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A diacylglycerol kinase inhibitor, R59022, stimulates glucose transport through a MKK3/6-p38 signaling pathway in skeletal muscle cells

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Abstract

Diacylglycerol kinase (DGK) is one of lipid-regulating enzymes, catalyzes phosphorylation of diacylglycerol to phosphatidic acid. Because skeletal muscle, a major insulin-target organ for glucose disposal, expresses DGK, we investigated in the present study a role of DGK on glucose transport in skeletal muscle cells. PCR study showed that C2C12 myotubes expressed DGKα, δ, ε, ζ, or θ isoform mRNA. R59022, a specific inhibitor of DGK, significantly increased glucose transport, p38 and MKK3/6 activation in C2C12 myotubes. The R59022-induced glucose transport was blocked by SB203580, a specific p38 inhibitor. In contrast, R59022 failed to stimulate both possible known mechanisms to enhance glucose transport, an IRS1-PI3K-Akt pathway, muscle contraction signaling or GLUT1 and 4 expression. All these results suggest that DGK may play a role in glucose transport in the skeletal muscle cells through modulating a MKK3/6-p38 signaling pathway.

Keywords: Diacylglycerol kinase; Glucose transport; Skeletal muscle cell; p38; R59022

Introduction

Lipid-regulating enzymes involve the glucose metabolism. For instance, acyl CoA:diacylglycerol acyltransferase 1 knockout mice exhibited increased insulin sensitivity [1]. Moreover, mitochondrial acyl-CoA:glycerol-sn-3-phosphate acyltransferase 1 knockout mice exhibited increased hepatic insulin sensitivity [2]. Thus, lipid-regulating enzymes are possible to be involved in glucose metabolism. Diacylglycerol kinase (DGK) is one of lipid-regulating enzymes, catalyzes phosphorylation of diacylglycerol (DG) to phosphatidic acid (PA) [3-7]. Ten mammalian isoforms have been identified, and are classified into five subtypes based on their primary structure. DGKs contain a conserved catalytic domain and an array of other conserved motifs that are likely to play a role in lipid-protein and protein-protein interactions in various signaling pathways dependent on DG and/or PA production. Although DGK is known as one of lipid-regulating enzymes, little is known about the implication of DGK for glucose metabolism. Because skeletal muscle expresses DGK isoforms [3] and is a major insulin-target organ for glucose disposal [8,9], we made a hypothesis that DGK, a lipid-regulating enzyme, plays a role in glucose metabolism in the skeletal muscle. To clarify the above hypothesis, we investigated in the present study a role of DGK on glucose transport in skeletal muscle cells.

Materials and Methods

Cell culture

C2C12 mouse myoblast cells were purchased from the American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/l glucose and 10% fetal bovine serum (Sigma, St. Louis, MO).

Confluent myoblasts were differentiated to myotubes by lowering the serum concentrations to 5% horse serum (Invitrogen, Carlsbad, CA). Cells were used for the experiments after 4-6 days of differentiation.

Chemicals

R59022, a DGK inhibitor, was purchased from Calbiochem (San Diego, CA) and was dissolved in dimethy sulfoxide (DMSO) with a final concentration of 0.1% DMSO in culture medium. Bovine serum albumin (BSA) used was free fatty acid-free grade (Sigma). [^{3}H]2-deoxyglucose was purchased from American Radioactive Chemicals (St. Louis, MO). 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside (AICAR) was obtained from Toronto research chemicals (North York, ON). SB203580 was purchased from LC Laboratories (Woburn, MA). Antibodies used were purchased from Cell Signaling technologies (Danvers, MA), except: insulin receptor substrate-1 (IRS1) and p85 subunit of phosphatidylinositol 3-kinase (PI3K) (Upstate Biotechnology, Lake Placid, NY); phosphotyrosine-RC20 (BD Biosciences, San Jose, CA); Akt substrate of 160 kDa (AS160) (Novus Biologicals, Littleton, CO); glucose transporter (GLUT) 1 and 4 (Santa Cruz Biotechnology, Santa Cruz, CA).

