucagon

respectively. Unlike what was observed in hearts administered with 10nM glucagon

via Langendorff perfusion, the average pressure of 10nM glucagon was less than that

of control in the working mode. Figures 5 and 6 are representative tracings of a heart

perfused with a supra-physiological glucagon concentration. It can be seen that an

increase in contractility did occur. However, the response eventually decreased as the

heart progressed through the perfusion which explains why the average pressure was

less than control (figure 7).

It has been determined in this laboratory that, not only does a physiological

concentration of glucagon mimic the same physiological action of insulin in the heart,

but it also shares the same signaling pathway (Harney and Rodgers, 2008). It is well

established that insulin binds to the insulin receptor which activates PI3K followed by

AKT to increase glycolysis (Lee and Pilch, 1994). Evidence from this laboratory

shows that glucagon at physiological concentrations also activates the PI3K/AKT

signaling pathway. Thus, we used this signaling pathway to assess the claim that changes in cardiac glycolysis could occur independently of changes in cardiac contractility with physiological concentrations of glucagon. The PI3K/AKT signaling pathway was quantified by measuring total amounts of AKT and the amount of phosphorylated AKT. A phosphorylated AKT:total AKT ratio was calculated to assess the activation of this signaling pathway when a hormone is given to the heart. For hearts perfused in Langendorff mode, an average P-AKT/T-AKT of 0.468 +/- 0.076 (mean +/- SEM) was observed for untreated hearts. The highest P-AKT/T-AKT ratio that was observed was when insulin was administered to the hearts (0.561 +/- 0.128). Supra-physiological glucagon concentrations produced the second highest ratio of 0.603 +/- 0.236. Administration of physiological glucagon produced the smallest a P-AKT/T-AKT ratio of 0.221 +/- 0.032.

AKT quantification was also assed in hearts perfused in the working mode. A value of 0.468 +/- 0.076 was observed for untreated hearts. Administration of insulin to hearts gave the highest P-AKT/T-AKT ratio compared to the other groups (1.168). Supra-physiological glucagon produced the second highest P-AKT/T-AKT ratio of 0.719 +/- 0.068. Finally, administering physiological glucagon produced an P-AKT/T-AKT ratio of 0.472. +/- 0.099.

CHAPTER 4

CONCLUSION

The quintessential action of glucagon is to increase contractility of the heart. However, studies proved that this only happens at supra-physiological concentrations. In the laboratory of Dr. Robert Rodgers, they were able to find a different action of glucagon at physiological concentrations. Glucagon increased glucose uptake, glucose oxidation, and decreased palmitate oxidation to a degree similar to insulin (Harney and Rodgers, 2008; Rodgers et al., 2001a). Furthermore, physiological concentrations of glucagon activated the PI3K/AKT pathway without any activation of AC/cAMP (Harney and Rodgers, 2008). Therefore, we believe that increasing cardiac contractility is not the main *in vivo* action of glucagon.

The first objective of this study was to determine whether a supra-physiological concentration of glucagon increases inotropy compared to physiological concentrations in Langendorff perfused hearts. A supra-physiological concentration of glucagon was able to produce an average LV pulse pressure that was greater compared to controls in Langendorff perfused hearts. Hearts perfused with a physiological concentration of glucagon or insulin produced LV pressures that were modestly elevated compared to control hearts. This result was consistent with previous reports (Harney and Rodgers, 2008; Tuncok et al., 1997). Hearts perfused with any of the hormone treated groups, in working mode, did not produce an average LV pulse pressure that was larger compared to controls. An inotropic response was expected to

occur in hearts treated with supra-physiological glucagon concentrations because it has been previously reported that high glucagon concentrations increase developed tension or pressure in rat hearts or cardiac muscle preparations (MacLeod et al., 1981; Rodgers et al., 1981). Although the average response was not different, it appears that a transient inotropic response was produced. This observation can be explained by glucagon's tachyphylaxis response. Tachyphylaxis response, in heart, occurs when there is a rapid increase in left ventricular pressure which is then decreases back to baseline shortly after (MacLeod et al., 1981). This response occurs in G-protein signaling due to either receptor desensitization or by activity of phosphodiesterases that degrades cAMP. Fita-Juan and colleagues (2004), studied the tachyphylaxis response in isolated right ventricle strips in rats. When glucagon was added to these hearts, a transient (< 2min) inotropic response was induced (Juan-Fita et al., 2004). In this study, it was observed that the inotropic response was at its highest point one minute after glucagon administration, then rapidly decreased.

The second objective was to determine whether a physiological concentration of glucagon was able to activate the PI3K/AKT signaling pathway. We evaluated activation of PI3K/AKT pathway by quantifying phosphorylated AKT and total AKT via western blots. For Langendorff and working mode perfused hearts, there appears to be no increase in AKT activation between physiological glucagon treated hearts compared to untreated hearts. This was contrary to Harney and Rodgers's report which showed PI3K/AKT activation when hearts were perfused with low concentrations of glucagon. A physiological concentration of insulin produced more activation in AKT compared to untreated hearts in either Langendorff or working mode perfusion

methods. Due to the small number of subjects in the insulin treated group, the values obtained cannot definitively show that insulin was capable of activating AKT at a higher magnitude compared to untreated hearts.

