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Peroxisome proliferator-activated receptors γ and α agonists stimulate cardiac glucose uptake via activation of AMP-activated protein kinase☆

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Abstract

Myocardial energy and glucose homeostasis are crucial for normal cardiac structure and function. Peroxisome proliferator-activated receptors (PPARs) play an important role in controlling transcriptional expression of key enzymes that are involved in glucose metabolism, and they have been demonstrated to significantly reduce tissue injury in cardiovascular diseases. Adenosine monophosphate (AMP)-activated protein kinase (AMPK) is a sensor that maintains intracellular energy homeostasis and mediates a number of physiological signals. It has been reported that AMPK promotes glucose uptake. We hypothesize that PPARγ and α agonists may play a role in the regulation of glucose metabolism through AMPK. We tested this hypothesis by using isolated papillary muscles of rat hearts treated with PPARγ and α agonists, troglitazone and GW7647, respectively. Our results demonstrated that both troglitazone and GW7647 significantly stimulated 2-deoxyglucose uptake of cardiac muscles. Interestingly, both agonists stimulated phosphorylation of AMPK and its downstream protein target acetyl-CoA carboxylase. Endothelial nitric oxide synthase (eNOS) was also activated by both agonists. In addition, AMPK activator 5-amino-4-imidazole-1-β-D-carboxamide ribofuranoside increased glucose uptake, while AMPK inhibitor compound C and NOS inhibitor, N^{ω}-nitro-L-arginine, significantly blocked troglitazone- and GW7647-stimulated glucose uptake in cardiac muscles. There was also a reduction of glucose uptake with a marked decrease in AMPK and eNOS phosphorylation. In conclusion, both PPARγ and α activation play a role in the regulation of glucose uptake in cardiac muscles and this regulation is mediated by the AMPK and eNOS signaling pathways.

Keywords: Peroxisome proliferator-activated receptors; Left ventricular papillary muscles; Glucose uptake; AMP-activated protein kinase; Endothelial nitric oxide synthase

1. Introduction

Peroxisome proliferator-activated receptors (PPARs), members of the nuclear hormone receptor superfamily of ligand-activated transcription factors, were demonstrated to modulate genes that regulate lipid and glucose metabolism [1]. More recently, PPARs have been shown to participate in the regulation of cell growth and migration [2], oxidant stress [3,4] and inflammation [5] in the cardiovascular system. Three PPAR subtypes, encoded by distinct genes, are designated as PPARα, PPARδ (also known as β) and PPARγ. PPARγ controls adipocyte differentiation and lipid storage [6] and is highly expressed in adipose tissue. Through its effects on adipose tissue and skeletal muscle, PPARγ regulates the action of insulin. Selective agonists of PPARγ are insulin-sensitizers thiazolidinediones or glitazones, such as troglitazone, pioglitazone and rosiglitazone. PPARα is found in tissues where fatty acid catabolism is important and is stimulated by natural ligands such as fatty acids and eicosanoids, lipid-lowering fibrates [7] and synthetic agonists (e.g., GW7647) [8]. The PPARγ and α agonists have recently received considerable attention for their involvement in cardiovascular diseases [9,10]. A growing body of evidence suggests they can decrease platelet aggregation and delay intra-arterial thrombus formation [11], reduce myocardial infarct size [12] and have diverse anti-inflammatory effects in coronary arteriosclerosis [13] and myocardial ischemia/reperfusion injury [14–17].

Adenosine monophosphate (AMP)-activated protein kinase (AMPK) is a heterotrimeric enzyme complex consisting of one catalytic α subunit, two regulatory β subunits and a γ subunit.

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AMPK was initially found to be an important regulator of fatty acid oxidation in heart [17] and skeletal muscle [18] but has also emerged as an important mediator of glucose metabolism [19]. It can be activated by stresses that deplete cellular adenosine triphosphate (ATP) and increase the AMP/ATP ratio, such as metabolic poisoning, hypoxia, exercise and nutrient deprivation in mammalian cells [20]. The importance of AMPK during hypoxic conditions is highlighted by recent findings that transgenic mice with deficient AMPK signaling have diminished glucose uptake in hypoxic skeletal muscles [21] in both the ischemic [22] and postischemic heart [23]. The downstream mechanisms through which AMPK mediates the activation of glucose uptake may potentially in part be dependent on its interaction with the nitric oxide pathway [24,25]. AMPK phosphorylates endothelial nitric oxide synthase (eNOS) on Ser^{1177} [26], leading to eNOS activation and nitric oxide (NO) production. NO produced in endothelial cells may have an important paracrine role to modulate myocyte metabolism and function in muscle tissues, resulting in the stimulation of glucose uptake. Diminished insulin-stimulated glucose uptake was observed in eNOS knockout mice [27].