RT-PCR analysis

Total RNA was extracted from C2C12 myotubes at day 6 after differentiation using TRIzol reagent (Invitrogen). RT-PCR was described previously [10]. Briefly, the amplification was carried out in a 50 μl of reaction mixture containing cDNA synthesized with oligo-dT primer (ReverTra Ace, TOYOBO, Osaka, Japan) correspondent to 50 ng of total RNA, 25 pmoles each of the forward and reverse

primer (except for DGKβ, 10 pmoles), 0.2 mM dNTPs and 1.25 units of KOD Dash Taq DNA polymerase (TOYOBO). The reaction conditions were: 30 cycles of 94°C for 30 s, 58°C for 2 s and 74°C for 30 s. The products were separated electrophoretically in a 2% agarose gel and stained with ethidium bromide. A mouse whole brain of C57Bl/6Ncrj (Charles River Japan, Tokyo, Japan) was used for positive control. Expression of 36B4 was confirmed to show the quality of cDNA. Intron-spanning primers were used in order to avoid false positive due to genomic contamination and described in Table 1.

Glucose transport assay

Glucose transport was assayed by measuring [^{3}H]2-deoxyglucose uptake as described [11]. Briefly, differentiated C2C12 myotubes (day 4-5) were treated for 24 h or indicated time with several concentrations of R59022 or vehicle (DMSO) in serum-free DMEM containing 0.2% BSA and then Krebs-Ringer-phosphate buffer containing 0.2% BSA for 40 min. For positive control, insulin (final concentration, 100 nM) was added for 15 min. Uptake of [^{3}H]2-deoxyglucose was measured for last 4 min. Uptake was measured routinely in triplicate or quadruplicate for each experiment.

Protein extraction, Immunoprecipitation and Western blot

C2C12 myotubes were treated with several doses of R59022 or vehicle for 24 h in DMEM containing 0.2% BSA. Insulin or AICAR was used for positive control as indicated. Protein extraction and Western blotting were performed as described previously [9,12]. Briefly, total proteins were extracted with lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and 10% glycerol) containing

protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany) and phosphatase inhibitors (Sigma). For the analyses of IRS1 phosphorylation and IRS1/PI3K association, 500 μg protein were immunoprecipitated with anti-IRS1 antibody using protein A/G agarose beads for 16 h at 4ºC, and then eluted using Laemmli buffer containing β-mercaptoethanol. For the other analyses, equal amount of protein (20-50 μg/lane) were resolved on 7.5 or 10% SDS-PAGE. The bands were visualized using a luminoimage analyzer (Fujifilm, Tokyo, Japan) and quantified using a MultiGauge software version 2.2 (Fujifilm).

Measurement of p38 mitogen-activated protein kinase (p38) activity

C2C12 myotubes were treated with several doses of R59022 or vehicle for 24 h and with 100 nM insulin for 10 min as a control. The activity of p38 was analyzed using an assay kit (Cat. #9820, Cell Signaling Technology) according to the manufacturer's instruction.

Statistical Analysis

Data are expressed as the means ± S.E. Statistical analysis was performed by analysis of variance and subsequent Fisher's LSD test. $P < 0.05$ was considered statistically significant.

Results

First, we examined and confirmed whether C2C12 myotubes express DGK by RT-PCR. The DGK has ten isoforms (α, β, γ, δ, η, κ, ε, ζ, ι and θ) and classified into five subtypes based on the functional domains (Type 1: DGKα, β, γ; Type 2: DGKδ, η, κ; Type 3: DGKε; Type 4: DGKζ, ι; Type 5: DGKθ). We analyzed in

this study at least one isoform out of each subtype of DGK. PCR studies revealed that C2C12 myotubes expressed DGKα, δ, ε, ζ, and θ isoforms (Fig. 1A, upper panel), whereas mouse brain as a positive control expressed all isoforms tested (Fig. 1A, lower panel).