Differences in methodology could explain the unexpected results that were observed in this experiment. One factor that may explain the discordant results is the difference in the age range of rats enrolled in each study. In the current experiment, the age was not recorded but, the age can be approximated using body weight (2017). Harney and Rodgers (2010), used rats that weighed between 200-400 grams which approximates to two to three months. In this experiment, it was noted that many of the animals that were used weighed over 400 grams with is approximately three months or older.

Another point that arises, due to the unexpected results, is whether or not the glucagon used was sufficient to activate AKT. The glucagon that was used may have lost intrinsic activity to activate the glucagon receptor, or, the dilution procedure was not accurate and low than expected concentrations of glucagon was administered to the perfused heart. A crucial experiment that can give evidence to the integrity of the glucagon solution was to perfuse hearts with $5\text{-}^3\text{H}$-glucose and measure the change in rate of glycolysis. If glucagon administration was able to activate glycolysis, but not PI3K/AKT, then it can be argued that glucagon's physiological actions are not mediated by PI3K/AKT.

Aging can affect the biochemical and physiological parameters of this experiment. First, the strength of the PI3K/AKT signaling pathway could have decreased due to using older rats. Griecsova and colleagues (2015), showed that three-

month-old rats had a lower AKT activation than 1.5 months old rats (Griecsová et al., 2015). In this study, several rats weighed over 400 grams which approximate to a 3-month-old rat. This may explain why AKT activation was not as robust as expected after insulin treatment. This could also be a factor that cause physiological glucagon treated hearts to have a lower than expected AKT activation.

A decline in cardiac performance can also be attributed to changes in the GPCR receptor signaling. These changes include reductions in the: 1) number of receptors, 2) sensitivity of the receptor and 3) downstream actions after receptor activation. In a study performed by Xiao et al (1998), they found that age related declines in LV contractility were due to decreases in the number of β-adrenergic receptors. It was also determined from this study that older rats have less AC activity compared to young rats when a ligand binds to the receptors (Xiao et al., 1998). Furthermore, heart contractility decreased due to changes in calcium handling after β-adrenergic stimulation. It was found that calcium levels were decreased in older animals compared to younger ones which will reduce the strength of contraction (Ferrell and Howlittt 2007). It was shown that decreases in calcium levels during diastole can decrease the contraction force at the next heartbeat. Since the glucagon receptor is also a GPCR associated with AC, it can be expected that similar changes can occur due to aging which may have affected the hearts ability to contract.

Fibrosis of heart tissue is another possible factor that may explain the lower than expected pressure produced by the hearts. Age-related fibrosis increases collagen deposits into the heart's extracellular matrix and causes a decrease in performance. In ideal conditions, the extra cellular matrix plays an important role in maintaining the

organs structure that allows it to work normally (Heeneman et al., 2003). For certain conditions (i.e. aging) there may be an overproduction of extracellular matrix proteins which decreases the hearts ability to perform. In an experiment conducted by Sangaralingham et al., they showed that collagen production in the left ventricle was higher in 20-month-old Fisher rats than in two- months old rats (Sangaralingham et al., 2016). This increase in fibrosis was accompanied by decreases in ejection fracture and decreases in diastolic wall tension.

Evidence is also present that shows young Sprague-Dawely rats are prone to develop spontaneous cardiac lesions. In a study conducted by Chanut et al. (2013), they looked at the presence of lesions in different regions of the heart and they found the highest occurrence of lesions to be within the myocardium of the left ventricle. It was also reported that the stress from handling the rats had no effect on changing the occurrence of the lesions (Chanut et al., 2013). This can have an effect on the pressure generated by the heart. The presence of cardiac lesion can cause an inflammation response that may release factors which can decrease LV function. For example, it has been shown that TNF (a pro-inflammatory agent) infusion resulted in reduced inotropic response which was reversed when TNF was removed (Mann, 2002). Depending on the size and number of lesions in the heart, this may have contributed to the lower than expected pressure development of the left ventricle.

If this experiment was to be redone, a different approach for identifying the PI3K/AKT signaling pathway would be necessary. One method that can be utilized are cell culture techniques followed by western blot analysis to probe the same proteins. Isolated cardiomyocytes can be obtained from whole heart by perfusing it with

digestive enzymes via Langendorff perfusion (Louch et al., 2011). The isolated cell

can then be plated onto a culture dish and be given culture media. In the culture media,

different concentrations of hormone can be given to the cardiomyocytes and protein

quantification can be determined via western blotting.

Western blot analysis have been performed on hepatocytes and probed for

AKT (Aw et al., 2014; Zhao et al., 2000). In these experiments, glucagon was not able

to activate the PI3K/AKT signaling pathway. This observation can be explained by the

fact that the receptors present in the liver are not linked to PI3K/AKT. However, this

author was not able to find any reports that performed cell culture experiments on

isolated ventricular cardiomyocytes treated with glucagon. Performing this method

may provide evidence on the physiological signaling cascade of glucagon.

