Improving type 2 diabetes through a distinct adrenergic signaling pathway involving mTORC2 that mediates glucose uptake in skeletal muscle

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Running title: Improving diabetes by adrenergic mTORC2 activation
Abstract
Type 2 diabetes is an increasing worldwide epidemic that poses major health problems. We have identified a novel physiological system that increases glucose uptake in skeletal muscle but not in white adipocytes. Activation of this system improves glucose tolerance in Goto-Kakizaki rats or mice fed a high fat diet, which are established models for type 2 diabetes. The pathway involves activation of β2-adrenoceptors that increase cAMP levels and activate PKA that phosphorylates mammalian target of rapamycin complex 2 (mTORC2) at S2481. The active mTORC2 causes translocation of GLUT4 to the plasma membrane and glucose uptake without the involvement of Akt or AS160. Stimulation of glucose uptake into skeletal muscle following activation of the sympathetic nervous system is likely to be of high physiological relevance since mTOR complex 2 activation was observed at the cellular, tissue and whole animal level in rodent and human systems. This signaling pathway provides new opportunities for the treatment of type 2 diabetes.
INTRODUCTION

The marked increase in type 2 diabetes worldwide (1) emphasizes the importance of novel treatments that rectify glucose homeostasis. Ideally this involves signaling pathways not dependent on insulin. Although activation of adrenoceptors in vitro has been shown to stimulate glucose uptake in skeletal muscle (2-10), the signaling pathways involved and the potential for treating type 2 diabetes have been unclear. In vivo, sympathetic effects on glucose homeostasis are complex being influenced by glucose outflow from the liver, insulin release from the pancreas, as well as glucose uptake into peripheral tissues such as white fat, brown fat and muscle. Long-term activation of β2-adrenoceptors in rats causes skeletal muscle hypertrophy (11) leading to decreased plasma insulin levels, increased insulin sensitivity and improved glucose tolerance (12, 13) and in man there are beneficial metabolic changes (14, 15) whereas some β-blockers exacerbate diabetes (16). Despite this circumstantial evidence (2-10), the mechanisms involved in adrenergic facilitation of glucose uptake in peripheral tissues and their potential therapeutic role are poorly understood.

Skeletal muscle is the most important organ in the body for glucose clearance and utilization (17). High sympathetic nervous system activity (e.g. exercise, fight or flight) is associated with enhanced glucose uptake into skeletal muscle, resulting primarily from norepinephrine release from adrenergic nerve terminals (18). Catecholamines released during sympathetic activation stimulate adrenoceptors at the cell surface. Mechanisms proposed to explain adrenergically-mediated glucose uptake include stimulation of transcription and translation of proteins, non-carrier mediated and unspecific effects and translocation or activation of glucose transporters (GLUTs) (10, 19, 20), emphasising the lack of a unifying concept for the mechanisms involved. Previously we showed that β-adrenoceptors profoundly affect glucose uptake by an AMPK-independent pathway (3) that is not secondary to lowering of ATP levels or energy deficiency since activation also increases glycogen formation in L6 skeletal muscle cells (4, 21). Based on
these studies and others (22), we have examined the mechanism utilized by β-adrenoceptors to increase glucose uptake in skeletal muscle and whether activation of this pathway could improve type 2 diabetes.

We describe here for the first time that specific activation of β2-adrenoceptors in skeletal muscle, but not white adipocytes, causes translocation of GLUT4 to increase glucose uptake, by a mechanism dependent on mTORC2. Stimulation of this pathway improves glucose tolerance in rat and mouse models of type 2 diabetes. These studies reveal a physiological pathway and a novel mechanism that challenges accepted views of the role of GPCR signaling in glucose metabolism. Furthermore, it identifies novel targets that could be exploited for the treatment of type 2 diabetes.
Research Design and Methods

Animals

129sv mice (Charles River Laboratories, USA) were bred at the Stockholm University animal facility. β1/β2-KO mice (Jackson Laboratory) (23) were of mixed background (129X1/SvJ,C57BL/6J, DBA/2 and FVB/N). Male C57BL/6J mice (45-57 grams on high fat diet for 6-months were provided by Dr. Natasa Petrovic; Stockholm University, Sweden. The high fat diet contained 45 % fat, 35 % carbohydrates and 20 % proteins (Research Diets (cat no. D12451)). Sprague-Dawley rats (male) (Scanbur (Sweden)) and 12-month old male Goto-Kakizaki rats (260-390 grams) were provided by Prof. Gustav Dallner (Stockholm University, Sweden).

Ethical approval

All studies were approved by the North Stockholm Animal Ethics committee (permission N123/09, N69/12, N51/12). Animals were sacrificed by CO2 under anesthesia.

Glucose tolerance test in Goto-Kakizaki rats and high fat diet fed C57BL/6J mice

The glucose tolerance test (24) was performed by intraperitoneal injection of glucose (2g/kg) (Riedel-de Haën AG, Germany) in fasted (6 h) GK-rats or high fat diet fed C57BL/6J mice. Blood glucose was measured in a drop of blood from the tail before and following 15, 30, 60, 90 and 120 minutes of glucose administration. Animals were treated with clenbuterol (Sigma) 30 mg/L in the drinking water for 4 days and the glucose tolerance test performed on the 5th day.

In Vivo Glucose Uptake

In vivo glucose uptake was performed according to the methods from Liu and Stock (5) with minor modifications. Wild-type (WT) 129sv mice or β1/β2 KO mice were fasted for 5h and anesthetized with pentobarbital (60 mg/kg i.p.). Mice were injected with KU0063794 (10 mg/kg) in DMSO or vehicle 10 min before treatment with insulin (1 mg/kg), isoproterenol (1 mg/kg) or saline. After 20 min, 130 μCi/kg
of \(^{3}\text{H}\)-2DG (Perkin Elmer, Waltham MA USA; 8 Ci/mmol) was injected i.p. Animals were sacrificed 1h later by CO\(_2\), and skeletal muscle (quadriceps) dissected, lysed in 0.5M NaOH and radioactivity measured by liquid scintillation counting.

**Glucose uptake in white adipocytes**

Epididymal white fat depots were isolated (25) from Sprague-Dawley rats and digested in buffer (HEPES with 0.2% collagenase, 0.9g L\(^{-1}\) glucose and 1.5% bovine serum albumin), at 37\(^{0}\)C with gentle shaking for 30 minutes, and filtered (nylon mesh 250\(\mu\)m). Mature adipocytes were collected from the top of the suspension. Cells were washed with DMEM containing 3% BSA. Mature adipocytes were incubated with agonists in glucose-free DMEM containing 3% BSA for up to 1h at 37\(^{0}\)C. After incubation, \(^{3}\text{H}\)-2-deoxy-D-glucose (50nM) was added to the cells for a further 60 seconds at 37\(^{0}\)C. Cells were then separated from the incubation medium by centrifugation though a layer of silicone oil, and frozen at -20\(^{0}\)C (26). Frozen cell cakes were removed and transferred to scintillation vials for counting.