Next, we examined the effect of a DKG inhibitor [13], R59022 on glucose transport in C2C12 myotubes to determine whether DGK affects glucose transport in the skeletal muscle cells. We used C2C12 myotubes in this study because the cells express multiple DGK isoforms as demonstrated in Fig. 1A. As demonstrated in Fig. 1B, insulin as a positive control significantly stimulated glucose transport in this experimental condition and R59022 similarly increased glucose transport measured using [^{3}H]2-deoxyglucose in C2C12 myotubes. Statistical significance was observed at a dose of 10 μM of R59022 and the stimulation of glucose transport by R59022 was dose-dependent. Fig. 1C shows the time-course change of glucose transport by a 30 μM dose of R59022. Stimulation of glucose transport by R59022 started at 8 h and the stimulation was observed until at least 24 h after the inhibitor.

In the next step, we attempted to clarify the mechanism by which R59022 stimulates glucose transport in C2C12 myotubes. Because it has been reported that glucose transport is deeply regulated by the IRS1-PI3K-Akt signaling pathway and the translocation of GLUT from cytosol to cell surface [14], we tested the above possibility in the increased glucose transport by R59022 in C2C12 myotubes. The effects of R59022 on phoshorylation of IRS1, association of IRS1/PI3K and phosphorylation of Akt in C2C12 myotubes were examined. As illustrated in Fig. 2A, 2B and 2C, R59022 failed to stimulate IRS1 phosphorylation, association of IRS1/PI3K or Akt phosphorylation whereas insulin as a positive control potently stimulated these three tested biochemical markers. Since muscle contraction is an

another stimulator for glucose transport independently of IRS1-PI3K-Akt pathway by activating AMP-activated protein kinase (AMPK) [15], we made a hypothesis that muscle contraction signal by activating AMPK plays a role in the increased glucose transport by R59022 in myotubes. As shown in Fig. 2D, R59022 did not stimulate phosphorylation of AMPK, whereas AICAR, a chemical activator for AMPK, used as a positive control, indeed enhanced its phosphorylation in C2C12 myotubes. Because AS160 has recently been identified as an integrated down-stream mediator for Akt and AMPK towards GLUT translocation [16], we examined the effect of R59022 on AS160 phosphorylation in C2C12 myotubes. As shown in Fig. 2E, AICAR but not R59022 activated AS160 phosphorylation in C2C12 myotubes.

To test a possibility that the increased glucose transport by R59022 may be resulted from up-regulation of GLUT, we examined the effect of R59022 on GLUT expression in C2C12 myotubes. As seen in Fig. 2F and 2G, R59022 did not affect the protein expression of GLUT1 and 4 respectively.

It has been reported that p38 is implicated in glucose transport not by translocation of GLUT but by activation of GLUT [17]. To investigate the possible involvement of p38 activation on R59022-induced stimulation of glucose transport in the skeletal muscle cells, we examined the effect of R59022 on phosphorylation of p38 in C2C12 myotubes. As demonstrated in Fig. 3A and 3B, immunoblotting clearly shows that R59022 dose-dependently stimulated phoshorylation of p38 in C2C12 myotubes. A significant increase in p38 phosphorylation was obtained by R59022 at 10 μM or larger doses in C2C12 myotubes. We next investigated whether phosphorylation of p38 by R59022 is dependent upon its kinase activity using *in vitro* immunoprecipitation-kinase assay. Fig. 3C shows that R59022 increased the phosphorylation of ATF-2, the substrate of p38.

To clarify whether R59022-induced stimulation of glucose transport is indeed caused by p38 activation in C2C12 myotubes, we examined the speculation using SB203580, an inhibitor of p38. As shown in Fig. 4A, insulin or R59022 significantly increased in glucose transport in C2C12 myotubes. Although SB203580 by itself did not change glucose transport, R59022-induced stimulation of glucose transport was blocked by SB203580 in C2C12 myotubes, suggesting that activation of p38 is functionally relevant in R59022-induced stimulation of glucose transport.

We next tried to investigate the mechanism of R59022-induced p38 activation in the skeletal muscle cells. Because two mitogen-activated protein kinase kinases, MKK3 and MKK6 are immediate upstream activators for p38 [18], the effect of R59022 on MKK3/6 phosphorylation in C2C12 myotubes was examined. R59022 as well as insulin significantly stimulated phosphorylation of MKK3/6 (Fig. 4B). The stimulation of MKK3/6 phosphorylation was dose-dependently observed by R59022.