Instead of using ex vivo hemodynamics to measure the contractility of the

heart, in vitro video edge detection of isolated ventricular cardiomyocytes could be

used to measure a single myocyte's ability to shorten (Bazan et al., 2010). There is

evidence that the cardiomyocyte has glucagon receptors on it so the changes in

contractility can be measure when cell are perfused with glucagon containing solution

(Ali et al., 2015; Méry et al., 1990; Tang and Insel, 2004). It has the advantage over *ex*

vivo measurements of contractility because the experimenter is directly measuring the

ability of a single myocyte to shorten when given a certain stimulus. The contractility

of many myocytes can be measured which may provide evidence for how the cells

will function in the intact organ. A limitation of this method is that the results may not

represent what is occurring at the organ level. Whole heart perfusion methods

incorporate preload and after load, both which influence the tension that can be

developed in the LV (Schechter et al., 2014). Furthermore, measuring contractility in isolated cardiomyocytes does not represent how the heart may act *in vivo* because the cells are not connected to each other. Gap junctions are channels which connect the intracellular content between two cardiomyocytes. These channels allow ionic current to pass directly from one cardiomyocyte to another which allow the whole heart to contract in a synchronized manner (Rook et al., 1992). Interruption of this current through each cardiomyocyte can prevent the ventricles from depolarizing and thus fail to contract. Contractility can be measured using cell based experiments. Three-dimensional cell culture techniques allow for an experimenter to grow cells that does not limit the cells to grow on a flat surface. Cells are able to form connections with each other that may closer replicate what happens *in vivo*. In a report by Shimizu et al., they were able to measure cell shortening in cultured cardiac tissue grafts by using a digital video camera (Shimizu et al., 2002).

Limitations:

The surgical procedure used in this study was similar to the procedure performed by Harney and Rodgers (2010). The experimenter tried to replicate the same conditions that Harney used when performing the heart isolation, but the experimenter was not there to observe Harney perform the procedure. It was thought that differences in experimenter's ability to isolate the heart could have contributed to the different results. Differences such as speed of heart isolation and location of cannula placed on the aorta could have affected the outcome of the procedure. Using anesthesia could have facilitated in the heart isolation process by reducing the stress

put on the animal. Minimizing the stress on the heart may reduce the damage put on the heart while the experimenter performs the heart isolation procedure.

Another limitation of this experiment is that absolute pressure values were recorded, rather than changes in pressure relative to baseline. Changes in LV pulse pressure from baseline is a better marker for contractility. The reason is that there are many factors that can influence the amount of force the heart can produce; such as changes in pre-load and afterload, health of the heart, or quality of solutions used to treat heart. Contractility is the heart's ability to increase the amount of force independent of pre-load and/or after load. Recording the change in LV pulse press will rule out some of the effects of extrinsic factors that may change the pressure the heart produces. With absolute pressures, we can see that different pressures are generated but we cannot fully conclude that the pressures generate was due to the heart's intrinsic ability to develop more force.

Conclusion

In summary, it cannot be determined if a high concentration of glucagon was able to induce an inotropic response. LV pressure was the highest in supra-physiological treated hearts perfused in Langendorff mode. However, LV pressure was similar between untreated and supra-physiological glucagon administered hearts. The data also shows that there were no changes in AKT activation among the four groups. A limitation in this study that can explain why difference in measurements were not detected were due to the number of rats in each group. Since some of the treatment groups had less than four animals, statistics were not able to be performed to

determine if there was a difference in values for LV pressure development and AKT
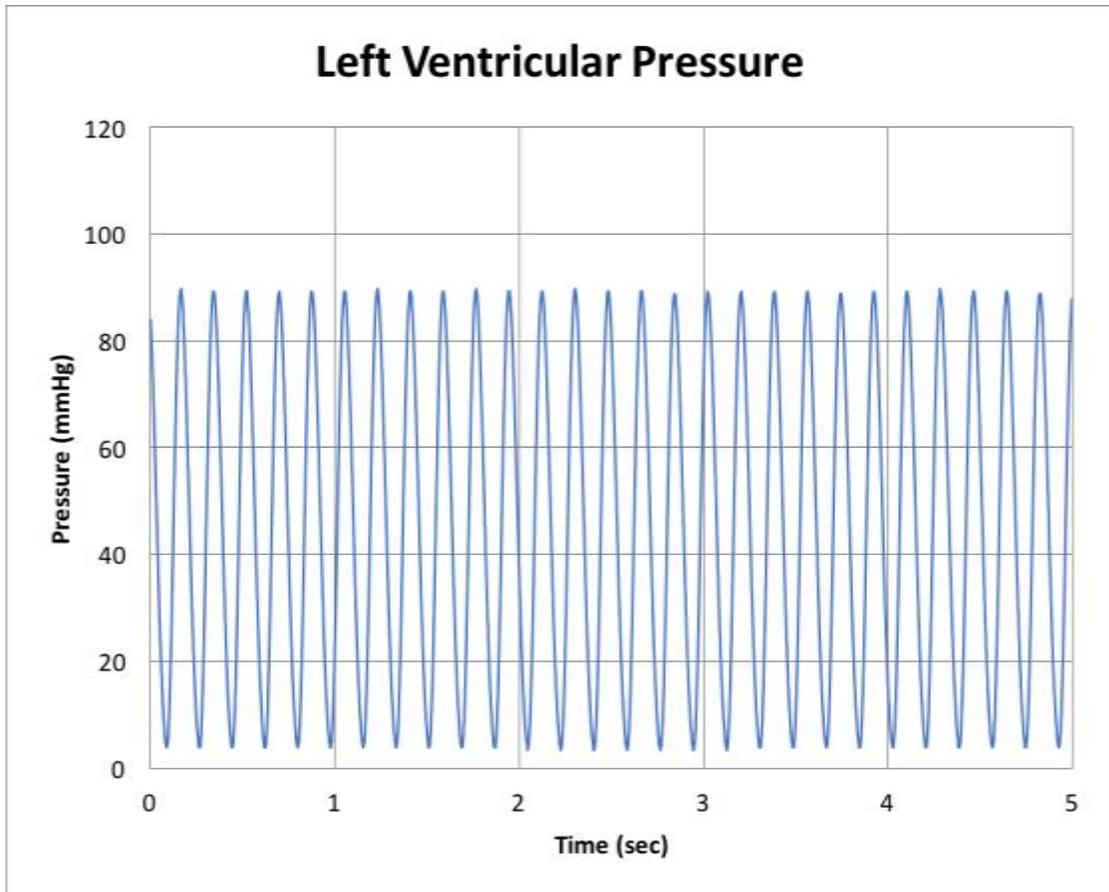
activation.