**Glucose Uptake in Soleus Muscle ex vivo**

Soleus muscle was dissected from Sprague-Dawley rats and suspended with hooks stretching the muscle very gently in water jacketed organ baths containing 30 ml Krebs-Henseleit bicarbonate (KHB) buffer (118.5mM NaCl, 4.7mM KCl, 1.2mM KH\(_2\)PO\(_4\), 25mM NaHCO\(_3\), 2.5mM CaCl\(_2\), 1.2mM MgSO\(_4\), 5mM HEPES pH 7.4) containing 5mM glucose and 15mM mannitol, bubbled with 95% O\(_2\)/5% CO\(_2\) and maintained at 37\(^{0}\)C. Tissues were equilibrated for 5 min before KU0063794 (100nM) or vehicle were added for 30 min, followed by either insulin (100nM) or isoproterenol (100nM) for 1h. Muscles were rinsed with KHB (containing 20mM mannitol) for 10 min, then incubated in KHB (containing 8 mM 3-O-methylglucose, 12 mM mannitol, 438\(\mu\)Ci/mmoll 3-O-methyl\(^{3}\text{H}\)glucose (80.2 Ci/mmoll; PerkinElmer) and 42 \(\mu\)Ci/mmoll \(^{14}\text{C}\) mannitol (58.8 mCi/mmoll; PerkinElmer) for 12 min. Muscles were rinsed with PBS, frozen in liquid nitrogen, weighed and dissolved in 1 mL of 0.5M NaOH at 60\(^{0}\)C. \(^{3}\text{H}\) and \(^{14}\text{C}\)
radioactivity was measured by liquid scintillation counting. Total muscle 3-O-methylglucose and extracellular space were measured as described previously (23) and expressed as a rate of 3-O-methylglucose transport per mL of intracellular water per hour.

**Cell culture**

L6 cells (ATCC; Manassas, VA USA) or L6-GLUT4myc cells (Prof. Amira Klip, Hospital for Sick Children, Toronto, Canada) were grown in DMEM supplemented with 4mM L-glutamine, 10% FBS, 100 Units/ml penicillin, 100µg/ml streptomycin and 10mM HEPES, in a 37°C incubator with 8% CO₂. Differentiation was induced by growing cells to ~90% confluence and lowering of FBS to 2% for 7 days.

Human primary skeletal muscle cells (SKMC) were purchased from Karocell AB (Stockholm, Sweden), Lonza (Basel, Switzerland) and Promocell GmbH (Heidelberg, Germany) and grown in Hams F-10 containing 20% heat-inactivated FBS, 2mM L-glutamine, 50U/ml penicillin and 50µg/ml streptomycin. Differentiation was initiated by growing cells to ~90% confluence and reducing FBS to 4% for 3 days then 2% for 4 days. Prior to experiments, cells were kept in serum-free medium overnight. All cell media was purchased from Sigma-Aldrich (St Louis MO, USA).

**Glucose Uptake in L6 and SKMC Cells**

Cells were incubated overnight in serum-free medium (0.5% BSA) and [3H]-2-deoxy-D-glucose (50 nM, 8 Ci/mmol, Perkin Elmer) uptake measured as previously described (6). Cells were pretreated with inhibitors for 30 min and then exposed to insulin, isoproterenol, or 8-Br-cAMP for 2h unless otherwise stated. Radioactivity was detected by liquid scintillation counting.

**Q-PCR**
L6 cells were harvested in 1mL Ultraspec\textsuperscript{TN} RNA isolation reagent (Biotecx Laboratories Inc. Houston, TX, USA) and RNA isolated according to the manufacturer’s instructions. Total RNA was dissolved in DEPC-water (Invitrogen, Carlsbad, CA, USA) and quantified in a DU-50 Beckman (Fullerson, CA, USA) spectrophotometer. Total RNA was reverse transcribed (Applied Biosystem, Carlsbad, CA, USA) using random hexamers for first-strand cDNA synthesis. Primers for GLUT1 (f:AGAACCGGGGCAAGAGT, r:GAACAGCTCCAAGATGGTGAC), GLUT4 (f:TTGCAGTGCTGCTGCTGA), and TFIIB (f:GGTCTGCTCCAACCTTGTGCT, r:CCAGTCACCTCGCTGCTGA) as endogenous control were designed by Universal Probe Library (Roche Applied Science, Penzberg, Germany) and purchased from Invitrogen. SYBR\textsuperscript{®} GREEN PCR Master Mix (Applied Biosystems) was mixed with reference dye and primers, and loaded onto 96-well MicroAmo plates (Applied Biosystems). 2\textmu l template cDNA was added in duplicate. TFIIB was used as a control.

**Immunoblotting**

L6 myotubes were used in all immunoblotting experiments. Cells were serum-starved overnight before experiment. Immunoblotting was performed as previously described (27, 28). Primary antibodies (Akt, phospho-Akt T308, phospho-Akt S473, phospho-AS160 T642, phospho-TBC1D1 (Thr590), GLUT4, GLUT1, Myc, phospho-mTOR S2448, phospho-mTOR S2481, phospho-p70S6K, raptor, and rictor diluted 1:1000) were all from Cell Signaling (Danvers, MA, USA) and detected using a secondary antibody (horseradish peroxidase-linked anti-rabbit IgG, Cell signaling) diluted 1:2000 and measured using enhanced chemiluminescence (ECL, Amersham Biosciences).

**Immunocytochemistry and immunofluorescence**

L6 or SKMC cells were grown in 4- or 8-well culture chamber glass bottom slides (BD Biosciences, Franklin Lakes, BJ, USA), treated with drugs, washed in DMEM, fixed with 4% formaldehyde in PBS for
5 min, quenched with 50mM glycine in PBS for 10min, blocked with 5% BSA in PBS for 1h and incubated with GLUT4 (Santa Cruz Biotech) or Myc-tag (Cell signaling) (1:200 dilution in 1.5% BSA in PBS) overnight at 4°C. Cells were washed in PBS and incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG or Alexa Fluor 555-conjugated goat anti-rabbit IgG or Alexa Fluor 488-conjugated rabbit anti-goat IgG secondary antibody (1:500 dilution, 1.5% BSA in PBS) for 1h. PIP3 was visualized in permeabilised L6 cells (0.5% Triton X-100 in water, 15 min) and blocked with 10% rat serum in TBS overnight at 4°C. Cells were incubated for 1h at 37°C with mouse anti-PIP3 conjugated to a biotinylated goat anti-mouse IgM (diluted 1:100), washed three times (10% rat serum in TBS) and secondary antibody added (streptavidin-AlexaFluor 488 1:2000 in TBS, 30min, 37°C). Slides were mounted in ProLong Gold antifade reagent (Invitrogen) and images observed in an inverted laser-scanning microscope (LSM 510META; Carl Zeiss, Advanced Imaging Microscopy, Jena, Germany). Laser power, offset and gain including other conditions were the same for each experiment. Stained cells were quantified using Image J (NIH).