Discussion

In the present study, we examined whether DGK may be implicated in glucose transport in skeletal muscle cells because the skeletal muscle expresses DGK [3]. Since several research groups used C2C12 cells to analyze glucose transport in the skeletal muscle cells [19,20], the C2C12 cells were selected in this study. We first examined whether C2C12 myotubes express several DGK isoforms mRNAs and showed that C2C12 myotubes expressed DGKα, δ, ε, ζ, and θ isoforms, indicating that C2C12 myotubes express all five subtypes (type 1-5) of DGK. Earlier investigators demonstrated that C2C12 mouse myoblasts expresses DGKδ and ζ

[21,22]. The present study therefore provided an evidence that C2C12 myotubes express not only DGKδ and ζ, but DGKα, ε and θ isoforms. In the following experiments, we used C2C12 myotubes as a model to investigate a role of DGK.

Next, we examined the effect of DGK inhibition by a specific inhibitor on glucose transport in the C2C12 myotubes to clarify a role of DGK in glucose transport in the skeletal muscle cells. As clearly demonstrated in this study, R59022 significantly stimulated glucose transport in skeletal muscle cells in dose- and time-dependent manners, suggesting that DGK inhibition would increase glucose transport in skeletal muscle cells. In other words, glucose transport would be suppressed by DGK in skeletal muscle cells. DGK phosphorylate the second-messenger DG to PA [3-6]. DGKs play a role in lipid-protein and protein-protein interactions in various signaling pathways dependent on DG and/or PA production. DGK is therefore believed to be activated at the plasma membrane where DG is generated. Strålfors et al. have demonstrated that DG enhanced glucose transport in adipocytes [23]. It has been furthermore shown that phorbol ester, a DG analogue [24], stimulates muscle glucose transport [25]. These results suggest that DG stimulates glucose transport. Because R59022 is an inhibitor of DGK, DG should be accumulated by treatment with R59022. Although the present study did not confirm that R59022 indeed increased DG level in the cells, these evidence led us to speculate that DG accumulation might mediate at least in part the R59022-induced stimulation of glucose transport in C2C12 myotubes. In any event, these evidences suggested that DGK might be involved in glucose transport. As we have clearly demonstrated in this study, C2C12 myotubes used in this study express at least five isoforms of DGK. Among these isoforms of DGK, further study should be needed to clarify which isoform(s) is involved in the regulation of glucose transport.

Next, we tried to clarify the mechanism by which R59022 stimulates glucose transport in C2C12 myotubes. Since the mechanism of glucose transport stimulated by insulin is well characterized [14], we tested a possibility whether R59022 may stimulate glucose transport through a mechanism similar to insulin signaling in skeletal muscle cells. An IRS1-PI3K-Akt pathway is well established as a signaling that mediates insulin-induced stimulation of glucose transport in skeletal muscle cells [14]. It has been demonstrated that muscle contraction is a potent stimulator for glucose transport independently of the IRS1-PI3K-Akt pathway by activating AMPK [15]. We therefore examined the above possibility that an IRS1-PI3K-Akt pathway or muscle contraction signal, or both might mediate the R59022-induced stimulation of glucose transport in C2C12 myotubes. The present study however failed to support the above possibility.

Marshall et al. have demonstrated that glucose transport is stimulated in GLUT 1 or 4 transgenic mice [26], indicating that increased expression of glucose transporter, GLUT, stimulates glucose transport. We therefore examined the effect of R59022 on expression of GLUT1 and 4. As demonstrated in this study, R59022 failed to increase GLUT1 and 4 expression in C2C12 myotubes, suggesting that the stimulated glucose transport by R59022 is not associated with the expression of GLUT in the skeletal muscle.

p38 is involved in glucose transport in the skeletal muscle cells [17]. For instance, activation of p38 by anisomycin, a specific activator for p38, stimulates p38 phosphorylation and increases glucose transport [27]. In contrast, inhibition of p38 by SB203580 suppresses glucose transport in skeletal muscle cells [28]. Thus, p38 plays a vital role in glucose transport in the skeletal muscle cells. The present study demonstrated that R59022 significantly stimulated p38 phosphorylation, its kinase

activity and glucose transport and SB203580 blocked the R59022-induced stimulation of glucose transport in C2C12 myotubes, strongly suggesting that p38 activation may mediate the R59022-induced stimulation of glucose transport.