Figures

Figure 1: Representative pressure measurement for a heart perfused in the Langendorff preparation. This graph was shows the pressure produced by the left ventricle after 1 minute of perfusion. The difference between the maximum and minimum pressure were averaged to determine pulse pressure. This heart was treated with no hormone.
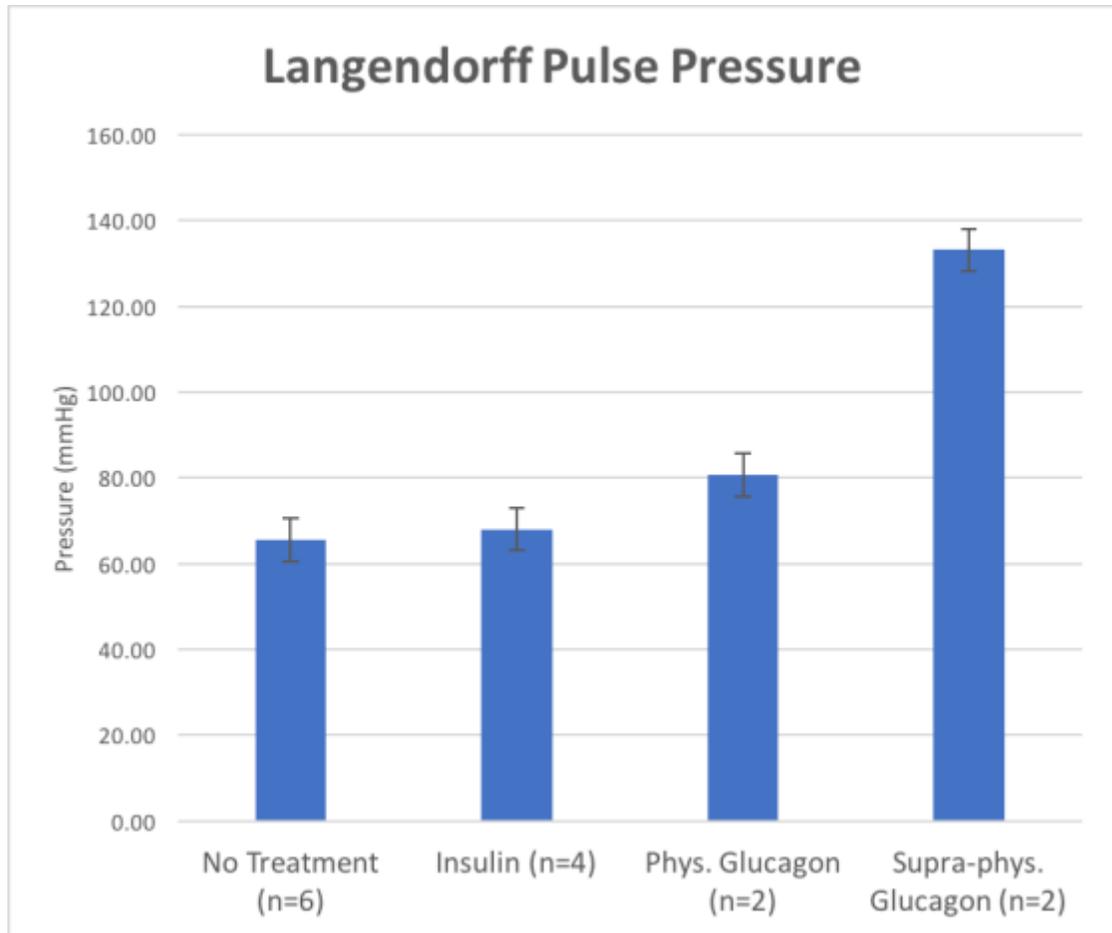
Figure 2: Average pulse pressure produced by each treatment. Pulse pressure was determine by taking the difference of the average maximal