**Transient Transfection of siRNA**

L6 cells were tranfected with GLUT4mycGFP (constructs from Prof. Jeffrey E. Pessin at the Albert Einstein College of Medicine, New York, NY, USA) or siRNA (Qiagen, Hilden, Germany) against rat GLUT4, rictor or raptor. Scrambled siRNAs were used as control. Myotubes or myoblasts were detached using trypsin/EDTA, transferred to Eppendorf tubes and centrifuged at 1000×g for 3 min, resuspended in 20 µl SE Cell Line Nucleofector solution for L6 cells and P1 primary Nucleofector solution for SKMC cells (Lonza, Basel, Swiss) and 100 pmol of siRNA (L6 myotubes) or 0.8 µg GLUT4mycGFP construct (L6 myoblasts and human SKMC) added. L6 cells were nucleofected by using the DS-137 program and SKMC cells with the DS-138 program in the Lonza 4D-nucleofector. Cells were electroporated in 16-well microcuvette plates (Lonza), pre-warmed RPMI1640 added (80µl), and then transferred to 1.5 ml microcentrifuge tubes or glass bottom slides (BD Biosciences) containing DMEM with 10% FBS for 8h.
Medium was changed to serum-free medium (0.1% BSA) and cells incubated overnight. The transfection did not affect cell viability assessed by trypan blue staining (data not shown). Since myotubes do not easily re-attach, these cells were kept in suspension in microcentrifuge tubes, while myoblasts (used for confocal experiments) were seeded on slides. 24h after transfection, glucose uptake, immunohistochemistry or western blotting was performed.

**Glucose uptake in cells in suspension**

On the day of the experiment, transfected cells were stimulated with insulin (1 µM) or isoproterenol (1 µM) for 2h in microcentrifuge tubes at 37°C. Cells were centrifuged and pelleted (1000xg, 2 min) and media carefully removed and changed to glucose free media and insulin and isoproterenol re-added. After incubation for 15 min, 50 nM 2-deoxy-D-[1-³H] glucose (8 Ci/mmol) was added for 10 min. 2-deoxy-D-[1-³H] glucose uptake was terminated by washing with ice-cold PBS twice with centrifugation (1000xg, 30sec), and 400 µl of 0.2 M NaOH was added to lyse the cells. Radioactivity was measured by liquid scintillation counting.

**Statistical Analysis**

Experiments were performed in duplicate, and the results expressed as mean ± s.e.m. Statistical significance of differences between groups was analysed by Student's paired two-tailed t-test or for >2 groups, one- or two-way ANOVA was used (*P<0.05, **P<0.01, ***P<0.001). Data is presented as the increase expressed as % of basal if not otherwise stated.
RESULTS

\( \beta_2 \)-Adrenoceptor activation increases glucose uptake in skeletal muscle \textit{in vitro} and \textit{in vivo}

Goto-Kakizaki (GK) rats and high-fat diet fed C57BL/6J mice are two established models of type 2 diabetes (Fig 1A, 1B) that were treated with the \( \beta_2 \)-adrenoceptor agonist clenbuterol (30mg/L in the drinking water, 4 days) or normal drinking water, prior to a glucose tolerance test. Clenbuterol-treated GK rats had greatly improved glucose tolerance after 60, 90 and 120 minutes and C57BL/6J mice after 30 and 60 minutes of glucose administration compared to untreated animals (Fig 1A, 1B) demonstrating that \( \beta_2 \)-adrenoceptor stimulation greatly improves glucose tolerance in two different models of type 2 diabetes. Clenbuterol, like isoproterenol and insulin also increased glucose uptake in L6 myotubes (Fig S1A). We then investigated whether this effect results from actions on glucose uptake in tissues such as white fat and skeletal muscle. The general \( \beta \)-adrenoceptor agonist isoproterenol was used to rule out effects on \( \alpha_1 \)-adrenoceptors which increase glucose uptake via an AMPK-mediated mechanism (3). Isoproterenol increased glucose uptake \textit{in vivo} in wild-type mouse skeletal muscle to a greater extent than insulin, and this effect was absent in \( \beta_1/\beta_2 \)-adrenoceptor KO mice (Fig. 1C) indicating that the effect of isoproterenol is mediated by \( \beta \)-adrenoceptors (almost certainly \( \beta_2 \)-adrenoceptors, see discussion). Glucose uptake was also stimulated by isoproterenol \textit{ex-vivo} in intact rat soleus muscle (Fig. 1D) but not in rat isolated white adipocytes showing that the pathway is present in skeletal muscle but not white adipocytes (Fig. 1E)

The potential relevance of these findings to humans was tested in human primary skeletal muscle cells (SKMC) where isoproterenol stimulation increased glucose uptake to a greater extent than insulin (Fig. 1F). L6 skeletal muscle cells were used to further investigate the mechanism of \( \beta_2 \)-adrenoceptor mediated glucose uptake. Isoproterenol increased glucose uptake in both L6 myoblasts and myotubes (Fig. 1G, 1H) with the effect in myotubes being more than twice that in myoblasts (41.5 vs 18 fmol per well). The effect
of isoproterenol was concentration-dependent and displayed the same efficacy and potency as insulin (Fig. S1B), with the effect measureable by 30 min and peaking by 2 h (Fig. S1C).

**β₂-Adrenoceptors stimulate glucose uptake independently of major components of the insulin signaling pathway**

Insulin-mediated glucose uptake occurs by activation of a well-characterized signaling pathway involving PI3K, Akt and AS160 (29). To investigate whether β₂-adrenoceptor-mediated glucose uptake requires PI3K, we used the PI3K-PIKK inhibitor PI-103. A role for PI3K or related kinases was suggested by inhibition of both insulin- and isoproterenol-stimulated glucose uptake by PI-103 (IC₅₀ 0.7 µM, Fig 2A). However, Akt, a downstream target of PI3K activation, was clearly not phosphorylated at T308 or S473 following β₂-adrenoceptor stimulation (in contrast to insulin that increased Akt phosphorylation at both sites at all time points examined) (Fig 2B). The Akt inhibitor X (10µM) or SH-6 (1µM) that prevent Akt activation by interfering with its phosphatidyl-binding domain (31, 32) both inhibited insulin-stimulated glucose uptake, Akt phosphorylation and AS-160 phosphorylation, but had no effect on β₂-adrenoceptor-mediated glucose uptake (Fig 2C,D,E,S2A,S2B). TBCD1 phosphorylation at Thr590 was increased only following insulin but not isoproterenol stimulation (Fig S2C). Insulin but not isoproterenol increased PIP₃ content and this effect was inhibited by LY294002 (Fig 2F). These results show that β₂-adrenoceptor-stimulated glucose uptake does not utilize major components of the insulin pathway.