Indeed, recent studies have proposed PPARγ agonists (e.g., rosiglitazone) protect endothelial cells with an AMPK-dependent mechanism [28]. Fenofibrate, a PPARα agonist, has also been showed to stimulate eNOS phosphorylation and NO production through AMPK activation in human umbilical vein endothelial cells [29]. In this present study, we hypothesize that PPARγ and α agonists may play a role in the regulation of glucose metabolism contributing to its homeostasis in cardiac tissues and AMPK-eNOS signaling may also play a key role in this process. Isolated rat left ventricular papillary muscles were utilized to assess these effects. Our results indicate that increased glucose uptake in cardiac tissue by PPARγ and α agonists is partially dependent on AMPK-eNOS signaling activation.

2. Methods and materials

2.1. Experimental animals

Adult male Sprague-Dawley rats (8-week-old) were purchased from Beijing Vital River Laboratory Animal Technology (Beijing, China) and maintained in the animal facility of the School of Medicine, Shandong University. All animals were housed with controlled temperature (22–26°C), humidity (50–60%) and lighting (12/12 circadian cycle) with free access to standard rat chow and sterile water throughout the study. All animal experimental procedures were performed in accordance with the Guidelines for Animal Experiments of the School of Medicine of Shandong University and were approved by the Institutional Authority for Laboratory Animal Care.

2.2. Isolated heart papillary muscles preparation

Rats were anesthetized by intra-peritoneal injection of sodium pentobarbital (60 mg/kg). After the atria were removed, the left ventricle was cut open and the anterior and posterior papillary muscles (3–6 mg) were dissected free. The isolated papillary muscles were initially incubated for 30 min at 37°C in oxygenated phosphate-buffered saline containing 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose and 1% bovine serum albumin, as described previously [24]. There was a constant oxygen flow through the sealed muscle incubation containers which were agitated in a water bath at 37°C. Papillary muscles were then incubated in buffers. These buffers contain AMPK inhibitor compound C (10 μM) [30] or NOS inhibitor N^{ω}-nitro-L-arginine (L-NAME, 0.1 μM) [31] or vehicles as controls for 30 min depending on the experiment before the addition of pharmacological agonists. Pharmacological agonists 5-amino-4-imidazole-1-β-D-carboxamide ribofuranoside (AICAR, 1 mM) [32], troglitazone or GW7647 was also added to the buffers with an incubation period of another 30–60 min depending on the experiment.

2.3. Glucose uptake

Papillary muscles were incubated as above, and 2-deoxy-D-[^{3}H] glucose (1 μCi/ml) was added during the final 30 min of incubation to measure the rate of glucose uptake.