Because two mitogen-activated protein kinase kinases, MKK3 and MKK6 are immediate upstream activators for p38 [18], we made a hypothesis that MKK3/6 phosphorylation may be a upstream signaling mechanism to activate p38 by R59022. As demonstrated in the present study, R59022 increased MKK3/6 phosphorylation in C2C12 myotubes, suggesting an involvement of MKK3/6 in p38 activation by R59022. The R59022-induced stimulation of glucose transport, activation of p38 and phosphorylation of MKK3/6 were all observed by R59022 in the same dose range (10-30 μM), furthermore supporting the speculation that the MKK3/6-p38 signaling pathway mediates the R59022-induced stimulation of glucose transport in C2C12 myotubes.

In summary, the present study suggested that DGK may play a role in glucose transport in the skeletal muscle cells through modulating a MKK3/6-p38 signaling pathway.

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Figure legends

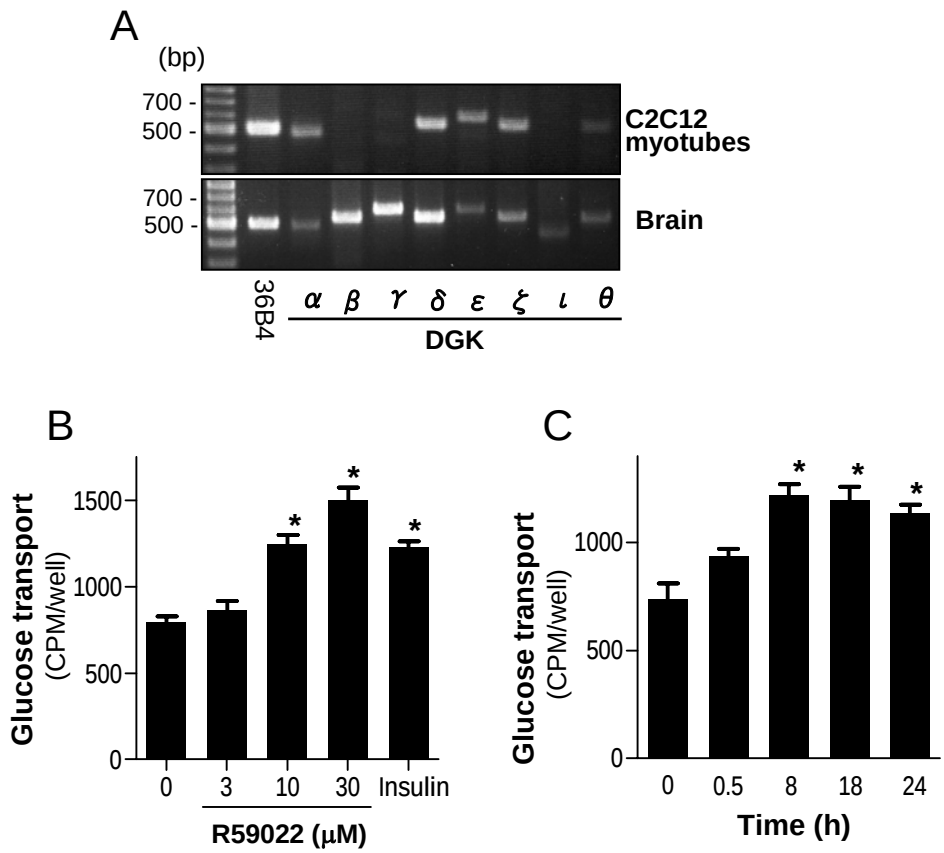
Fig. 1. (A) mRNA expression of DGK isoforms (α, β, γ, δ, ε, ζ, ι, or θ) in C2C12 myotubes (day 6) and mouse brain as a positive control was detected by RT-PCR. Dose-response effect of R59022 on glucose transport (B) and time-course change of glucose transport by R59022 in a dose of 30 μM (C) in C2C12 myotubes were shown. Insulin treatment (100 nM) was performed as a positive control. Glucose transport was assayed using [^{3}H]2-deoxyglucose for last 4 min. Data are expressed as the mean ± S.E. (n = 3-4). *P < 0.05, when compared vehicle only.