**β-Adrenoceptor-stimulated glucose uptake involves cAMP and mTOR**

To further investigate β-adrenoceptor-mediated glucose uptake, we used cAMP analogues since β₂-adrenoceptor activation increased cAMP levels in skeletal muscle cells (Fig. S2D). Glucose uptake was increased by the cell-permeable cAMP analogues, 8-bromoadenosine 3′,5′-cAMP (8-Br-cAMP, 1mM), N6,2′-O-dibutyryladenosine 3′,5′-cAMP (db-cAMP, 1mM), and cAMP (2mM), whereas the cell-impermeable analogue 8- Hydroxyadenosine- 3′, 5′-cAMP (8-OH, 1mM) had no effect (Fig. 3A). This
suggested that increases in cAMP levels were associated with increased glucose uptake in L6 cells. Since the PI3K inhibitor-PI-103 also inhibits other PIKK family kinases (33, 34), including mTOR, we examined more selective inhibitors. The mTOR inhibitors Torin-1 and KU0063794 (KU) blocked β2-adrenoceptor and cAMP-mediated glucose-uptake in L6 myotubes (Fig. 3B, 3C). KU also inhibited isoproterenol-mediated glucose uptake in intact rat soleus muscle \textit{ex vivo} and in mouse skeletal muscle \textit{in vivo} (Fig. 3D, 3E).

**mTORC2 is obligatory for β2-adrenoceptor-stimulated glucose uptake**

mTOR exists as two complexes, mTORC1 that is associated with raptor and phosphorylated at S2448, and mTORC2 that is associated with rictor and phosphorylated at S2481, with these two phosphorylation sites reflecting mTOR activity (35). In L6 cells, isoproterenol increased phosphorylation only at S2481 in a time dependent manner (Fig. S3A), whereas insulin phosphorylated mTOR at both S2448 and S2481. While S2448 was phosphorylated within 5 min of insulin stimulation, isoproterenol did not affect phosphorylation at this site at any time point examined (Fig. S3A). mTOR phosphorylation in response to isoproterenol or insulin was blocked by KU (Fig. 4A). A downstream target of mTORC1 (p70S6K) was phosphorylated slightly after isoproterenol, although to a lesser extent than insulin (Fig S3B). Akt inhibitor X decreased insulin-induced phosphorylation of mTOR at S2448, but not isoproterenol- or insulin-induced phosphorylation of mTOR at S2481 (Fig. S3C) indicating that Akt is not upstream of mTOR S2481. 8-Br-cAMP (1mM) or the PKA-selective cAMP-analogue 6-Benz-cAMP (1mM) also caused phosphorylation of mTOR at S2481 (Fig. 4A, 4B), mimicking the effect of isoproterenol and indicating that this site is regulated by the cAMP-PKA pathway. The PKA inhibitor 14-22 (PKI) partially blocked the isoproterenol effect and completely blocked the effect of 8-Br-cAMP on S2481, while not affecting insulin-mediated S2481 phosphorylation (Fig 4C). Since mTOR is a key factor in the β2-adrenoceptor pathway, we examined the complex involved. mTORC1 contains the regulatory-associated-protein of mTOR (Raptor), while mTORC2 contains the rapamycin-insensitive-companion of mTOR.
(Rictor). Short-term (30min) treatment with the mTORC1 inhibitor rapamycin had no effect on isoproterenol and 8-Br-cAMP-stimulated glucose uptake (Fig. 4D) but long-term exposure (24h), that inhibits mTORC2 assembly, inhibited isoproterenol-stimulated glucose uptake, suggesting involvement of mTORC2 (Fig. 4E). Rictor siRNA treatment markedly depleted Rictor (Fig. 4F) and abolished isoproterenol-stimulated glucose-uptake (Fig. 4G), whereas Raptor siRNA or control siRNAs had no effect. This clearly demonstrated that mTORC2 is a key factor involved in β₂-adrenoceptor-mediated glucose uptake in skeletal muscle.

**β₂-Adrenoceptor activation increases glucose uptake independently of transcription and translation**

Glucose uptake can be increased by *de novo* synthesis of GLUTs associated with increases in transcription and/or translation. The general transcription inhibitor actinomycin D (2µg/ml) did not significantly affect basal glucose uptake or cell viability (not shown), or insulin- or isoproterenol-mediated glucose uptake at 2h (Fig 5A), indicating that changes in transcription are not involved. Accordingly, GLUT1 and GLUT4 mRNA levels were not changed by isoproterenol (1µM) (Fig 5B, 5C). The general translational inhibitor cycloheximide (50µM) did not affect basal glucose uptake or cell viability, or either insulin- or isoproterenol-mediated glucose uptake at 2h (Fig 5D). Neither GLUT1 nor GLUT4 protein levels were altered by isoproterenol (Fig 5E, 5F) suggesting that β₂-adrenoceptor-stimulated glucose uptake is not dependent upon *de-novo* GLUT1 and GLUT4 synthesis at time-points up to 2h.

**β₂-Adrenoceptor-stimulated glucose uptake depends on GLUT4**

We next examined whether β₂-adrenoceptor-stimulated glucose uptake involved GLUTs. Both GLUT inhibitors, cytochalasin B (10µM) and phloretin (100µM) (36), reduced basal glucose uptake (Fig. S4A) and inhibited glucose uptake to isoproterenol and insulin (Fig. S4B, S4C), suggesting that GLUTs were involved. The selective GLUT4 inhibitor indinavir (100µM), blocked basal glucose uptake by ~40% (Fig.
S4A) consistent with other studies in L6 myotubes (37) and markedly reduced both β2-adrenoceptor and insulin-stimulated glucose uptake, both after 45 and 120 min stimulation, (Fig. S4D, S4E) suggesting a role for GLUT4.

