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α_1-Adrenergic Receptors Increase Glucose Oxidation Under Normal and Ischemic Conditions in Adult Mouse Cardiomyocytes

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Abstract

The role of catecholamine receptors in cardiac energy metabolism is unknown. α_1-adrenergic receptors (α_1-ARs) have been identified to play a role in whole body metabolism but its role in cardiac energy metabolism has not been explored. We used freshly prepared primary adult mouse cardiomyocytes and incubated with either ^{14}C-palmitate or ^{14}C-glucose tracers to measure oxidation rates in the presence or absence of phenylephrine, an α_1-AR agonist (with β and α_2-AR blockers) under normal cell culture conditions. $^{14}CO_2$ released was collected over a 10 min period in covered tissue culture plates using a 1M hyamine hydroxide solution placed in well cups, counted by scintillation and converted into nmoles/hr. We found that phenylephrine stimulated glucose oxidation but not fatty acid oxidation in adult primary cardiomyocytes. α_1-AR stimulated glucose oxidation was blocked by the AMPK inhibitor, dorsomorphin dihydrochloride, and the PKC inhibitor, rottlerin. Ischemic conditions were induced by lowering the glucose concentration from 22.5mM to 1.375 mM. Under ischemic conditions, we found that phenylephrine also increased glucose oxidation. We report a direct role of α_1-ARs in regulating glucose oxidation under normal and ischemic conditions that may lead to new therapeutic approaches in treating ischemia.

Keywords

Glucose; oxidation; metabolism; alpha1-adrenergic; G-protein coupled receptor

1. Introduction

α_1-adrenergic receptors (ARs) regulate the sympathetic nervous system by binding the neurotransmitter, norepinephrine and the hormone, epinephrine. They are G-protein coupled receptors known to regulate the cardiovasculature system, particularly in hypertrophy and blood pressure [1]. Epinephrine can produce a variety of effects on energy metabolism by altering glucose and lipid oxidation and metabolic rate [2-3]. There are nine AR subtypes that are highly homologous and bind epinephrine with similar affinities but by coupling to different G-proteins, regulate many diverse functional roles. Epinephrine regulates

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metabolism through AR subtypes but the roles of each receptor system in this process are varied and past studies focused on β-ARs or α_2-ARs, not α_1-ARs.

Little is known about the role of α_1-ARs in metabolism. Previous metabolic studies suggested that α_1-ARs stimulate gluconeogenesis and ketogenesis [4] and suppress triglyceride secretion [5] using rat hepatocytes. However, these earlier studies used non-selective antagonists to block responses. α_{1B}-AR KO mice appeared to develop metabolic syndrome [6], while transgenic mice over expressing constitutively active mutants of the α_{1A}-or α_{1B}-AR subtypes increased whole body lipid oxidation [7]. α_1-ARs can regulate glucose uptake into cell lines [8-9], but never shown to regulate glucose metabolism. The β-ARs can regulate adipocyte metabolism through cAMP levels in fat [10] and are lipolytic [11]. α_{2A}-ARs inhibits insulin secretion [12] and are anti-lipolytic [13]. Therefore, α_1-ARs can regulate whole body and tissue-specific metabolism that was distinct from other AR subtypes.

We have previously published that α_{1A}-ARs but not α_{1B}-ARs can regulate glucose uptake into the adult heart *ex vivo* [14]. In the normal adult heart, metabolism relies heavily on fatty acids to generate the bulk of its ATP requirements. On the other hand, the fetal heart utilizes mostly glucose [15]. Glucose oxidation in the adult heart may play an important role as an alternative and protective form of energy needed in specific pathological conditions, such as ischemia [16-18]. The ischemic heart depends heavily on glycolysis for energy production because of the anaerobic conditions of the disease. If α_{1A}-ARs regulate glucose metabolism in the heart, they may play an important role as a favorable metabolic guard against ischemic injury. However, there are no previous reports of α_1-ARs regulating glucose oxidation. This study's objective is to show a direct role of α_1-ARs in regulating glucose oxidation under normal and ischemic conditions.

2. Materials and Methods

2.1. Animals and Euthanasia.

C57BL/6WT were purchased from Jackson Laboratory then bred at the Cleveland Clinic. Mice were maintained on a normal chow diet (Harlan, #2918) and kept on a 12-hour light/dark cycle in a facility controlled at 70°F. The mice had free access to food and water. Equal numbers of male and female mice at 2 months of age were used in each experiment. Mice were provided veterinary care in an AAALAC-approved animal facility. Mice were euthanized by CO_2 exposure followed by decapitation. The animal procedures used in this study conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication NO. 85-23, revised 1996), and was approved by the Animal Care and Use Committee at our institution (Protocol 0844).