Fig. 2. Effect of R59022 on (A) IRS1 tyrosine-phosphorylation, (B) IRS1/PI3K association, (C) Akt phosphorylation, (D) AMPK phosphorylation, (E) AS160 phosphorylation, (F) GLUT1 protein and (G) GLUT 4 protein expression. C2C12 myotubes were treated with several doses of R59022 for 24 h. For positive control, insulin (100 nM for 15 min) or AICAR (100 μM for 30 min) was used. The protein was extracted for later analysis. Data are calculated by fold change versus vehicle and expressed as the mean ± S.E. (n = 3). *P < 0.05, when compared vehicle only. Representative blot was shown on the upper side of the graph.

Fig. 3. Effect of R59022 on p38 activity in C2C12 myotubes. C2C12 myotubes were treated with several doses of R59022 for 24 h. Insulin was used at 100 nM for 15 min for positive control. (A) Representative immunoblot with phospho-p38 (Thr180/Tyr182) antibody (upper panel) or total p38 antibody (lower panel) were shown. (B) Results from A were quantified by densitometry and data represent mean ± S.E. (n = 3). (C) *In vitro* kinase assay of p38. Total cell lysates were immunoprecipitated with phopsho-specific p38 antibody and then the products were

performed kinase assay with recombinant ATF-2, a substrate of p38. Activity of p38 was determined by blotting the assay products with phospho-ATF-2 (Thr71) antibody. Representative blot was shown on the upper side of the graph. Data are calculated by fold change versus vehicle and expressed as the mean ± S.E. (n = 3). *$P < 0.05$, when compared vehicle only.