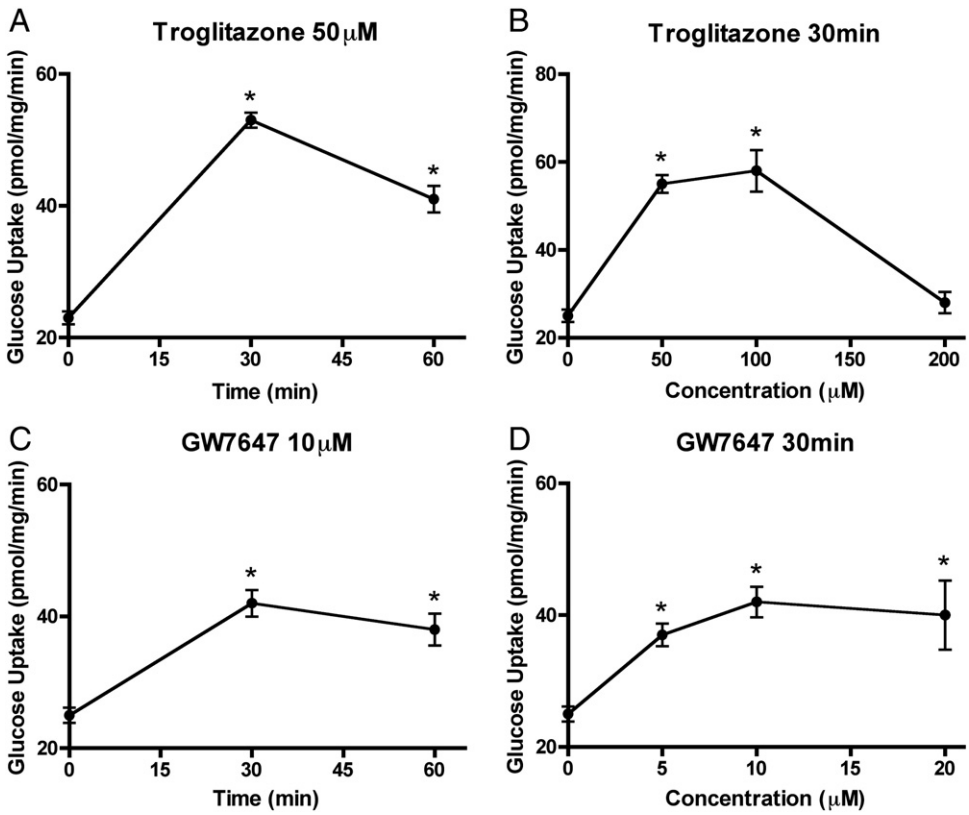

Fig. 1. Time and dose effects of troglitazone and GW7647 on glucose uptake. Isolated papillary muscles were exposed to 50 μM troglitazone or 10 μM GW7647 for 0, 30 and 60 min (A and C). Isolated papillary muscles were exposed to 50, 100 and 200 μM troglitazone or 5, 10 and 20 μM GW7647 for 30 min (B and D). 2-Deoxy-[1-^{3}H] glucose was then added to each solution for an additional 30 min incubation after which glucose uptake was measured. Values are means±S.E.M. for five experiments. *P<.05 versus controls.

In addition, [U-^{14}C] mannitol (0.1 μCi/ml) was added to measure the muscle extracellular space to correct for extracellular deoxyglucose. After the incubation, papillary muscles were washed three times with ice-cold saline, blotted dry and weighed. Muscles were then solubilized at 60°C with Soluene-350 (Packard Instrument, Meriden, CT, USA) and subjected to liquid scintillation counting [32].

2.4. High-performance liquid chromatography assay

Rat papillary muscles were transferred to a 1.5 ml microcentrifuge tube and 10 μl of 0.4 M ice-cold perchloric acid was added per milligram wet weight. The tissue was then immediately homogenized. The acidic homogenate was kept on ice for 30 min and then centrifuged at 14,000 rpm at 4°C for 10 min. An aliquot of the pellets was set aside for protein concentration measurements, which was done by using a protein assay reagent (Bio-Rad, Hercules, CA, USA). The resulting supernatant was neutralized with potassium carbonate (K_2CO_3). Before centrifugation, the supernatant was kept on ice for 10 min and at −80°C for 1–2 h to promote precipitation of the perchlorate. The supernatant was stored at −80°C until a high-performance liquid chromatography (HPLC) assay was done [33]. Prior to the HPLC assay, the supernatant was filtered and then an aliquot of 50 μl was injected into the HPLC. The chromatographic separation of AMP and ATP was performed using a C18 reversed-phase column (250×4 mm injected dose, particle size 5 μm). The mobile phase was composed of 215 mM potassium dihydrogen phosphate, 2.3 mM tetrabutylammonium hydrogensulfate (TBAHS), 4% acetonitrile, 0.4% potassium hydroxide (1 M) and the flow rate was 1 ml/min. During isocratic acquisition, components were monitored at 254 nm. Standard stock solutions were AMP (3 mg/ml), Ad (1 mg/ml) and ADP (1 mg/ml) prepared in 0.4 M perchloric acid. Fresh dilutions were made before each assay; a mobile phase was added in order to obtain 60 μg/ml AMP, 4 μg/ml Ad and 5 μg/ml ADP concentrations. Standard solutions of ATP (0.4 mg/ml) and a dilution (20 μg/ml) were prepared daily in the same way as described for AMP preparation.