2.2. Adult Mouse Myocyte Isolation.

The isolation was performed using the adult rat cardiomyocyte isolation kit (ac-7031) from Cellutron Life Technologies (Baltimore, MD). A simplified Langendorff system was used. The system was sterilized using 70% ethanol for 5 minutes and was then rinsed with autoclaved water thoroughly. An adult C57Bl6 mouse (2 months old, equal use of male and

female) was injected with 500 U/kg heparin (Sigma, St. Louis, MO) intraperitoneally and then anesthetized with one dose of Ketamine HCl (100 mg/kg) and Xylazine HCl (10 mg/kg) in a dose volume of 100 ul intraperitoneally. The heart was excised and placed in ice cold water bath. The aorta was cannulated using a 23G blunt needle and then tied with sterile 6-0 silk suture. The heart was then attached to the perfusion system under a laminar flow hood. The heart was perfused with 40 ml buffer 1 (from kit) at a flow rate of ~8 ml/min. This flow through was discarded. The heart was then perfused with 1X buffer 2 (digestion buffer from kit) and the flow through was collected in a 50 ml tube. 100 ul SB buffer (from kit) was added to the flow through and mixed and then recirculated. A second aliquot of 100 ul SB buffer was added and then the heart was perfused with recirculated buffer 2 for 35 minutes. The heart was then removed from the perfusion system and was very soft and uniformly pink. It was gently teased into small pieces using a #10 scalpel and the tissue transferred to a 50 ml tube using 5 ml buffer 2 enzyme solution and a 5 ml pipettor tip with a wide bore tip (used throughout procedure). This mixture was placed on a microplate shaker at 37°C at lowest rotation setting for 2 minutes with circular motion. The tissue was triturated with a wide bore pipette and allowed to settle. The cell suspension was collected and centrifuged to yield CM pellets. Another aliquot of 5 ml buffer 2 was added and the procedure was repeated but this time the supernatant was transferred to a 17 × 100 mm tube and centrifuged at 1000 rpm for 1 minute. The cells were resuspended in a tube containing 30 ml buffer 3 (from kit) and kept at room temperature. The tissue was again suspended in buffer 2 and rotated on the shaker as before for 10 minutes. The supernatant was then removed, centrifuged and the cells added to the buffer 3. This was repeated every 10 to 20 minutes until the tissue was dissociated. The cell suspension was passed through sterile 300 um Nylon mesh and then centrifuged at 1200 rpm for 3 minutes and suspended in AS serum-containing media. The cells (100,000/well) were pipetted into a 12-well plate coated with 10 ug/ml mouse laminin (Invitrogen, Grand Island, NY) for several hours at 37°C and each containing a 10mm diameter center well incubation flask (Kimble Chase #8832320-0000). The cells are counted using a hemocytometer assessed for viability by trypan blue inclusion. Viable cardiomyocytes will be large (~ 100 - 200 μm in length), have a square-edged rod-shaped morphology with clear striations and appear to have a sharp outer membrane under brightfield illumination. The cells were incubated at 37°C overnight with 5% CO_2. The cells resulting from one mouse heart was used for each experiment or N.

2.3. Ischemic Conditions.

After myocytes were prepared and equilibrated overnight at atmospheric O_2 levels and with normal non-ischemic S media (which contains 22.5mM glucose), the media was drawn off and replaced with Ischemia Media (118mM NaCl, 16mM KCl, 1.2mM $MgCl_2$, 1mM NaH_2PO4 , 2 mM $NaHCO_3$, 2.5mM $CaCl_2$, 20mM sodium lactate and 1.375 mM glucose, pH 6.2). pH was monitored before and after ischemia and no changes in pH was noted.

2.4. Glucose Oxidation.

The procedure to measure oxidation rates in cell lines is a modification of published protocols (20) with the major modification being the insertion of a center well cup containing 1M hyamine hydroxide as a CO_2 trap instead of filter paper disks. The experiments were performed by first removing rounded cells by washing once with AS