Fig. 4. (A) Effect of SB203580, a p38 inhibitor, on the R59022-induced stimulation of glucose transport in C2C12 myotubes. Glucose transport was assayed using [^{3}H]2-deoxyglucose for last 4 min. Data are expressed as the mean ± S.E. (n = 4). *$P < 0.05$, when compared vehicle only. (B) Effect of R59022 on MKK3/6 phosphorylation. C2C12 myotubes were treated with several doses of R59022 for 24 h. Insulin was used at 100 nM for 15 min for positive control. Phosphorylation of MKK3/6 was analyzed by immunoblot using phospho-MKK3/6 (Ser189/207) antibody. Representative blot was shown on the graph. The bands were quantified and data are calculated by fold change versus vehicle and expressed as the mean ± S.E. (n = 3). *$P < 0.05$, when compared vehicle only.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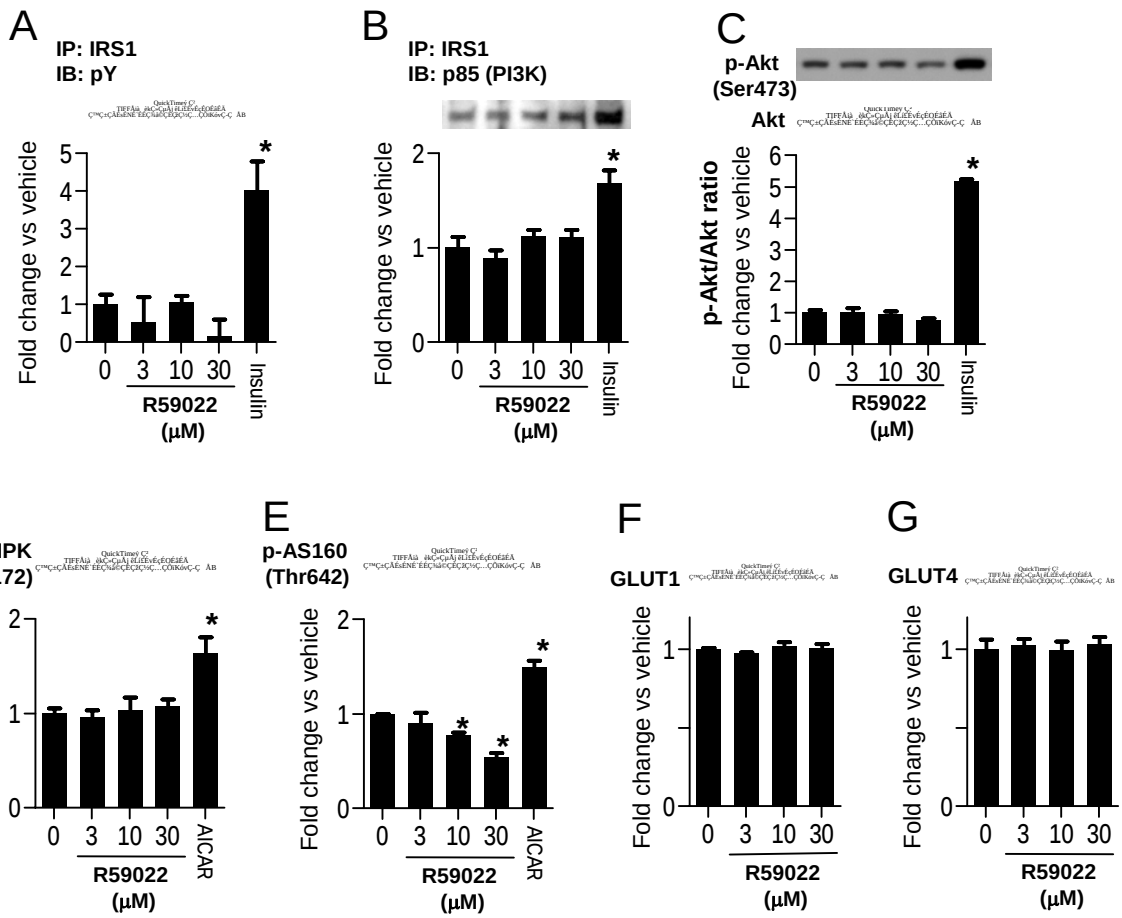
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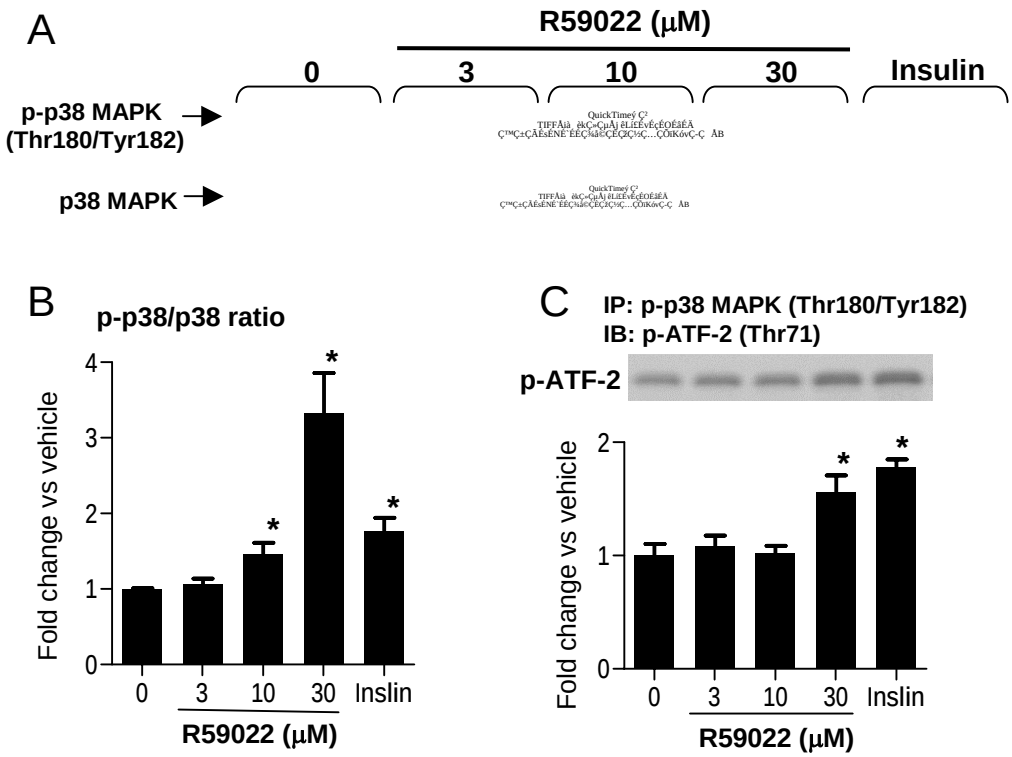
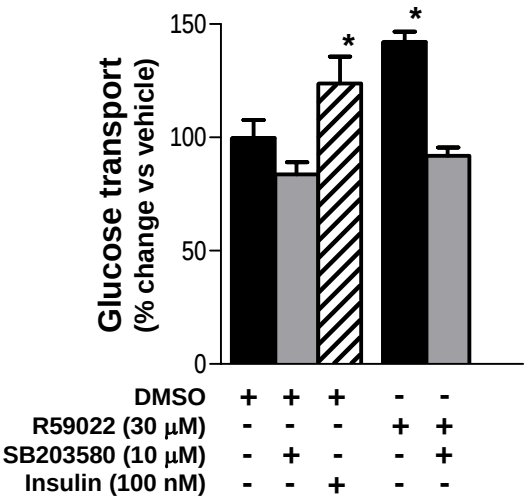
F
GLUT1
Fold change vs vehicle
0
1
0
3
10
30
R59022
(μM)
G
GLUT4
Fold change vs vehicle
0
1
0
3
10
30
R59022
(μM)