2.5. Western blot analysis

Rat papillary muscles were homogenized in a lysis buffer containing 20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 0.1% sodium dodecyl sulfate (SDS) and a 1% protease inhibitor cocktail. Samples were then sonicated for 15 s and centrifuged at 12,000×*g* for 10 min at 4°C. The protein concentration of the supernatant was evaluated using a protein assay reagent (Bio-Rad, Hercules, CA, USA). Equal amounts of protein (30 μg protein/lane) and a prestained molecular weight marker (GIBCO, Gaithersburg, MD, USA) were loaded onto 7–12% SDS-polyacrylamide gels in a minigel apparatus (Mini-PROTEAN II, Bio-Rad), separated and transferred to nitrocellulose membranes (0.2 μm pore size, Bio-Rad). The membranes were then incubated for 1 h in a blocking solution containing 5% nonfat milk in a phosphate-buffered saline 0.05% Tween solution (TBS). After incubation, the membranes were washed in TBS and incubated overnight at 4°C with anti-phospho-AMPK (1:1000; Cell Signaling, Beverly, MA, USA), anti-AMPK (1:1000; Cell Signaling), anti-phospho-Acetyl-CoA Carboxylase (Upstate Biotechnology, Lake Placid, NY, USA), anti-Acetyl-CoA Carboxylase (ACC) (1:1000;Cell Signaling), anti-phospho-eNOS (1:1000;Cell Signaling) and anti-eNOS (1:1000;Cell Signaling). After incubation with the primary antibodies, blots were incubated with either antimouse or antirabbit IgG horseradish peroxidase-linked antibodies at a dilution of 1:5000 for 60 min at room temperature. Immunoreactive bands were detected using the Super Signal West Dura Extended Duration Substrate (Pierce, Milwaukee, WI, USA). The intensity of each band was measured with a scanning densitometer (model GS-800; Bio-Rad) coupled with Bio-Rad analysis software.

2.6. Statistical analysis

Data were presented as Means±S.E.M. Differences between groups were assessed using analysis of variance followed by Newman–Keuls post hoc test. $P<0.05$ was considered statistically significant.

3. Results

3.1. Effects of PPARγ and α agonists on glucose uptake

Cardiac muscles were exposed to PPARγ or PPARα agonists for 30 or 60 min, and then, 2-deoxyglucose uptake of cardiac muscles was evaluated using 2-deoxy-D-[^{3}H] glucose. Both PPARγ and PPARα agonists, troglitazone and GW7647 significantly stimulated 2-deoxyglucose uptake of cardiac muscles. Troglitazone (50 μM) and GW7647 (10 μM) significantly enhanced glucose uptake with an incubation period of 30 or 60 min (Fig. 1A and C). There was also a significant elevation of glucose uptake in cardiac muscles treated with 50 or 100 μM troglitazone for 30 min. Similar results were observed when cardiac muscles were treated with 5, 10 or 20 μM GW7647 for 30 min (Fig. 1B and D).

3.2. Effects of PPARγ and α agonists on AMPK activation

AMPK has been emerged as an important mediator of glucose metabolism, and we assessed AMPK activation in cardiac muscles using western blot and HPLC assay. Treatment of isolated left ventricular papillary muscles with troglitazone or GW7647 resulted in AMPK activation. This was shown by the phosphorylation of AMPK and ACC,