To confirm that β2-adrenoceptor-stimulated glucose uptake was carrier-mediated, kinetic studies were performed with increasing concentrations of 2-deoxyglucose (38). Insulin increased glucose uptake (Fig. 5G) with $K_m$ values (Eadie-Hofstee plot) in agreement with previously reported values (10, 39). β2-Adrenoceptor-stimulation increased glucose uptake $V_{\text{max}}$ following classical saturable Michaelis–Menten kinetics with a $K_m$ value (2.5 ± 0.3mM) similar to insulin (Fig. S4F) suggesting that β2-adrenoceptor-stimulated glucose uptake involves transporter proteins (40). To confirm the involvement of GLUT4, siRNA directed against GLUT4 was utilized and markedly reduced β2-adrenoceptor mediated glucose uptake compared to a scrambled siRNA (Fig. 5H). Thus, GLUT4 is the transporter responsible for β2-adrenoceptor-mediated glucose uptake. Since GLUT4 is involved, we investigated the role of actin and cytoskeletal rearrangement using latrunculin B (Fig. 5I), which inhibited both insulin and isoproterenol-mediated glucose uptake, indicating that actin polymerization involved, and suggesting a role for GLUT4 translocation.

**Direct demonstration of GLUT4 translocation following β2-adrenoceptor-stimulated glucose uptake**

Direct visualization of GLUT4 translocation to the plasma membrane was examined by immunohistochemistry in non-permeabilized L6 cells and was observed following β2-adrenoceptor stimulation in native L6 myoblasts, L6 myoblasts transiently transfected with GLUT4mycGFP or stably transfected with GLUT4myc (Fig. 6A, 6B and S5A). This was confirmed in L6 myotubes and in stably transfected GLUT4myc L6 myotubes (Fig. S5B, S5C). β-Adrenoceptor stimulation also increased GLUT4 translocation in human native primary skeletal muscle cells (SKMC) (Fig. 6C) and in SKMC transiently transfected with GLUT4mycGFP (Fig. 6D). GLUT4 translocation to isoproterenol was
abolished by mTOR inhibition (Fig. 6E). β-Adrenoceptor-stimulated glucose uptake is therefore dependent on mTORC2 and on GLUT4 translocation.
DISCUSSION

There is increasing recognition that glucose homeostasis can be regulated independently of insulin receptors by activation of GPCRs. Typical GPCRs such as adrenoceptors facilitate glucose uptake in skeletal muscle and we have demonstrated that activation of $\alpha_1$ and $\beta_2$-adrenoceptors in skeletal muscle increases glucose uptake although the mechanisms are not fully understood (6, 7). This has led us to determine the signaling pathways utilized by $\beta_2$-adrenoceptors in skeletal muscle to increase glucose uptake since their activation improves glucose tolerance in diabetic GK-rats and obese C57BL/6J mice (Fig 1). Although this could result from a variety of mechanisms in different tissues our combined results suggest that the major part of the improvement emanates from $\beta_2$-adrenoceptor activation of glucose uptake in skeletal muscle (with $\beta$-adrenoceptors not increasing glucose uptake in mature white adipocytes). We suggest that sympathetic stimulation shunts glucose from liver to skeletal muscle to provide energy for muscular action rather than for storage in white fat. We believe that this event has high physiological and pathophysiological importance given the major role of skeletal muscle in glucose uptake.

We ruled out a role for $\beta_1$- or $\beta_3$-adrenoceptors as the in vivo effect of $\beta$-adrenergic agonists on glucose uptake in skeletal muscle is absent in $\beta_1/\beta_2$-KO mice and the selective $\beta_2$-adrenoceptor agonist clenbuterol displayed the same efficacy as isoproterenol in L6 muscle cells. Previous studies show that $\beta_2$-adrenoceptors are the primary target for catecholamines in skeletal muscle (6, 41) and studies in L6 cells demonstrate that $\beta$-adrenoceptor agonist effects are mediated by $\beta_2$-adrenoceptors (6), clearly suggesting that the effects of $\beta$-adrenergic agonists on glucose uptake are mediated primarily by $\beta_2$-adrenoceptors.

We show here that $\beta_2$-adrenoceptor agonists acutely stimulate glucose uptake with similar efficacy, potency and time course to insulin in L6 myotubes (Fig. S1B, S1C). Initially our assumption was that $\beta$-adrenoceptors increase glucose uptake by pathways similar to those utilized by insulin, since responses
were blocked by PI3K inhibitors. However, unlike insulin, β2-adrenoceptor stimulation failed to
phosphorylate Akt at either Ser473 or Thr308 at all time points examined, selective Akt inhibitors failed
to inhibit β2-adrenoceptor mediated glucose uptake and β2-adrenoceptor stimulation failed to increase
PIP3 levels in L6 cells suggesting that PI3K was not involved in the β-adrenoceptor pathway.
Examination of the selectivity of these inhibitors shows that they also inhibit other PIKK family kinases
(33, 34) including mTOR that also plays an important role in insulin signaling (Fig. 7). Specific inhibition
of mTOR by KU0063794 confirmed its role in insulin signaling but also revealed it was necessary for β-
adrenoceptor-stimulated glucose uptake. However, the pattern of mTOR phosphorylation in response to
β-adrenoceptor stimulation differed from that of insulin, with phosphorylation only at S2481, whereas
insulin caused phosphorylation at both S2448 and S2481. Phosphorylation at S2481 is associated with
mTORC2 whereas phosphorylation at S2448 is associated with mTORC1 (35) suggesting that insulin
regulates both mTOR complexes, whereas β-adrenoceptor activation regulates only mTORC2. Treatment
with rapamycin and siRNA knock down of raptor or rictor reveals the involvement of mTORC1 and
mTORC2, and we show that knock down of rictor but not raptor markedly inhibits both insulin and β-
adrenoceptor mediated glucose uptake, confirming mTORC2 as a key regulator of glucose uptake in
skeletal muscle. Thus, although the β-adrenoceptor pathway differs from the insulin pathway at the level
of Akt and PIP3, both pathways involve mTORC2. The involvement of mTOR was confirmed ex vivo
and in vivo where KU0063794 inhibited both insulin and β-adrenoceptor stimulated glucose uptake.
Recent studies indicate a role of mTORC2 (rictor) in glucose homeostasis, as either skeletal muscle or
adipose-specific ablation of rictor depresses insulin-stimulated glucose uptake in skeletal muscle (42) or
adipose tissue (43) respectively, and in both mice models impairs glucose tolerance in vivo.