A
R59022 (μM)
0
3
10
30
Insulin
p-p38 MAPK
(Thr180/Tyr182)
p38 MAPK
B
p-p38/p38 ratio
Fold change vs vehicle
0
1
2
3
4
0
3
10
30
Inslin
R59022 (μM)
C
IP: p-p38 MAPK (Thr180/Tyr182)
IB: p-ATF-2 (Thr71)
p-ATF-2
Fold change vs vehicle
0
1
2
0
3
10
30
Inslin
R59022 (μM)

A


Glucose transport
(% change vs vehicle)
150
100
50
0
*
*
DMSO + + + - -
R59022 (30 μM) - - - + +
SB203580 (10 μM) - + - - +
Insulin (100 nM) - - + - -

B

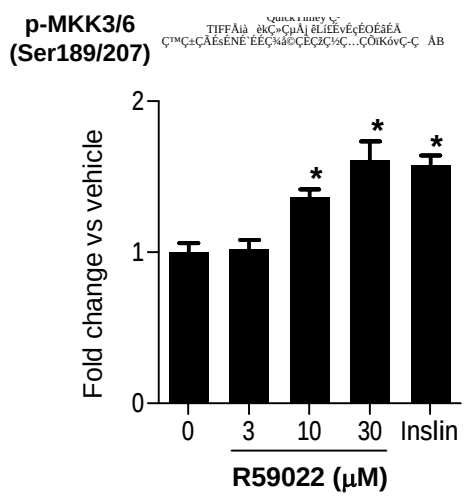

p-MKK3/6
(Ser189/207)
Fold change vs vehicle
2
1
0
*
*
*
0
3
10
30
Inslin
R59022 (μM)

Table 1

Primers used for PCR.

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')	Amplicon size (bp)
36B4	TCCTTCTTCCAGGCTTTGGG	TCCTCCGACTCTTCCTTTGC	512
DGK α	TCAGCCCAGAAGACTTCGCT	TGCCGTCCTGATCCATCTCT	495
DGK β	TTCTGGAAGCTGAGCTGCCT	TGAGTCGCCACACATGCTGT	537
DGK γ	TCATGAGGGCATACCTGGAG	TTCCCAGGAGGACCAGCAAA	600
DGK δ	TCCAGCCACCTGGGTACATT	TCTTCCTCACCAGGCTCCAA	541
DGK ε	TCCCCCTGTGCTCTTACTGT	TCTCCAGCATAGCCTGTACC	592
DGK ζ	TCTGAGGAGCAGATCCAGAG	TTGCTGGCCTTGAGGGTGTT	545
DGK ι	TTCCTGTGGCTAACGGTCCA	TGCAGCATGAAGCAGGTCAC	451
DGK θ	TCCAGCTGATTGAGGTGCTC	TAGCTCAAAGACCTGGTGCG	533