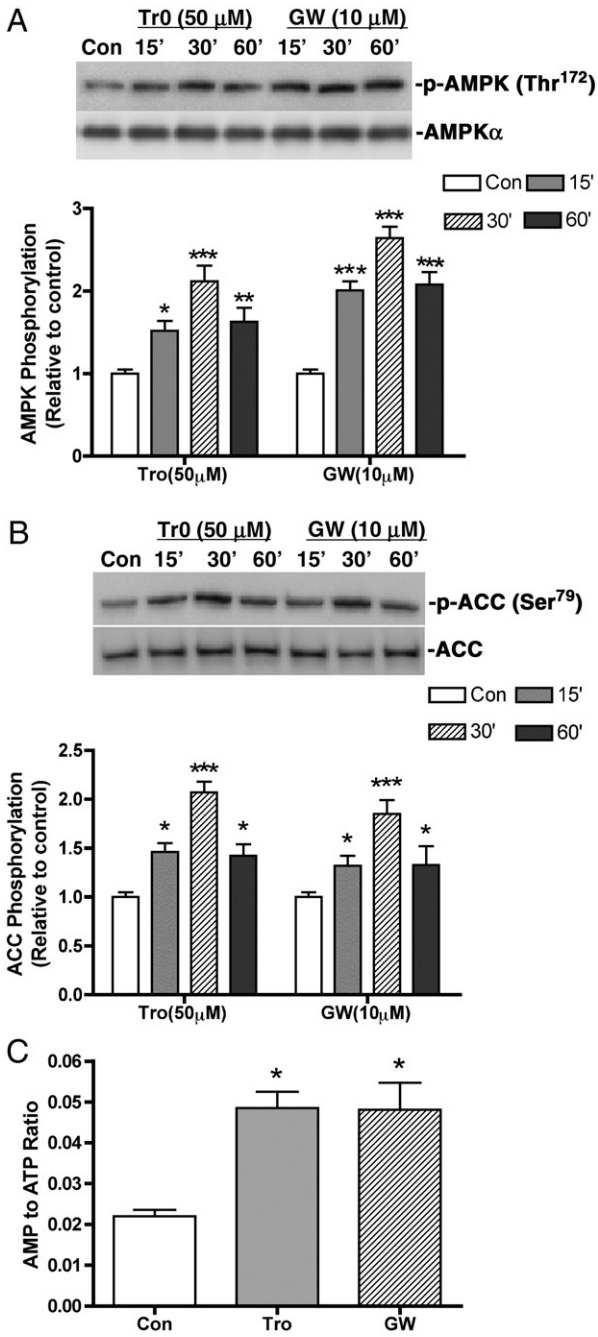

Fig. 2. Time effects of troglitazone (Tro, 50 μM) and GW7647 (GW, 10 μM) on AMPK and ACC phosphorylation in papillary muscles. Isolated papillary muscles were exposed to 50 μM troglitazone or 10 μM GW7647 for 0, 15, 30 and 60 min. Control (Con) muscles were not exposed to troglitazone or GW7647. Western blot analyses were performed for AMPK (A) and ACC (B). HPLC assay was performed to assess the ratio of AMP to ATP using cardiac papillary muscles with incubation with (Tro, 50 μM) or GW7647 (GW, 10 μM) for 30 min (C). Data were compiled from five independent experiments in each condition and presented as means±S.E.M. *$P<.05$ versus controls. **$P<.01$ versus controls. ***$P<.001$ versus controls.

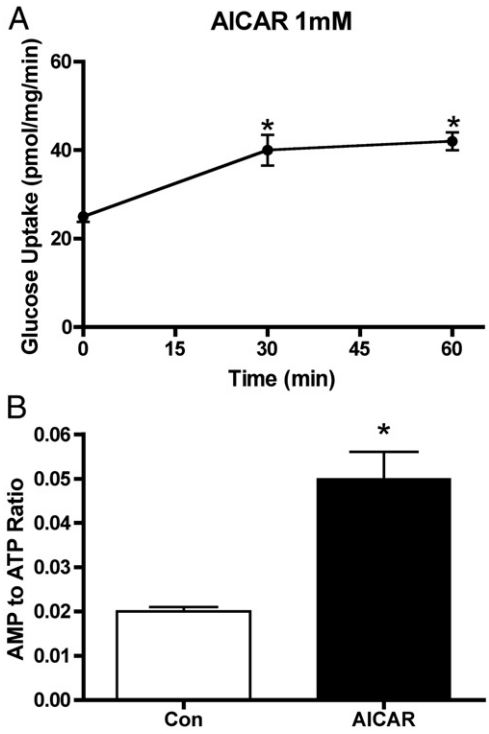

Fig. 3. Effects of AICAR on glucose uptake and change of AMP/ATP. (A) Effects of AICAR on glucose uptake. Isolated heart muscles were pre-incubated for 30–60 min with 1 mM AICAR before addition of 2-deoxy-[1-^{3}H] glucose for an additional 30 min to measure glucose uptake. (B) Effects of AICAR on change of AMP/ATP ratio. AMP/ATP ratio was assessed using HPLC assay on the supernatants of centrifuged heart muscles incubated for 60 min with or without 1 mM AICAR. Values are means±S.E.M. for three experiments, with two to four independently analyzed muscles per experiment. *P<.05 versus controls.

which is a down-stream target of AMPK. Troglitazone and GW7647 significantly increased AMPK phosphorylation to approximately two- and 2.5-fold within an incubation period of 30 min, respectively (Fig. 2A). Troglitazone and GW7647 also significantly increased ACC phosphorylation (Fig. 2B), suggesting that the increased activity of AMPK by the two agonists resulted in ACC phosphorylation. Total AMPK and ACC amounts did not change when cardiac muscles were stimulated by troglitazone or GW7647. AMPK is an energy-sensing enzyme that can be activated by an increase in the AMP/ATP ratio, and our experiments also revealed an elevated ratio of AMP/ATP when cardiac muscles were activated by troglitazone or GW7647 (Fig. 2C).