serum-free media, then washing once with buffer before the addition of the test reagents. A 1ml media blank was included on the tissue culture plate as a control. ^{14}C-Glucose (250mCi/mmole, Perkin Elmer #NEC042X250UC) is used at 0.212 uCi/well in the presence or absence of the α_1-AR agonist phenylephrine (100uM) with or without an inhibitor. The plate is incubated for 2 hour in a tissue culture incubator at 5% CO_2 and 37°C to equilibrate, then the plate was sealed with microplate sealing tape (Thermo Scientific #15036). A large dish containing 1M hyamine hydroxide was placed inside the tissue culture incubator to scrub $^{14}CO_2$ released into the atmosphere. 300ul of 1M hyamine hydroxide solution was added to the center well cup using a syringe to puncture the tape, followed by 150ul of 1M sulfuric acid and mixed by swirling the plate This was followed by injecting 100ul of 0.5M sodium bicarbonate, mixed by swirling, then incubated for 10 minutes in the tissue culture incubator. The center well cup is then removed using forceps and dropped directly into a glass scintillation vial containing 10 ml of scintillation fluid. After counting, the total number of nmoles of $^{14}CO_2$ gas released is calculated based upon the specific activity of the ^{14}C-glucose and the total nanomoles of glucose (cold and hot) in the mixture.

2.5. Fatty Acid Oxidation.

The same experiment is performed as above except that uniformly -labeled ^{14}C-palmitate (850mCi/mmole, Perkin Elmer #NEC534050UC) was added to each well (0.212uCi/well).

2.6. Inhibitors.

To assess the signal transduction pathway mediated by α_1-ARs, the following list of reagents were used: α_1-AR agonist 100μM phenylephrine HCl (Sigma-Aldrich, St. Louis, MO, P-6126) in the presence of 1 μM propranolol and 0.1μ M rauwolscine to block β -and α_2 -ARs respectively, non-selective α_1-AR antagonist prazosin (1μ M; Sigma- Aldrich, P-7791), Etomoxir (250uM, Tocris 4539); dorsomorphin dihydrochloride (50uM, Santa Cruz sc-361173); Rottlerin (5uM, Tocris 1610); 2 Deoxy-D-Glucose (50mM, Sigma D3179).

2.7. Statistical Analysis.

Data is presented as individual points with the mean ± SEM and analyzed using GraphPad Prism software (GraphPad, San Diego, CA). The two-sided Grubbs test was used to identify outliers which were excluded from the data set. Differences in variables were examined by one-way analysis of variance (ANOVA) followed by a Newman-Keuls post-test and used to compare data sets in the different experimental conditions. A probability value $P< 0.05$ was set as statistically significant.

3. Results

3.1. Time-course of glucose and palmitate oxidation levels.

To establish conditions for the oxidation assays, we performed time-course studies varying the time of incubation of the myocytes with the reagents and fresh media before the rate of oxidation was determined. We found that glucose oxidation (Fig 1) achieved its highest rate after 2 hours of incubation. The rate of palmitate oxidation is much greater than glucose oxidation and at its highest level after 1 hour of incubation. As time of incubation progresses, the rate of palmitate oxidation rapidly decreases, while glucose oxidation

displayed a slower rate of decay. To be able to compare the palmitate versus glucose oxidation rate directly, all subsequent oxidation assays were then performed after 2 hour of incubation.

3.2. α_1-AR stimulation increased glucose oxidation in adult mouse myocytes.

We previously published that α_1-ARs increased the rate of glucose uptake into the heart through stimulation of GLUT transporters GLUT-1 and GLUT-4 and conferred protection against low glucose induced apoptosis [14]. Since glucose uptake and its subsequent phosphorylation is rate-limiting in glucose metabolism [19], we tested directly the ability of the α_1-AR to stimulate glucose oxidation in the adult myocyte. The addition of ^{14}C-glucose to freshly prepared adult myocytes and measuring the amount of $^{14}CO_2$ gas released over a 10 minute period measured after a 2 hour incubation provides more of a steady-state rate of glucose oxidation. We found that α_1-AR stimulation using phenylephrine (100uM in the presence of β-and α_2-AR blockers) increased the rate of glucose oxidation by two-fold and the effect was blocked by prazosin, an α_1-AR antagonist (1uM)(Fig 3). To confirm that we were directly measuring glucose oxidation, we used the inhibitor 2-deoxyglucose (50mM). 2-Deoxyglucose (2DG) blocks glucose oxidation by phosphorylating hexokinase, the first enzyme in glycolysis, resulting in 2DG-P which is non-hydrolyzable and accumulates resulting in the inhibition of the pentose phosphate pathway and glycolysis [20]. We found that 2DG inhibited both the basal rate as well as the phenylephrine-stimulated rate of glucose oxidation (Fig 3).

3.3. α_1-AR stimulation did not increase palmitate oxidation in adult mouse myocytes.