The regulation and cross talk between insulin, mTORC1 (raptor), mTORC2 (rictor) and Akt is complex
(Fig. 7). Insulin mediates increases in PI3K activity, phosphatidylinositol (3,4,5)-trisphosphate (PIP3)
recruits inactive Akt and phosphoinositide-dependent kinase-1 (PDK1) to the plasma membrane via their
N-terminal PH domain, allowing Akt phosphorylation at Thr308 by PDK1, and in parallel PI3K phosphorylates mTORC2 at Ser2481. The subsequent conformational change in Akt allows mTORC2 to phosphorylate Akt at Ser473, thereby fully activating Akt, resulting in mTORC1 phosphorylation at Ser2448 and subsequent AS160 and GLUT4 translocation. In contrast, β2-adrenoceptors activate mTORC2 in a PI3K-Akt independent manner. Since β2-adrenoceptors fail to activate PI3K (observed as no increases in PIP3 levels), PDK1 and Akt cannot, as discussed for insulin above, be recruited to the plasma membrane. This prevents phosphorylation of Akt at Thr308 that is absolutely required for mTORC2 mediated phosphorylation of Akt at Ser473, and subsequent phosphorylation of AS160. Our results demonstrate that β2-adrenoceptor activation leads to phosphorylation of mTOR at Ser2481 via a PKA mediated mechanism that is independent of PI3K-Akt (Fig. 7).

This is the first demonstration that GPCRs can specifically stimulate mTORC2 in mammalian cells. The only other evidence that GPCRs can activate mTORC2 is found in the soil-living amoeba dictyostelium discoideum, where chemotactic activation of GPCRs and mTORC2 (44-46) occurs suggesting that GPCR stimulation of mTORC2 could be of ancient origin.

There is however some evidence that other GPCRs can activate mTORC1 independently of Akt/PI3K, including the Gaq/11-coupled α1-adrenoceptors in cardiomyocytes (47), the Gaq/11-coupled prostaglandin F2α receptor and Gas-coupled luteinizing hormone receptor in the corpus luteum (48). There has been great attention focused on the regulation and downstream targets of mTORC1 that can be pharmacologically inhibited by rapamycin. mTORC1 regulates protein translation and synthesis by activating proteins such as p70-S6 kinase (p70S6K). While our study shows, both pharmacologically and by gene knockdown, that β2-adrenoceptors do not utilize mTORC1 for glucose uptake they may activate mTORC1 to some degree (as measured as phosphorylation of p70 S6K). The activation of mTORC1
might reflect the long-term hypertrophic effects of β2-adrenergic agonists on skeletal muscle that was not a focus of this study.

Compared to mTORC1, little is known of downstream targets of mTORC2, but they do include pathways involved in actin cytoskeleton organization. There is evidence for a Rac-dependent (Akt-independent) arm in skeletal muscle that regulates cytoskeletal rearrangement involved in insulin signaling to glucose uptake (49, 50). Such pathways should be investigated further in connection with mTORC2 and adrenergic signaling. Both β2-adrenoceptor (51) and mTORC2 activation cause acute actin cytoskeleton reorganization (52) and inhibition of this process by Latrunculin B blocks β2-adrenoceptor stimulated glucose uptake. Taken together our findings identify a pathway involving activation of mTORC2 by a mammalian GPCR, independently of PI3K/Akt and that mTORC2 is an important master regulator of adrenergic mediated glucose uptake, likely through cytoskeletal rearrangement.

Glucose uptake in skeletal muscle is the rate-limiting step for whole body glucose metabolism (17). Glucose uptake in response to β-adrenoceptor stimulation has similar efficacy to insulin, is saturable and is blocked by GLUT inhibitors and by pretreatment with GLUT4 siRNA. These results show for the first time that GLUT4 is required for β-adrenoceptor stimulated glucose uptake and occurs independently of changes in transcription or translation (again suggesting that mTORC1 is not involved), and involves translocation of GLUT4 to the plasma membrane, a process generally considered to be predominantly insulin-regulated. Thus the adrenergic pathway utilizes two crucial components of the insulin pathway: mTORC2 and GLUT4. However, in contrast to insulin signaling, β2-adrenoceptor activation does not lead to activation of mTORC1, Akt or the downstream phosphorylation of the Rab-GTPase-activating protein AS160.
Glucose uptake in type 2 diabetes is associated with defects in PI3K activity, insulin receptor tyrosine, IRS and Akt phosphorylation, causing impairment of GLUT4 trafficking and glucose uptake. Thus there is normally no defect per se with GLUT4 trafficking, rather defects in the insulin signaling mechanisms regulating GLUT4 trafficking (53) and these defects are bypassed by the β2-adrenoceptor-mTORC2 pathway. This was illustrated in two different diabetic muscle models in vivo where β2-adrenoceptor agonists improved glucose tolerance. However, current β2-adrenoceptor agonists normally display a number of side effects (including myocardial hypertrophy (54)), making them unsuitable for the treatment of type 2 diabetes. Insights obtained from the β2-adrenoceptor mediated pathway to mTORC2 and glucose uptake opens up new avenues in the search for novel approaches and drugs to treat type 2 diabetes.

In conclusion, we describe a novel physiological pathway involving β2-adrenoceptors, PKA, mTORC2 and GLUT4 that promotes glucose uptake in both healthy and type 2 diabetic skeletal muscle. This study reveals new and exciting approaches for our understanding of the role of adrenergic mechanisms in metabolic health and disease.
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FIGURE. LEGENDS

**Fig. 1.** Activation of β-adrenoceptors improves glucose tolerance in diabetic animals and increases glucose uptake *in vivo, ex vivo and in vitro.*

(A) In Goto-Kakizaki (GK) rats, a model of type 2 diabetes, clenbuterol (30 mg/L) administration in the drinking water for 4 days reduced blood glucose levels at 60, 90 and 120 minutes following glucose (2g/kg, i.p.) compared to untreated GK-rats (n=4). (B) C57BL/6J mice were high fat diet-induced type 2 diabetic and treated with clenbuterol (30mg/L) in the drinking water for 4 days which reduced blood glucose levels at 30 and 60 minutes following glucose (2g/kg, i.p.) compared to untreated mice (n=5). (C) The effects of isoproterenol (1mg/kg) and insulin (1mg/kg) on glucose uptake *in vivo* in mouse WT and β₁/β₂-AR KO skeletal muscle, (n=4). (D) Isoproterenol (100nM, 2 h) and insulin (100nM, 2 h) both increased glucose uptake in rat isolated soleus muscle *ex vivo* (n=3); whereas (E) treatment with insulin (1µM, 1h) but not isoproterenol (1µM, 1 h) significantly increased glucose uptake in mature adipocytes (n=7) (F) Isoproterenol (1µM, 2 h) or insulin (1µM, 2h) increased glucose uptake in human SKMC cells (n=5) and the same treatments increased 2-deoxy-³H-glucose uptake in L6 myoblasts (n=5) and (G) in myotubes (H), (n=5). *(p<0.05), ** (p<0.01), *** (p<0.001).