3.3. Effects of AICAR on glucose uptake

AICAR acts as an AMPK agonist and stimulates glucose uptake. We investigated AICAR-induced AMPK activation. Incubation with AICAR stimulated heart muscle deoxyglucose uptake (P<.05; Fig. 3A). An elevated AMP/ATP ratio was also observed when cardiac muscles were activated by AICAR (Fig. 3B).

3.4. Effects of compound C on PPARγ and α agonist-stimulated glucose uptake

To examine the extent that AMPK mediates its effect on glucose uptake, we used compound C to inhibit AMPK in cardiac muscles. As described previously, treatment of cardiac muscles with troglitazone (50 μM, 30 min) and GW7647 (10 μM, 30 min) resulted in approximately 2- and 2.5-fold increases in AMPK phosphorylation, respectively (Fig. 2). Western blot analysis of AMPK and ACC revealed that AMPK activation was inhibited by compound C, a selective AMPK

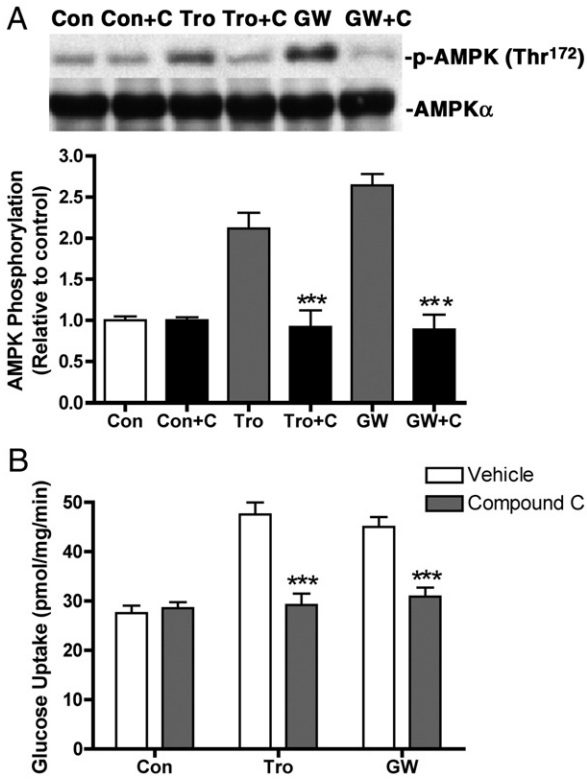

Fig. 4. Effects of compound C inhibition on AMPK activation and glucose uptake induced by troglitazone and GW7647 in cardiac muscles. Papillary muscles were incubated with vehicle or compound C (10 μM) for 30 min before the addition of troglitazone (Tro, 50 μM) or GW7647 (GW, 10 μM) and incubated for 30 min. Western blot analyses were performed for AMPK. 2-Deoxy-[1-^{3}H] glucose was then added to each solution and they were incubated for an additional 30 min to measure glucose uptake. (A) Representative blots and densitometric analysis are shown for each experimental condition for AMPK. (B) Effects of compound C on glucose uptake. Data were compiled from five independent experiments in each condition and presented as means±S.E.M. ***P<.001 compound C versus vehicle controls.

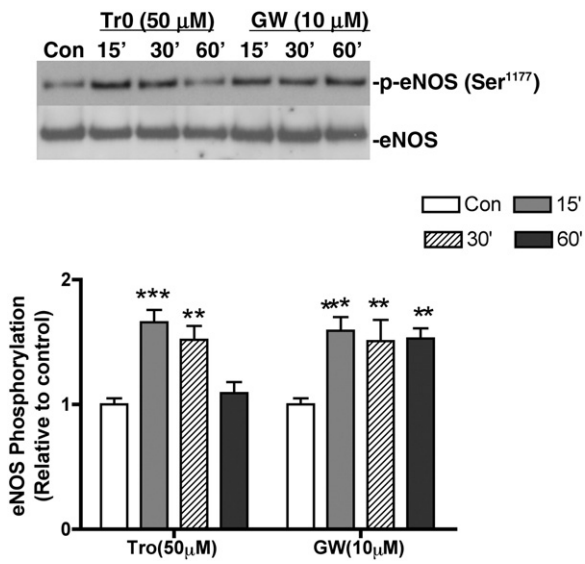

Fig. 5. Effects of troglitazone (Tro, 50 μM) and GW7647 (GW, 10 μM) on eNOS phosphorylation in papillary muscles. Isolated papillary muscles were exposed to 50 μM troglitazone or 10 μM GW7647 for 0, 15, 30 and 60 min. Control (Con) muscles were not exposed to troglitazone or GW7647. Western blot analyses were then performed for eNOS. Data were compiled from 5 independent experiments in each condition and presented as means±S.E.M. **P<.01 versus controls. ***P<.001 versus controls.