To measure the steady-state rate of fatty acid oxidation, we used ^{14}C-palmitate in place of ^{14}C-glucose and measured the amount of $^{14}CO_2$ gas released over a 10 minute period after 1 hour of incubation as previously described for cell Lines [21]. We also confirmed that we were directly measuring fatty acid oxidation by using the inhibitor Etomoxir (250uM), an irreversible inhibitor of carnitine palmitoyltransferase-1 that combines fatty acyl-CoA with carnitine. Etomoxir inhibits the formation of acyl carnitines, which are required for the transport of fatty acids from the cytosol into the intermembrane space of the mitochondria [22] and needed for the production of ATP. We found that Etomoxir inhibited the basal rate of palmitate oxidation (Fig 4). However, there was no statistical increase in the rate of palmitate oxidation after stimulation with phenylephrine (Fig 4) and there was no inhibition of the signal with prazosin.

3.4. α_1-AR stimulation of glucose oxidation is through PKCδ and AMPK.

We next explored the signaling pathway regulating glucose oxidation by α_1-ARs. Using the PKCδ inhibitor, Rotterin and siRNA, we previously established that GLUT1/4 translocation was regulated through PKCδ [14]. Rottlerin (1.25 μM, ROT) also inhibited the phenylephrine-mediated rate of glucose oxidation (Fig 5). α_1-ARs have been shown to activate AMP-activated protein kinase (AMPK) in the heart and in skeletal muscle cells to regulate glucose uptake [23-26]. AMPK is a sensor that regulates the homeostasis of cellular energy by monitoring the ratio of ATP:ADP, adjusting the rates of fatty acid or glucose oxidation accordingly. Using the AMPK inhibitor, dorsomorphin dihydrochloride (also

known as Compound C)(50uM), we show that α_1-AR stimulated glucose oxidation may also proceed through a AMPK pathway (Fig 5).

3.5. α_1-AR stimulation increases glucose oxidation during ischemia.

We next tested the ability of α_1-AR stimulation to regulate glucose oxidation under the ischemic conditions of low-glucose concentrations that would occur during reduced blood flow. When the glucose concentration in the media is reduced from 22.5mM to 1.375mM pH 6.2, the rate of glucose oxidation is decreased by over 50%. However, when the cells are stimulated with phenylephrine, the rate of glucose oxidation is significantly increased (Fig 6).

4. Discussion

We have demonstrated for the first time that α_1-ARs directly increased glucose oxidation in adult cardiomyocytes under normal and ischemic conditions. This is also the first report of adrenergic effects on energy metabolism in the adult heart. The pathway involved in this metabolic regulation appears to involve PKCδ and AMPK as rottlerin and dorsomorphin dihydrochloride blocked α_1-AR stimulation of glucose oxidation. This is the same pathway previously shown involved in α_1-AR mediated glucose uptake through GLUT-1/4 in the mouse adult heart [14]. As ischemia does not invoke an increase in glucose uptake [26-27], this would suggest that α_1-AR stimulation not only increases glucose delivery to the heart but is also the driving force for increased glucose oxidation.

Several studies previously demonstrated that glucose oxidation in the heart is altered in many cardiovascular diseases, including ischemia, heart failure, and diabetic cardiomyopathy [17-18, 28]. During episodes of ischemia, the reduction in glucose and oxygen to feed oxidative phosphorylation accelerates the uptake of glucose to provide a needed source of ATP to maintain cardiac function. Etomoxir and other inhibitors show beneficial effects in patients with heart failure by inhibiting fatty acid oxidation, resulting in increased glucose oxidation in the heart [29-31]. Drugs that directly enhance glucose oxidation such as dichloroacetate improve the coupling between glycolysis and glucose oxidation in the heart and benefited patients with coronary artery disease [32].

There are several previous studies indicating that the α_{1A}-AR subtype can impart beneficial effects during ischemia and heart failure. α_1-ARs were first indicated to confer protection was in the Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial (ALLHAT) trial [33-34]. An α_1-AR antagonist increased adverse cardiovascular incidents which stopped the trial early. α_1-AR stimulation also prevented a maladaptive heart response during pressure overload [35]. Transgenic mice using the endogenous promoter for the α_1-AR subtype and overexpressing the α_{1A}- but not α_{1B}-AR preconditioned the ischemic heart [36] and prevented damage through glucose uptake utilizing the PKCδ and GLUT1/4 pathways [14]. A cardiac-targeted α_{1A}-AR transgenic model also limited post-infarct remodeling [37] and cardiac dysfunction [38]. Our results suggest that α_{1A}-AR mediated glucose uptake and glucose oxidation may also play a role in this cardioprotection.