pressure minus the average minimal pressure. Bars represent average values and error bars represent the SEM.
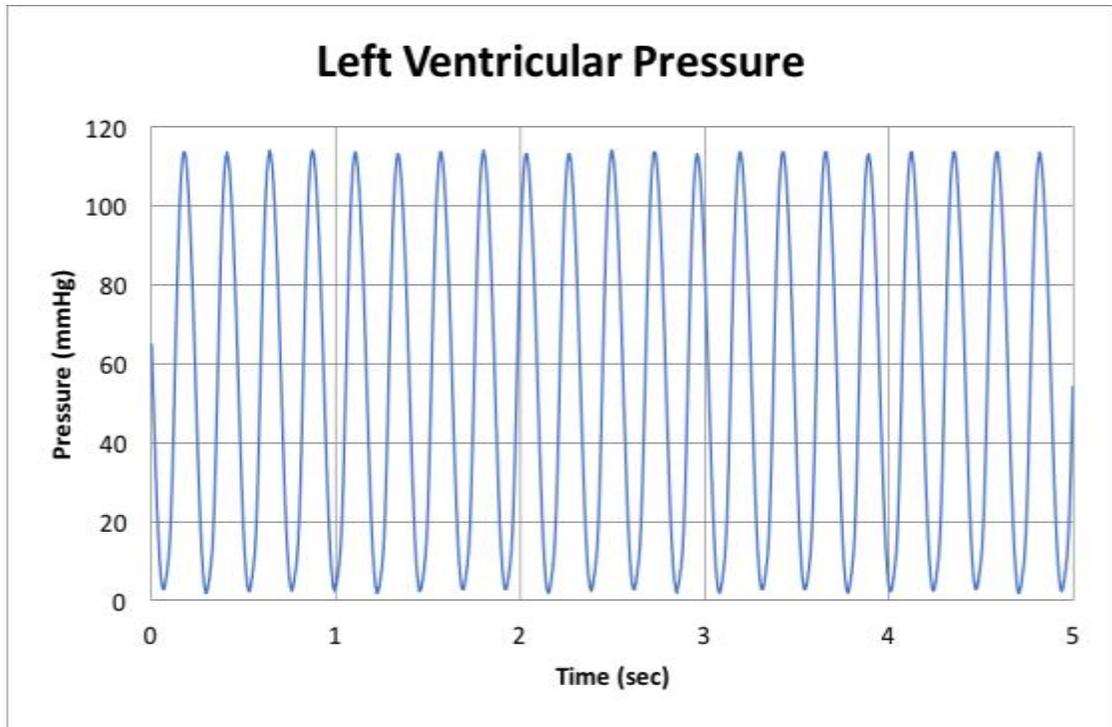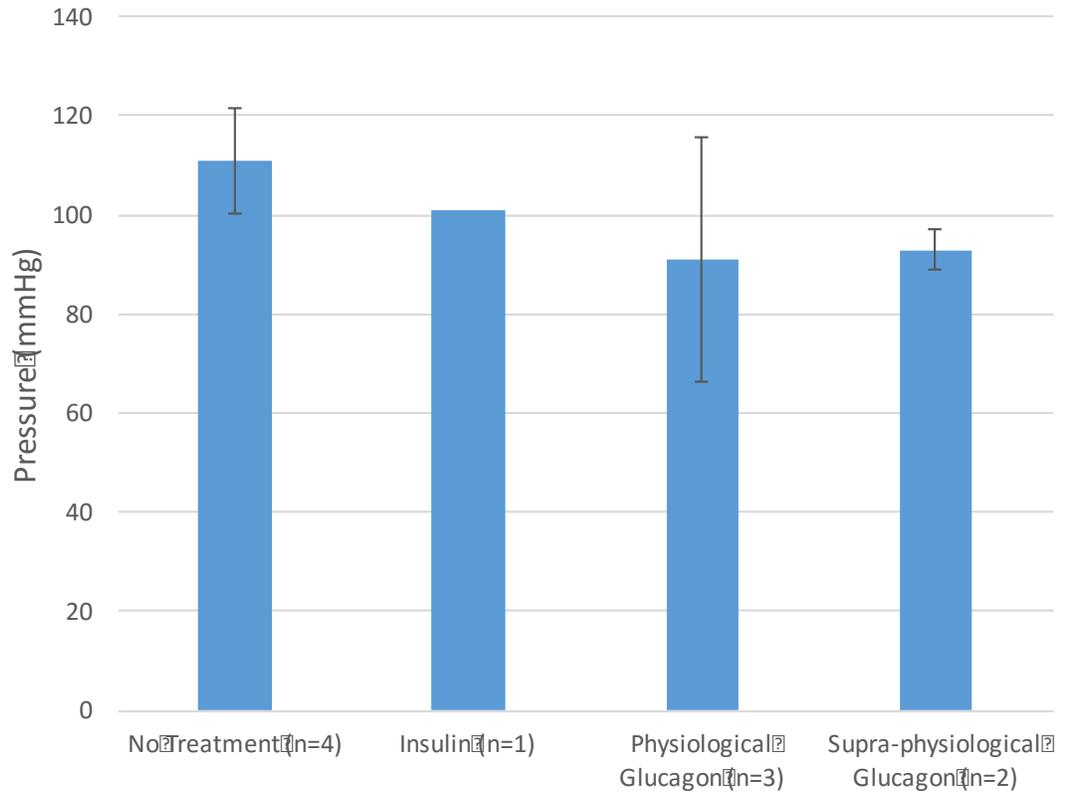
Figure 3: Representative pressure measurement for a heart perfused in the working mode. This graph was shows the pressure produced by the left ventricle after 10 minute of perfusion. The difference between the maximum and minimum pressure were averaged to determine pulse pressure. This heart was treated with no hormone.