inhibitor. Compound C significantly reduced the troglitazone- and GW7647-induced AMPK phosphorylation and had no effect on controls (Fig. 4A). Also interestingly, treatment of cardiac muscles with compound C prevented troglitazone- and GW7647-induced glucose uptake without affecting basal glucose uptake (Fig. 4B).

3.5. Effects of PPARγ and α agonists on eNOS activation

One possible downstream mediator in AMPK role in stimulating glucose uptake is the NO pathway. eNOS is highly expressed in heart capillary endothelial cells. Phosphorylation of eNOS at Ser^{1177} by AMPK is known to increase its enzymatic activity [26]. To examine the extent to which AMPK mediates glucose uptake through activation of the NO signaling pathway in isolated heart muscles, we incubated cardiac muscles with PPARγ or α agonists. Our study showed that troglitazone significantly increased eNOS phosphorylation within an incubation period of 15 min or 30 min; however, phosphorylated eNOS level did not change significantly within a 60-min incubation period. GW7647 significantly increased eNOS phosphorylation when the incubation periods were 15, 30 and 60 min. Total eNOS level did not change in both treated groups (Fig. 5).

3.6. Effects of L-NAME on PPARγ and α agonists stimulated glucose uptake

To determine the extent to which the eNOS pathway might contribute to the stimulation of glucose uptake by PPARγ and α agonists, cardiac muscles were incubated with NOS inhibitor before treatment with troglitazone or GW7647. Western blot analysis of eNOS revealed that eNOS activation was inhibited by addition of L-NAME, a non-selective NOS inhibitor (Fig. 6A). Also, L-NAME significantly inhibited troglitazone- and GW7647-stimulated glucose uptake, however, it had no effect on baseline glucose uptake, suggesting that nitric oxide production is not required to maintain basal glucose uptake in cardiac muscles (Fig. 6B).

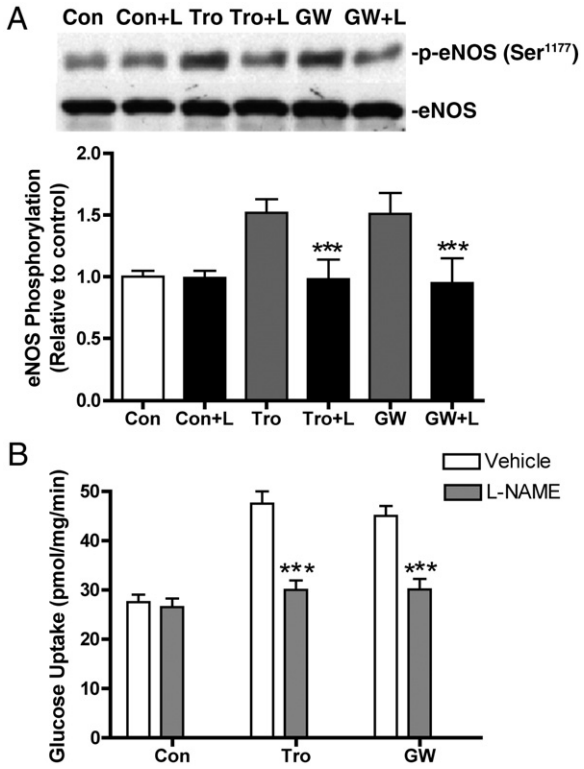

Fig. 6. Effects of L-NAME inhibition on eNOS activation and glucose uptake induced by troglitazone and GW7647 in cardiac muscles. Papillary muscles were incubated with vehicle or L-NAME (0.1 μM) for 30 min before the addition of troglitazone (Tro, 50 μM) or GW7647 (GW, 10 μM) and incubated for 30 min. Western blot analyses were then performed for eNOS. 2-Deoxy-[1-^{3}H] glucose was then added to each solution for an additional 30 min incubation after which glucose uptake was measured. (A) Representative blots and densitometric analysis are shown for each eNOS experimental condition. (B) Effects of L-NAME on glucose uptake. Data were compiled from 5 independent experiments in each condition and presented as means±S.E.M. ***P<.001 L-NAME versus vehicle controls.