While prior research shied away from the translational use of α_1-AR agonists because of its effects on blood pressure, α_{1A}-AR imidazoline partial agonists compounds have been shown in principle to mediate functions that are uncoupled from blood pressure [39-40]. The α_{1A}-ARs are preferentially activated by imidazolines [41] and imidazolines have biased-signaling towards the cAMP response when compared to the blood pressure inducing inositol triphosphate/Ca^{+2} pathway [42-43]. cAMP and its cAMP response element-binding protein/CREB are known mediators of metabolism and glucose homeostasis [44-46]. In addition, while all three α_1-AR subtypes are activated by norepinephrine and generate a cAMP response when β-ARs are blocked, the α_{1A}-AR displayed the greatest efficacy in generating cAMP in the presence of norepinephrine compared to phenylephrine or methoxamine [47]. This suggests that active conformations of the norepinephrine-mediated α_{1A}-AR response are biased towards cAMP and may be achievable for therapeutics. Confirming this hypothesis, our laboratory has recently developed novel positive allosteric modulators that are specific for potentiating the norepinephrine-mediated cAMP response of the α_{1A}-AR and do not increase blood in mice (provisional patent # 62837565). The ability of the α_{1A}-AR to increase glucose oxidation during ischemia reinforces the concept that stimulation of this receptor may confer a cardioprotective phenotype and provide a novel treatment for ischemia and heart failure.

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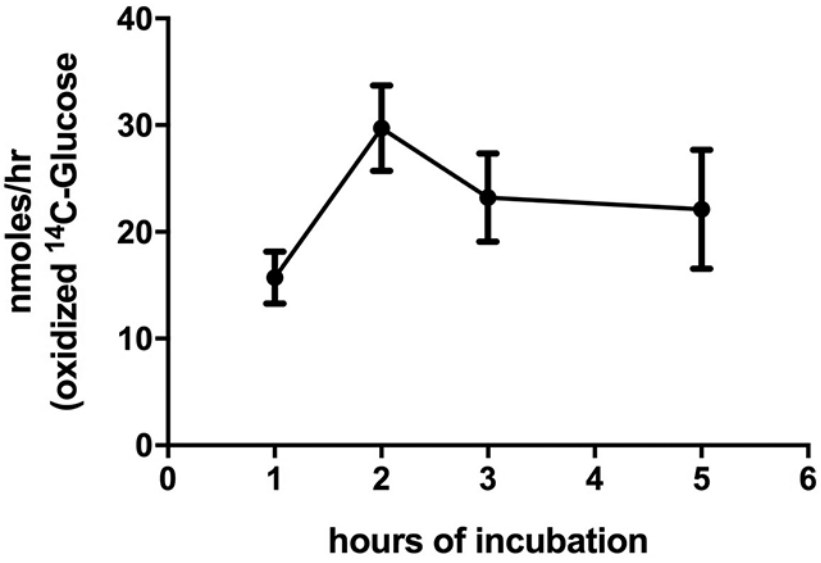

Figure 1. Time of incubation versus glucose oxidation rate in adult mouse myocytes.

Freshly prepared adult mouse cardiomyocytes were incubated with fresh serum-free media and ^{14}C-glucose for 1, 2, 3 and 5 hour time points to determine steady-state conditions. After the indicated incubation times, 300ul of 1M hyamine hydroxide solution was added to the center well cup using a syringe to puncture the tape, followed by 150ul of 1M sulfuric acid and mixed by swirling the plate This was followed by injecting 100ul of 0.5M sodium bicarbonate, mixed by swirling, then incubated for 10 minutes in the tissue culture incubator to capture released $^{14}CO_2$. The center well cup is then removed using forceps and dropped directly into a glass scintillation vial containing 10 ml of scintillation fluid. After counting, the total number of nmoles of $^{14}CO_2$ gas released is calculated based upon the specific activity of the ^{14}C. Results indicate that glucose oxidation reached a maximum after 2 hours then slowly decreased upon longer incubation times. The results are based upon N=4 independent experiments performed in duplicate.