Figure 4: Average pulse pressure produced by each treatment. Pulse pressure was determined by taking the difference of the average maximal pressure minus the average minimal pressure. Bars represent average values and error bars represent SEM
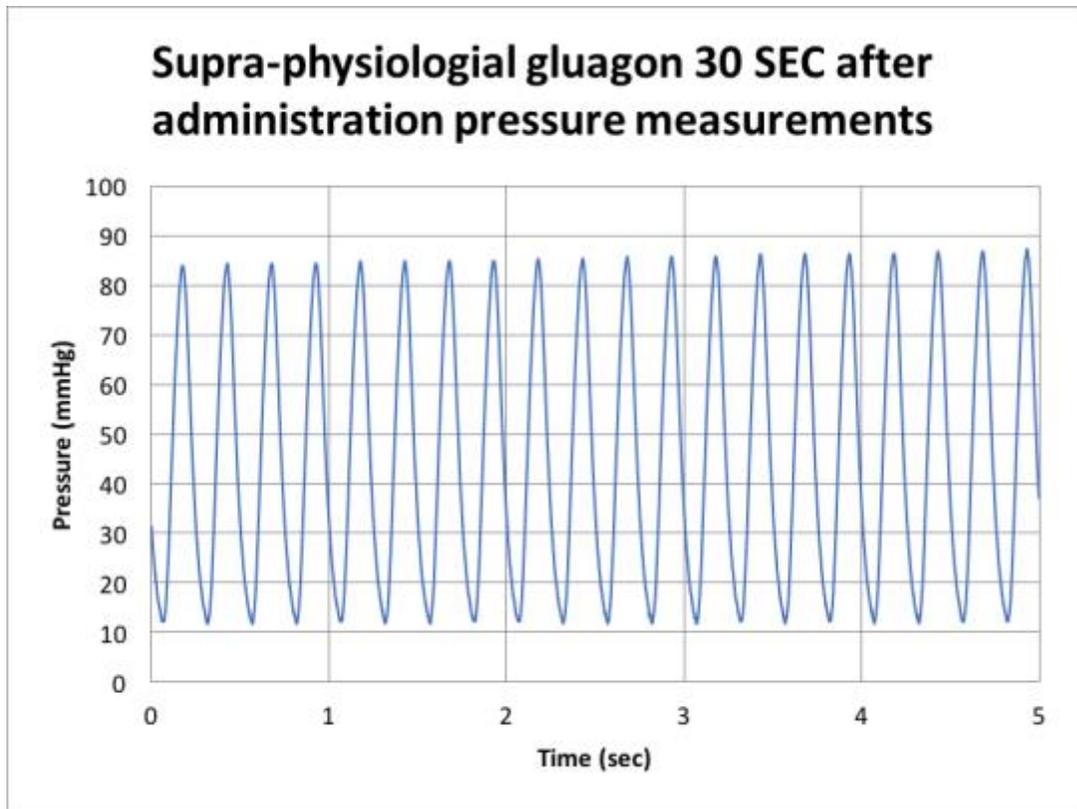
Supra-physiologial gluagon 30 SEC after administration pressure measurements
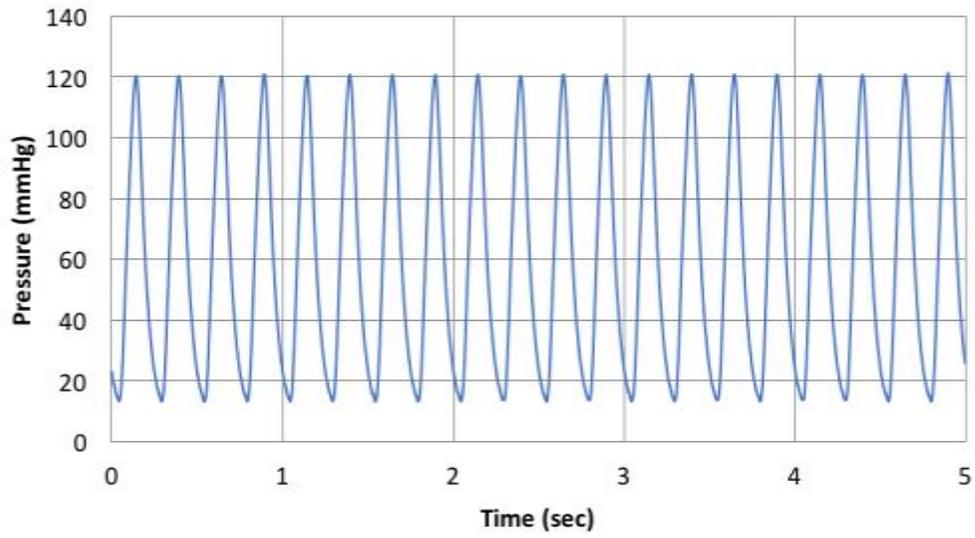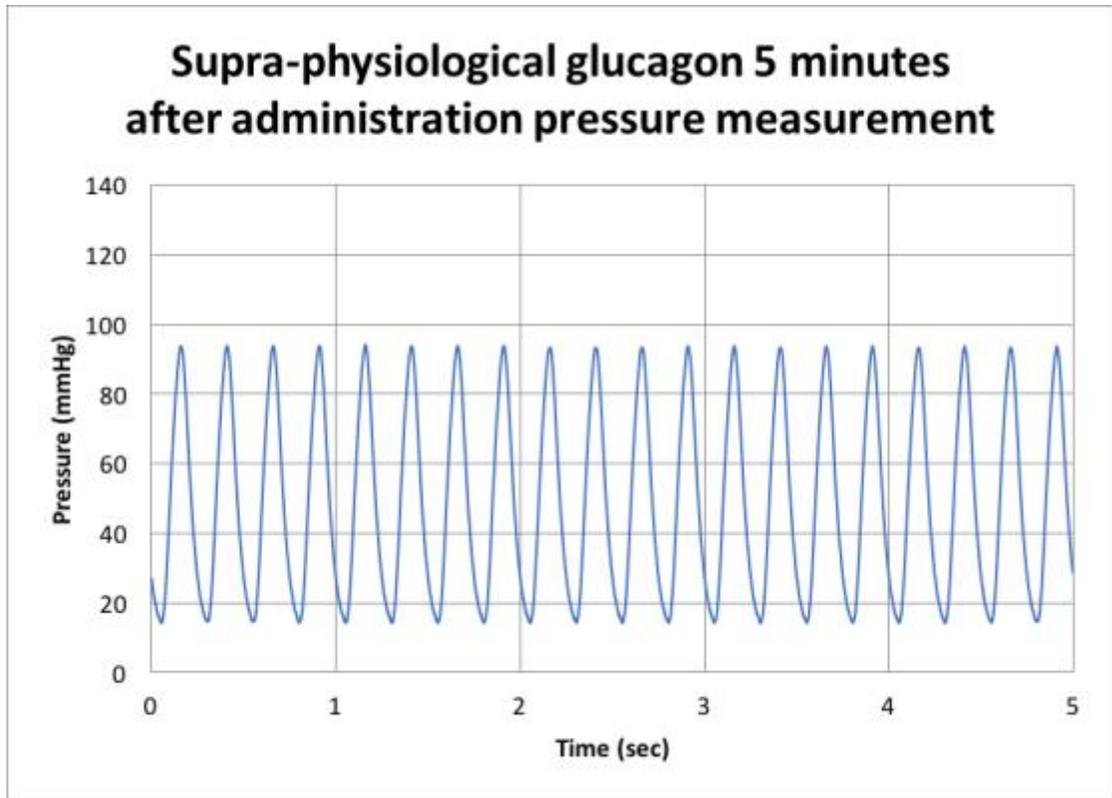
Figure 5