4. Discussion

Our results showed that treatment of isolated papillary muscles with troglitazone or GW7647 resulted in a significant increase of AMPK and ACC phosphorylation and activation of the eNOS signaling pathway. Our results are supported by other studies which showed a resulting significant increase in AMPK and ACC phosphorylation when L6 myotubes were treated with troglitazone for 1 h [34]. Other studies using isolated rat muscles have also reported that a 30 min exposure to troglitazone (5–100 μM) resulted in the rapid activation of AMPK [35]. Our HPLC results also revealed an elevated ratio of AMP/ATP when isolated papillary muscles were activated by troglitazone or GW7647. Treatment of isolated papillary muscles with AICAR, an AMPK agonist, led to increased glucose uptake and an elevated AMP/ATP ratio. The increase in glucose uptake possibly resulted from the activation of the AMPK-eNOS signaling pathway. Interestingly, inhibition of AMPK and eNOS activation by their respective inhibitors resulted in the abolishment of the increased glucose uptake induced by troglitazone or GW7647.

It has long been observed that pathologic cardiac hypertrophy and congestive heart failure caused by blood pressure overload are examples of states in which the myocardium switches to predominantly use glucose as the chief energy substrate [36]. We provided evidence to explain the mechanisms by which PPARγ and α agonists promote glucose uptake involving AMPK activation and activation of its downstream target ACC in the heart. AMPK is activated by rising AMP and falling ATP. Once AMPK is activated by low energy, it switches on ATP-producing catabolic pathways (such as fatty acid oxidation and glycolysis) and switches off ATP-consuming anabolic pathways (such as lipogenesis) [37]. AMP activates the system by binding to the γ subunit of AMPK that triggers phosphorylation of the AMPK catalytic α subunit by the upstream kinases and calmodulin-dependent protein kinase kinase. AMPK activation results in increased glucose uptake. It has been shown that the degree of AMPK activation and phosphorylation at Thr^{172} paralleled the extent of ischemia in the left ventricle with low-flow ischemia resulting in increased AMPK phosphorylation and stimulated glucose uptake [38]. This is of interest in light of recent studies showing that AMPK has an important role in the activation of glucose uptake in ischemic hearts. Transgenic mice expressing dominant negative α catalytic subunits of AMPK have reduced glucose uptake response during both ischemia [22] and postischemic reperfusion [23]. AMPK deficiency also blocks hypoxia-stimulated glucose transport in skeletal muscles [21]. These findings may be important in the understanding of the pathogenesis of diseases such as ischemic heart disease, heart failure and diabetes mellitus.

We also observed that troglitazone and GW7647 increased eNOS Ser^{1177} phosphorylation via a PPARγ- [39]and PPARα-dependent pathways [29,40], respectively. eNOS produces NO, and cardiac NO production has been shown in previous studies to play a pivotal role in regulating myocardial glucose uptake [41]. NO also stimulates glucose uptake in isolated skeletal muscle [42]. In our study, NOS inhibition had no effect on basal glucose uptake, indicating that NO may not be required to maintain basal glucose uptake in isolated heart muscles. Previous studies have demonstrated that increased NOS activity in cardiomyocytes and NO exerts cardioprotective effects in human

ventricular heart cells after low-volume anoxia and reoxygenation [43]. Another PPARγ agonist, rosiglitazone, also induces phosphorylation of eNOS which protects cardiac muscles from contractile dysfunction following ischemia/reperfusion [44].

In summary, exposure of isolated papillary muscles to troglitazone and GW7647 significantly increased glucose uptake and activated both AMPK and eNOS signaling. Pharmacological inhibition of AMPK and eNOS in our experiments prevented the effects of troglitazone- and GW7647-stimulated glucose uptake. This study provides evidence that both PPARγ and PPARα activation play a role in the regulation of glucose uptake in cardiac muscles, and this regulation is mediated by AMPK and eNOS signaling pathways. The findings presented here provide additional insight into the role of PPARγ and PPARα in cardiac glucose uptake. There is still a need for more studies to be done to further investigate these pathways.

Acknowledgments

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