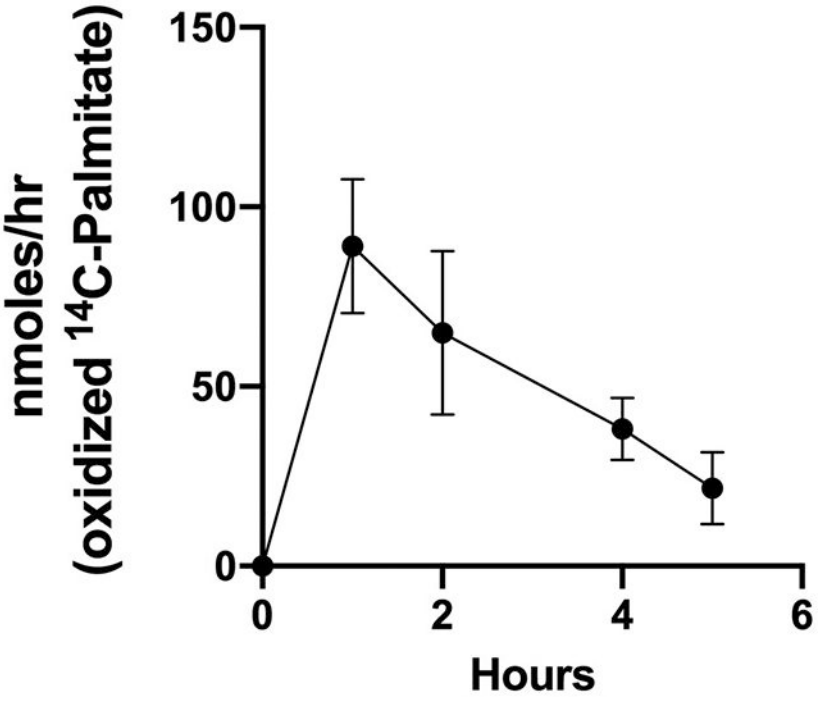

Figure 2. Time of incubation versus palmitate oxidation rate in adult mouse myocytes.

Freshly prepared adult mouse cardiomyocytes (N=1 is equivalent to one mouse heart) were incubated with fresh serum-free media and ^{14}C-palmitate for 1, 2, 4 and 5 hour time points. After the indicated incubation times, 300ul of 1M hyamine hydroxide solution was added to the center well cup using a syringe to puncture the tape, followed by 150ul of 1M sulfuric acid and mixed by swirling the plate This was followed by injecting 100ul of 0.5M sodium bicarbonate, mixed by swirling, then incubated for 10 minutes in the tissue culture incubator to capture released $^{14}CO_2$. The center well cup is then removed using forceps and dropped directly into a glass scintillation vial containing 10 ml of scintillation fluid. After counting, the total number of nmoles of $^{14}CO_2$ gas released is calculated based upon the specific activity of the ^{14}C. Results indicate that the rate of palmitate being oxidized is maximal after 1 hour then rapidly decreases upon longer incubation times. Results are based upon 3-4 independent experiments performed in duplicate.

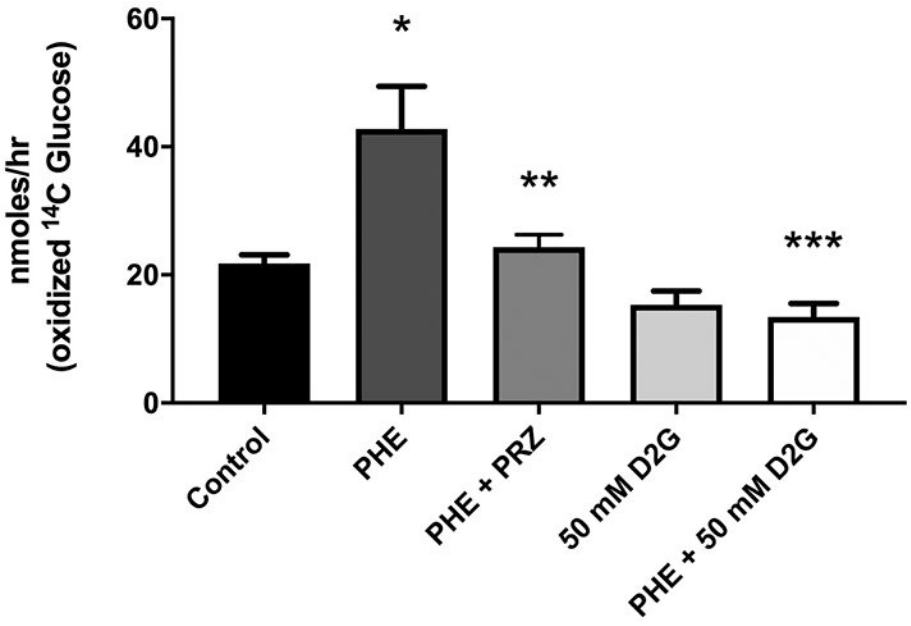

Figure 3. Glucose oxidation is stimulated by α_1-ARs in adult mouse myocytes.