Figure 6

Figure 7

Figures 5, 6, and 7 are representative pressure tracings of one heart perfused via working mode. A supra-physiological glucagon concentration was administered at time zero. Each figure represents a different time point on the same heart. The pressure was determined by taking the difference of maximum value and the minimum value.
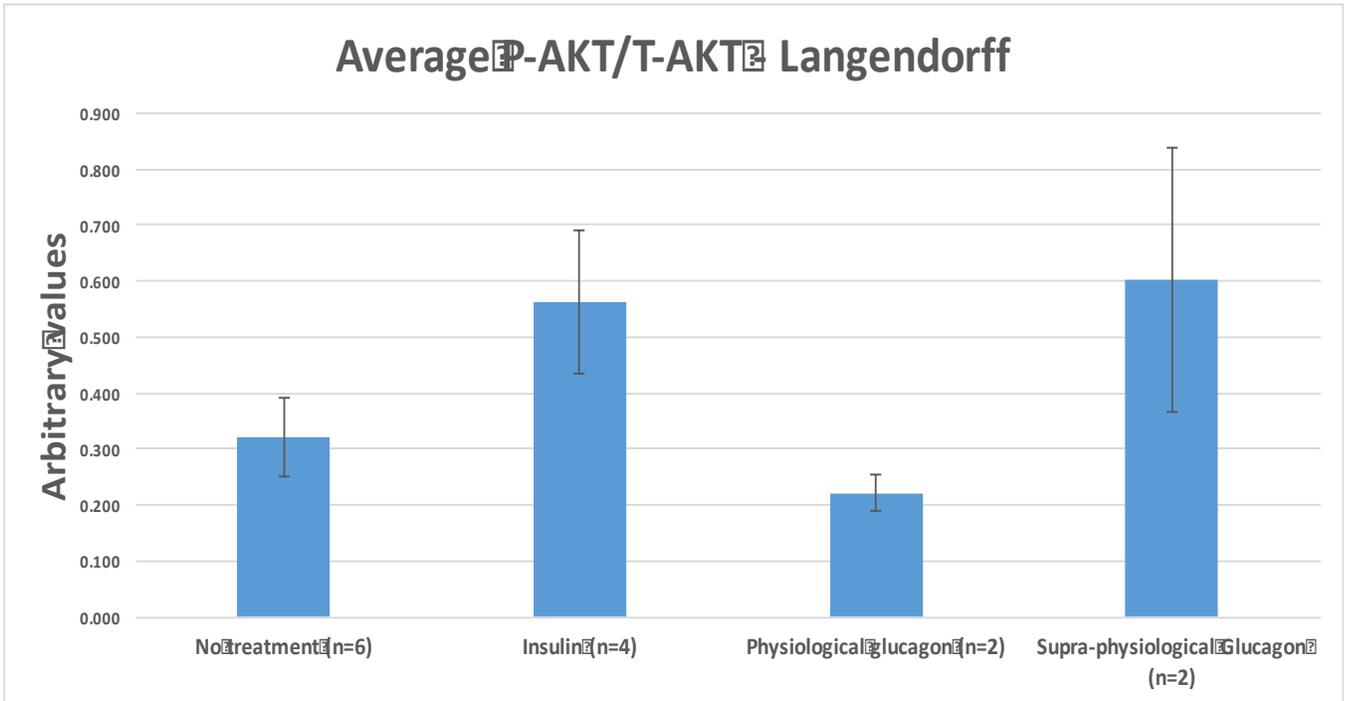
Figure 8: Average ratio of P-akt/T-akt for hearts perfused in the Langendorff Mode. Ratios represent the activation in PI3K/akt pathway. Error bars represent the SEM.
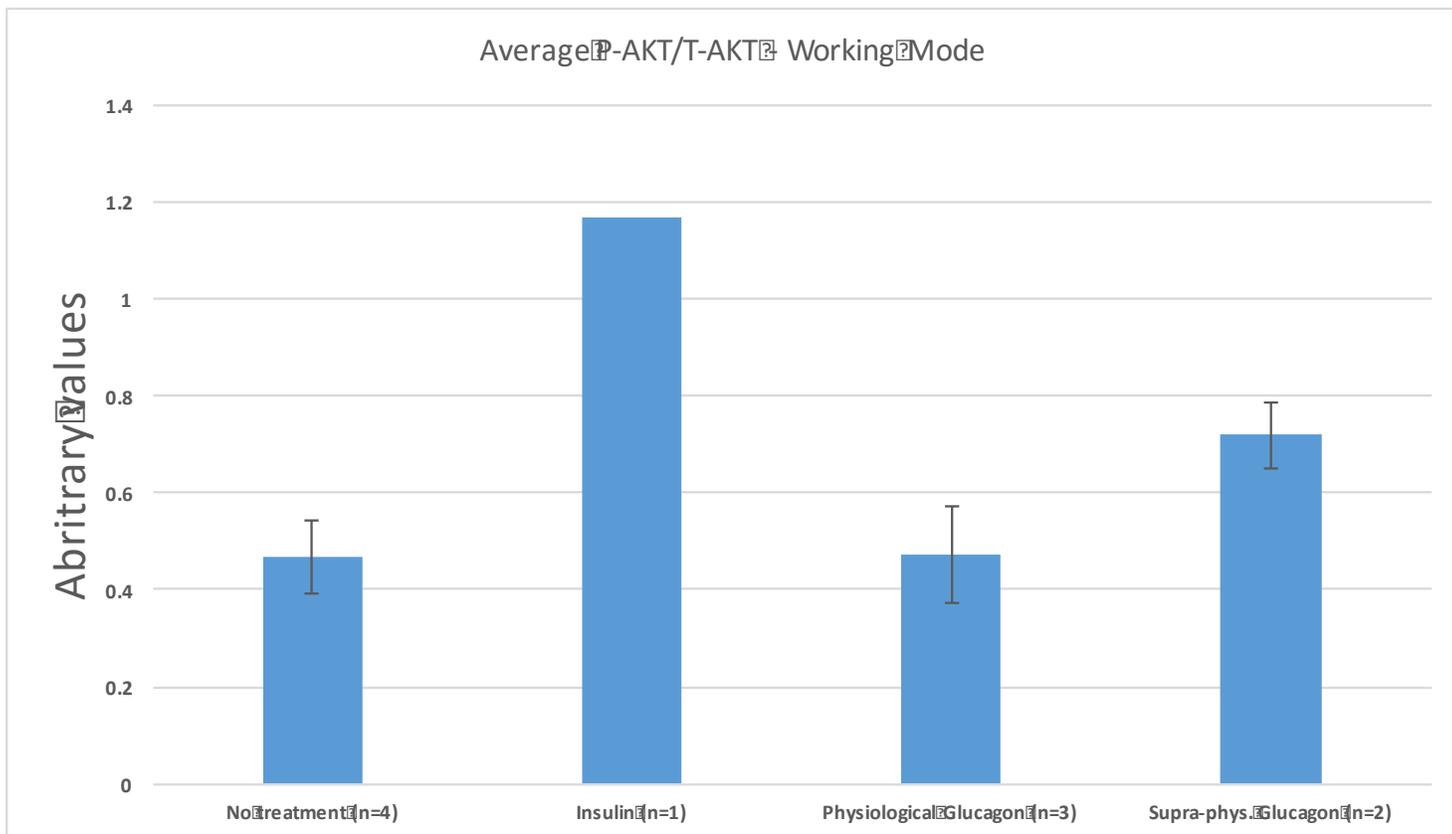
Figure 9: Average ratio of P-akt/T-akt for hearts perfused in the working Mode. Ratios represent the activation in PI3K/akt pathway. Error bars represent the standard deviation. Values represent means and error bars represent SEM

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