**Fig. 2. Akt is not involved in isoproterenol-mediated glucose uptake in skeletal muscle**

(A) The PI3K/PIKK inhibitor PI-103 concentration-dependently reduced insulin (1µM) and isoproterenol (1µM) stimulated glucose uptake (n=4). (B) Akt phosphorylation on threonine 308 (T308) and serine 473 (S473) in response to insulin (1µM) but not isoproterenol (1µM; n=3). (C) The Akt inhibitor X (10µM) did not affect isoproterenol (1µM) but reduced insulin (1µM) stimulated glucose uptake (2h; n=8). (D) The Akt inhibitor X (1µM, 30 min pretreatment) abolished Akt T308 or S473 phosphorylation in response to insulin (1µM, n=4). (E) The Akt inhibitor X (1µM, 30 min pretreatment) abolished AS160 phosphorylation at T642 in response to insulin (1µM) (n=3). (F) PIP3 immunoflorescence staining in L6...
skeletal muscle cells treated with isoproterenol (1µM, 5 min) or insulin (1µM, 5 min) in the absence or presence of LY294002 (1µM, 30 min).

**Fig. 3. mTOR is a key factor in isoproterenol-mediated glucose uptake**

(A) Glucose uptake in response to isoproterenol (1µM) or the cell permeable cAMP analogues 8-bromo-adenosine 3’,5’-cyclic monophosphate (8-bromo, 1mM), N6,2’-O-dibutyladenosine 3’,5’-cyclic monophosphate (db-cAMP, 1mM), cAMP (2mM), the non-cell permeable analogue 8-Hydroxyadenosine-3’, 5’-cyclic monophosphate (8-OH, 1mM) or insulin (1µM) (n=4-10). (B) The mTOR inhibitor Torin-1 (10nM, 30 min) inhibited glucose uptake in response to insulin (1µM, 2 h), isoproterenol (1µM, 2h), or 8-Br-cAMP (1mM, 2h) (n=4). (C) The mTOR inhibitor KU0063794 concentration-dependently inhibited insulin (1µM, 2h), isoproterenol (1µM, 2h) or 8-Br-cAMP (1mM, 2h) mediated glucose uptake (n=4). (D) In *ex vivo* experiments with rat soleus muscle, KU0063794 (100nM) blocked glucose uptake in response to isoproterenol (100nM, 2h) and insulin (100nM, 2h) (n=4). (E) KU0063794 (10mg/kg) inhibited isoproterenol (1mg/kg) and insulin (1mg/kg) stimulated glucose uptake in WT mouse skeletal muscle (n=5). Both *ex vivo* and *in vivo* glucose uptake was detected by [³H]-2DG.

**Fig. 4. Role of mTORC1 and mTORC2 in isoproterenol-mediated glucose uptake** (A) Western blots of mTOR phosphorylation at S2448 and S2481 in response to insulin (1µM, 2h), isoproterenol (1µM, 2h) or 8-Br-cAMP (1mM, 2h) in the presence or absence of KU0063794 (100nM). Insulin caused mTOR phosphorylation at S2448 and S2481 whereas isoproterenol and 8-Br-cAMP caused phosphorylation solely at S2481. Phosphorylation at both sites is blocked by the mTOR inhibitor KU0063794 (100nM; n=4). (B) mTOR phosphorylation in response to the PKA-selective cAMP-analogue N⁶-Benzoyladenosine-3’, 5’-cyclic monophosphate (6-Benz-cAMP, 1mM 2h). (C) Quantification of western blots detecting mTOR phosphorylation at S2481 in response to insulin (1µM, 2h), isoproterenol (1µM,
2h) or 8-bromo-cAMP (1 mM) the presence or absence of the PKA Inhibitor fragment 14-22 (PKI, 5 µM, 30 min pre-incubation). PKI partly significantly reduced mTOR phosphorylation in response to isoproterenol and 8-bromo-cAMP. (D) Acute (30 min) treatment of L6 myotubes with rapamycin inhibits mTORC1 and had no significant effect on glucose uptake in response to isoproterenol (1µM, 2h), 8-Br-cAMP (1mM, 2h), or insulin (1µM, 2h) (n=4). (E) Long-term pretreatment with rapamycin (1nM, 24h) down regulates mTORC2 and inhibited glucose uptake to isoproterenol (1µM) and insulin (1µM) (n=4). (F) Western blots from cells treated with rictor siRNA, raptor siRNA or a scrambled siRNA showing selective knockdown of rictor and raptor (n=4). (G) Glucose uptake in L6 myotubes transfected with scrambled, rictor or raptor siRNA (n=4-5). Only transfection with rictor siRNA inhibited glucose uptake in response to isoproterenol(1µM, 2h) or insulin(1µM, 2h).

**Fig. 5. Isoproterenol stimulated glucose uptake is not dependent on transcription or translation** (A) Actinomycin D (2µg/ml) treatment had no effect on insulin (1µM) and isoproterenol (1µM) mediated glucose uptake (2h) in L6 myotubes (n=4). (B) Quantification of GLUT1 mRNA levels by Q-PCR following 2h stimulation with either insulin (1µM) or isoproterenol (1µM) (n=6). Basal levels at 0 h were set as 100% (ct: 27.4). (C) GLUT4 mRNA levels (Q-PCR) were not changed following 2 h stimulation with either insulin (1µM) or isoproterenol (1µM). Basal levels at 0h were set as 100% (GLUT1 ct: 26.2, GLUT4 ct: 27.4). (D) Cycloheximide (50µM) treatment had no significant effect on insulin (1µM) or isoproterenol (1µM) mediated glucose uptake (2h) (n=4). (E) Western blot and quantification of GLUT1 protein levels in L6 myotubes treated with insulin (1µM, 2h) or isoproterenol (1µM, 2h). The levels in unstimulated cells were set as 100 %. (n=3). (F) GLUT4 protein levels were unchanged by treatment (2h) with insulin (1µM) or isoproterenol (1µM). The levels in unstimulated cells were set at 100 % (n=3). (G) Eadie-Hofstee plot of glucose uptake. Vmax (Y-axis intercept) and -Km (slope); values were 7947±536 pmol/min/µg and 2.5±0.3mM in isoproterenol-treated myotubes; 4045±702 pmol/min/µg and 1.5±0.6 mM in control; and 8280±504pmol/min/µg and 3.2±0.4M in insulin-treated L6 myotubes.
(n=5). (H) At right, Western blot analysis of GLUT4 and Akt after GLUT4 siRNA or scrambled siRNA treatment (n=5). *(p<0.05), ** (p<0.01), *** (p<0.001). (I) Glucose uptake in response to isoproterenol or insulin (1µM, 2h) in the absence or presence of the action inhibitor Latrunculin B (10µM, 30 min pre-incubation).