^{14}C-glucose is added to adult mouse myocytes in the presence or absence of phenylephrine (100uM, PHE in the presence of β-and α_2-AR blockers) with or without various inhibitors, incubated for 2 hours in a tissue culture incubator at 5% CO_2 and 37°C to equilibrate. The rate of glucose oxidation is then measured. α_1-AR stimulation increased the rate of glucose oxidation by two-fold which was blocked by the α_1-AR antagonist prazosin (1uM, PRZ) or the glycolysis inhibitor 2-deoxyglucose (50mM, D2G). Results are based upon 4-6 experiments performed in duplicate. Each N is equivalent to one mouse heart. * $p<0.05$ compared to control; **$p<0.05$ compared to PHE; ***$p<0.05$ compared to PHE.

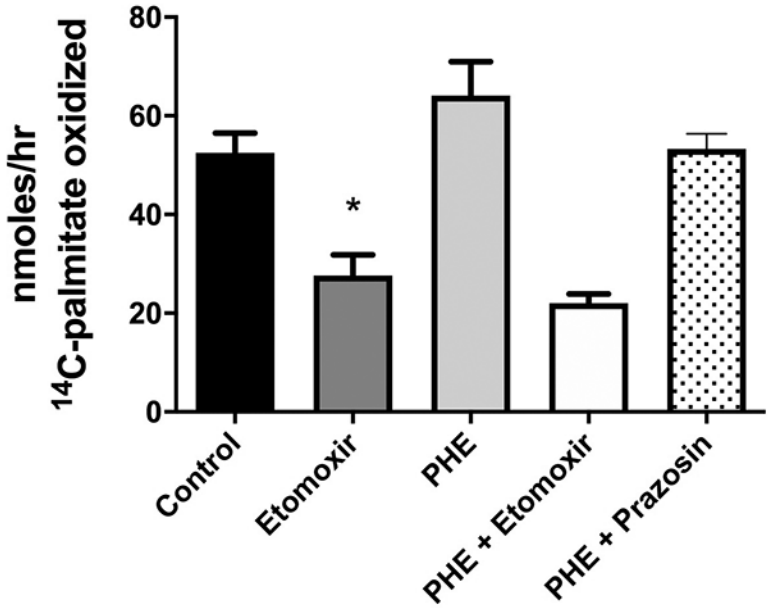

Figure 4. α_1-ARs do not stimulate palmitate oxidation in adult mouse myocytes.

^{14}C-palmitate is added to adult mouse myocytes in the presence or absence of phenylephrine (100uM, PHE in the presence of β-and α_2-AR blockers) with or without various inhibitors, incubated for 2 hours in a tissue culture incubator at 5% CO_2 and 37°C to equilibrate. The rate of palmitate oxidation is then measured. We found that α_1-AR stimulation did not affect the rate of palmitate oxidation. Etomoxir (250uM), an irreversible inhibitor of carnitine palmitoyltransferase-1, inhibited the basal rate of palmitate oxidation, confirming that we were indeed measuring fatty acid oxidation. Results are based upon 3 independent experiments performed in duplicate. Each N is equivalent to one mouse heart. *$p<0.05$ compared to control; **$p<0.05$ compared to PHE.

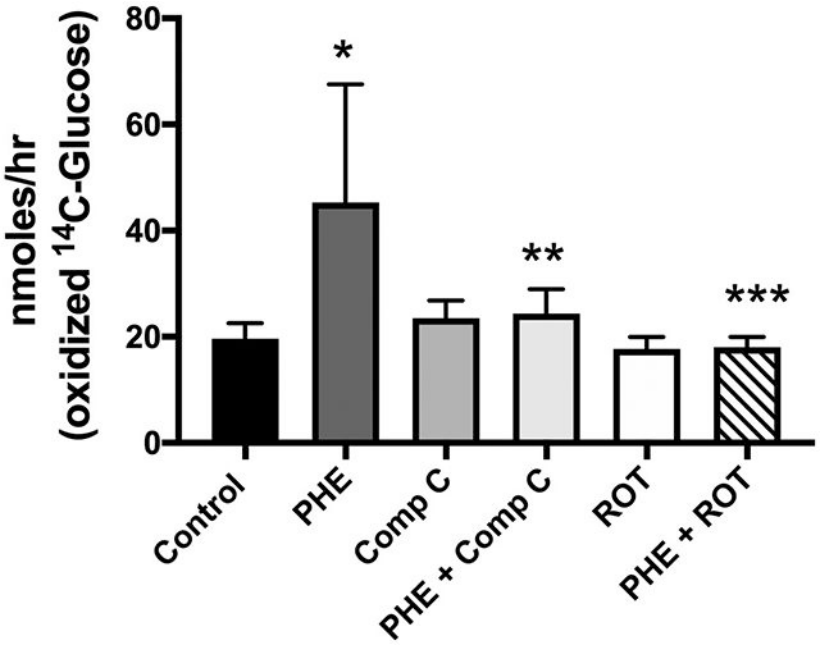

Figure 5. α_1-AR mediated glucose oxidation is blocked by AMPK and PKCδ inhibitors in adult mouse myocytes.

^{14}C-glucose is added to adult mouse myocytes in the presence or absence of phenylephrine (100uM, PHE in the presence of β-and α_2-AR blockers) with or without various inhibitors, incubated for 2 hours in a tissue culture incubator at 5% CO_2 and 37°C to equilibrate, then the rate of glucose oxidation measured. We found that the AMPK inhibitor, dorsomorphin dihydrochloride (also known as Compound C)(50uM, Comp C), and the PKCδ inhibitor Rottlerin (1.25 μM, ROT) inhibited the phenylephrine-mediated rate of glucose oxidation, suggesting the involvement of PKCδ and the AMPK pathway. Results are based upon 3-4 experiments performed in duplicate. Each N is equivalent to one mouse heart. *$p<0.05$ compared to control; **$p<0.05$ compared to PHE; ***$p<0.05$ compared to PHE.

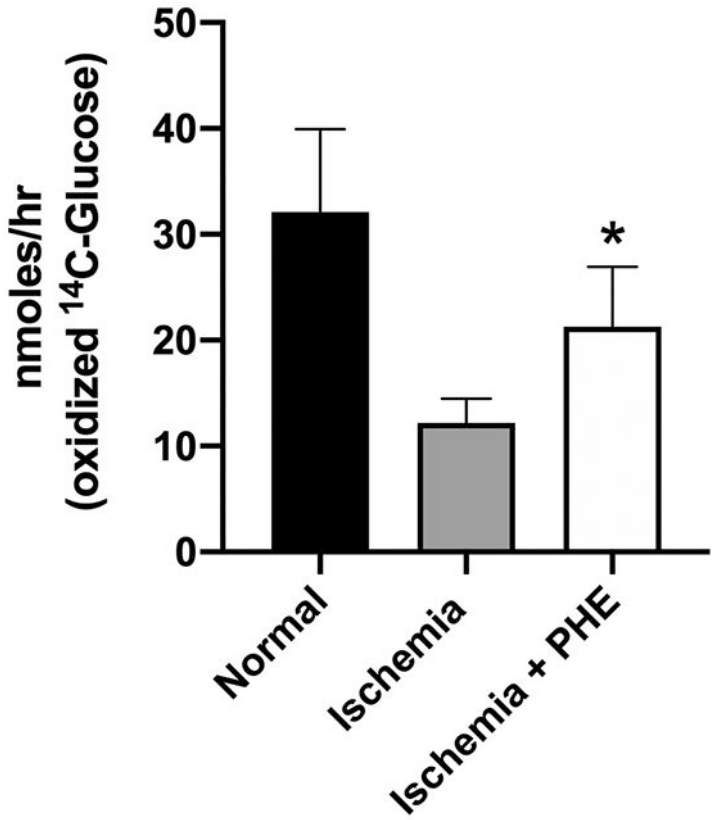

Figure 6. α_1-AR stimulation increases glucose oxidation during ischemia in adult mouse myocytes.

After myocytes were prepared and equilibrated overnight with normal non-ischemic S media (which contains 22.5mM glucose), the media was drawn off and replaced with Ischemia Media (with 1.375 mM glucose, pH 6.2). ^{14}C-glucose is added to the myocytes in the presence or absence of phenylephrine (100uM, PHE containing β-and α_2-AR blockers), incubated for 2 hours in a tissue culture incubator at 5% CO_2 and 37°C to equilibrate. The rate of glucose oxidation is then measured. When the glucose concentration in the media is reduced from 22.5mM to 1.375mM pH 6.2, the glucose oxidation rate decreased by over 50%. However, when the cells in ischemic buffer are stimulated with phenylephrine, the rate of glucose oxidation is significantly increased. Results are based upon 4-6 experiments performed in duplicate. Each N is equivalent to one mouse heart. *$p<0.05$ compared to Ischemia alone.