**Fig. 6. Isoproterenol stimulates GLUT4 translocation** (A) L6 myoblasts treated with isoproterenol (1µM) or insulin (1µM) for 2h show translocation of GLUT4 to the plasma membrane. Non-permeabilized L6 myoblasts were probed with GLUT4 antibody (green) targeting exofacial epitopes and visualized by confocal microscopy; plasma membrane GLUT4 is expressed as % control (n=36). (B) L6 myoblasts transiently transfected with GLUT4mycGFP and stimulated with isoproterenol (1µM) or insulin (1µM) for 2h also show translocation of GLUT4 to the plasma membrane (GFP fluorescence was similar in the cells examined). GLUT4 was detected with myc antibody (red); plasma membrane GLUT4 is expressed as % control (n=36). (C) and (D) Confocal micrographs of human SKMC (n=36) and SKMC transiently transfected with GLUT4mycGFP (n=36) probed with either GLUT4 (green) or myc (red) antibody, respectively, following isoproterenol (1µM) or insulin (1µM) treatment for 2h. Quantification of the images was performed with untreated plasma membrane GLUT4 as % control. (E) KU0063794 (100nM) blocked GLUT4 translocation in L6 myotubes. Cell surface GLUT4 was visualized with GLUT4 antibody (green) and detected with confocal microscopy. Histograms show that KU0063794 significantly inhibited the plasma membrane translocation of GLUT4 in response to isoproterenol (1µM, 2 h), insulin (1µM, 2h) or 8-Br-cAMP (1mM, 2h).

**Fig. 7. Proposed mechanisms of glucose uptake in response to activation of β2-adrenoceptors or insulin.**

The β2-adrenoceptor pathway when activated, couples to Gs to stimulate adenylate cyclase (AC) resulting in the production of cAMP, which in turn activates PKA that phosphorylates mTORC2 at S2481.
phosphorylated mTORC2 stimulates actin re-organization and GLUT4 translocation to the plasma membrane to increase glucose uptake.

Insulin binds to the insulin receptor (IR) resulting in interaction between IRS-1 and PI3K. PI3K activation leads to increased PIP3 levels and plasma membrane recruitment of Akt and PDK1 where Akt is phosphorylated at T308 by PDK1. PI3K also phosphorylates mTORC2 at S2481 and mTORC2 phosphorylates Akt at S473. mTORC2 stimulates actin re-organization and GLUT4 translocation to the plasma membrane and activated Akt dissociates from the membrane and phosphorylates mTORC1 at S2448 and AS160 at T642 which also promotes translocation of GLUT4 to the plasma membrane.
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**Fig. S1. β-adrenergic–stimulated glucose uptake in skeletal muscle cells.** (A) The effect of clenbuterol (1µM), isoproterenol (1µM) or insulin (1µM) on glucose uptake in L6 myotubes (n=3). *(p<0.05), ** (p<0.01). (B) Concentration-response curve for glucose uptake in L6 myotubes (2h) in response to isoproterenol and insulin (n=6). (C) Time course of isoproterenol (1µM) or insulin (1µM) (n=5) mediated glucose uptake in L6 myotubes.

**Fig. S2. Akt is not involved in isoproterenol mediated glucose uptake in L6 myotubes**
(A) Effect of Akt inhibitor SHE6 (1µM, 30min) on isoproterenol (1µM, 2h) and insulin (1µM, 2h) stimulated glucose uptake (n=5). *(p<0.05). (B) Akt T308 or S473 phosphorylation in response to insulin (1µM, 2h) or isoproterenol (1µM, 2h) in the presence or absence of SH-6 (1µM, pretreatment for 30min) (n=4). (C) Western blot showing phosphorylation on TBC1D1 (Thr590) after isoproterenol (1µM, 2h) or insulin stimulation (1µM, 2h) (n=4), ** (p<0.01). (D) cAMP accumulation assay in L6 myotubes. cAMP accumulation was analyzed at 5, 15, 30, 60, and 120min time points in response to isoproterenol (1µM). Data were expressed as a percentage of forskolin (100µM) response. Values of cAMP represent the mean +/- SEM (n=5).

**Fig. S3. Effect of Akt inhibitor X on insulin and isoproterenol mediated phosphorylation of mTOR.**
(A) Western blot showing mTOR phosphorylation after isoproterenol (1µM) or insulin (1µM) treatment after 0, 5, 10, 20 minutes and 1, 2 hours with quantification graph of mTOR phosphorylation levels (n=3). (B) Western blot showing the effect of insulin (1 µM, 2h) and isoproterenol (1 µM, 2h) on p-70S6K phosphorylation in the presence or absence of mTOR inhibitor KU0063796 (100 nM, pretreatment for 30 min). n=3, *(p<0.05) (C) Western blot showing the effect of Akt inhibitor X (1µM, pretreatment for 30min) on mTOR phosphorylation in response to insulin (1 µM, 2h) or isoproterenol (1µM, 2h), with quantification graph of mTOR phosphorylation levels (n=3). *(p<0.05).

**Fig. S4. Isoproterenol stimulates glucose uptake in L6 myotubes via GLUTs.** (A) Effect of the GLUT inhibitors cytochalasin B (10µM, n=6), phloretin (100µM, n=5) and indinavir (100µM, n=4) on basal glucose uptake and the effect of cytochalasin B (B), phloretin (C) and indinavir (D) on insulin (1µM, 2h) and isoproterenol (1µM, 2h) mediated glucose uptake (n=4-6). Insulin and isoproterenol-stimulated glucose uptake is shown as increase over basal. (E) The effect of indinavir (100µM, n=4) on glucose uptake after 45 or 120 min stimulation with insulin (1µM) or isoproterenol (1µM, 2h). (F) Glucose uptake measured in the presence of increasing concentrations of unlabelled 2-deoxy-glucose showing the effect of isoproterenol (1µM) or insulin (1µM) on the velocity of glucose uptake in L6 myotubes (n=5).

**Fig. S5. GLUT4 translocation in L6 cells** (A) Stably transfected GLUT4myc L6 myoblasts were stimulated with insulin (1µM) or isoproterenol (1µM) for 2h and GLUT4 at the plasma membrane visualized with myc antibody. (B) Untransfected L6 myotubes were stimulated with insulin (1µM) or isoproterenol (1µM) for 2h and GLUT4 at the plasma membrane was visualized with GLUT4 antibody. (C) Stable
transfected GLUT4myc L6 myotubes were stimulated with insulin (1µM) or isoproterenol (1µM) for 2h. GLUT4 at the plasma membrane was visualized with GLUT4 myc antibody and nucleus with DAPI. All images were detected with confocal microscopy and images were quantified and expressed as percentage of control (n=36).
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