Metabolism
Clinical and Experimental

VOL 51, NO 3 MARCH 2002

PRELIMINARY REPORT

The Effects of Phospholipase C Inhibition on Insulin-Stimulated Glucose Transport in Skeletal Muscle

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Previous research has demonstrated that phospholipase C (PLC) is involved in insulin-stimulated glucose transport in 3T3-L1 adipocytes. The purpose of the current investigation was to determine if PLC is also involved in insulin-stimulated glucose uptake in rat skeletal muscle. To that end, we used an in vitro muscle preparation of the rat soleus muscle to test the effects of the PLC inhibitor, U73122, on glucose transport. The PLC inhibitor, U73122, led to a concentration-dependent inhibition of insulin (0.6 nmol/L)-stimulated glucose transport, whereas it had no effect on basal glucose transport. Specifically 10, 20, 50, and 150 μmol/L U73122 inhibited insulin (0.6 nmol/L)-stimulated glucose transport approximately 17%, 20%, 26%, and 38%, respectively, while an equal molar concentration of U73343 (inactive form of U73122) and/or carrier media (dimethyl sulfoxide [DMSO]) did not influence glucose uptake. A secondary aim of this investigation was to determine if increasing the concentration of insulin from a physiologic concentration (0.6 nmol/L) to a supraphysiologic concentration (6.0 nmol/L) could ameliorate the inhibitory effects of U73122. A 10-fold increase in insulin eliminated the inhibitory effects of U73122 on insulin-stimulated glucose uptake in soleus muscle. In summary, this preliminary report provides evidence to suggest that a PLC signaling mechanism modifies insulin-stimulated glucose uptake in skeletal muscle via its influence on insulin sensitivity.

THE INSULIN SIGNALING pathway in skeletal muscle involves a complex array of intracellular signaling events initiated by insulin receptor autophosphorylation, tyrosine kinase activation, and the stimulation of signaling factors such as insulin receptor substrate-1 and phosphatidylinositol 3-kinase (PI 3-kinase).[1-3] It has recently been suggested that phospholipase C (PLC) may also be involved in this pathway. In support of this argument, it has been shown that insulin-stimulated glucose transport is attenuated in the presence of U73122, a PLC inhibitor, in rat[4] and 3T3–L1 adipocytes.[5] The interaction of the gamma isoform of this enzyme with the insulin receptor,[5] the activation of PLCγ via phosphatidyl (3,4,5) triphosphate $(PIP)_3$,[6] a known product of PI 3-kinase activation, and the expression of PLCγ in mature skeletal muscle[7] suggests a role for PLC in insulin signaling in skeletal muscle.

PLC mediates the hydrolyses of phosphatidylinositol glycans leading to the production of diacylglycerol (DAG), which in turn, can activate novel and conventional protein kinase C (PKC) isoforms. The activation of DAG-sensitive PKC isoforms may lead to the phosphorylation/activation of L-type calcium channels, which may then produce a slight, but relevant, increase in intracellular calcium concentration. Three lines of evidence support this theory. First, L-type calcium channel blockers[8,9] and PKC inhibitors[10] have been shown to attenuate insulin-stimulated glucose transport in skeletal muscle. Secondly, mouse skeletal muscle L-type calcium channels have been shown to be phosphorylated upon insulin-like growth factor-1 (IGF-1) stimulation via a PKC-dependent mechanism.[11] Lastly, it has recently been demonstrated that insulin results in an increase in calcium concentration that is localized to the plasma membrane.[12] The role of PLC in insulin-stimulated glucose transport has been investigated in adipocytes,[4,5] but has not been tested in intact skeletal muscle. The purpose of the present investigation was to explore the effects of U73122 on in vitro insulin-stimulated 3-*O*-methylglucose transport in rat skeletal muscle.

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Submitted April 7, 2001; accepted September 28, 2001.

Supported in part by an internal grant from the Office of Academic Research and Sponsored Programs, Ball State University and a grant from the Indiana Space Grants Consortium, Ball State University Affiliate.

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0026-0495/02/5103-0003$35.00/0
doi:10.1053/meta.2002.30500

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (125 ± 5 g) were group caged, fed standard rat chow and water ab libitum, and maintained on a 12:12 light-dark cycle. Food was removed 12 hours prior to experiments. All protocols were approved by the Ball State University Animal Care and Use Committee.

Muscle Strip Preparation

Glucose uptake was determined using an in vitro muscle preparation. Animals were anesthetized with a 0.1 mL/100 mg body weight intraperitoneal injection of a ketamine mixture. The soleus was surgically isolated, split in situ, clamped at its resting length in plexiglass clamps, and removed.

Determination of Glucose Transport

Glucose transport was determined as described previously.[13] Briefly, clamped muscles were placed in sealed vials containing Krebs Henseleit Buffer (KHB) supplemented with mannitol (18 mmol/L) and pyruvate (2 mmol/L). The vials were gassed with 95% 0_2 and 5% CO_2 and maintained at 30°C in a shaking water bath (120 cycles/minute) throughout the experiment. After a 10-minute recovery period, the muscles were transferred to preincubation vials containing KHB supplemented with 4 mmol/L glucose and 16 mmol/L mannitol with or without insulin and/or inhibitors for 30 minutes. Prior to the final incubation step, the muscles were rinsed for 10 minutes in fresh KHB supplemented with 20 mmol/L mannitol and the required experimental condition and then placed in an incubation media containing 3-0-[^{3}H]-methylglucose (437 μCi/mmol), [^{14}C] mannitol (8 μCi/mmol) and the presence or absence of insulin and inhibitors as indicated in the figures and tables. Following the final incubation, muscles were removed from their clamps, blotted, and quick-frozen immediately in liquid nitrogen (-80°C) until further analysis. Muscle samples were processed as described previously[9] and analyzed in a liquid scintillation counter with channels preset for simultaneous [^{3}H] and [^{14}C] readings for the determination of glucose uptake. All data are expressed as mean ± SE. Differences between control and experimental conditions were determined using unpaired Student's *t* test. When comparisons were made between more than 2 groups, an analysis of variance was used. An LSD post hoc analysis was used to locate the source(s) of significant variance.

Table 1. The Effects of Varying Concentrations of U73122 (10, 20, and 50 μmol/L), U73343, and DMSO on Insulin-Stimulated (0.6 nmol/L) Glucose Transport in Rat Skeletal Muscle

Experimental Condition	3-*O*-Methylglucose Transport
Basal (4 mmol/L glucose)	1.41 ± 0.18 (18)
Insulin (0.6 nmol/L)	4.35 ± 0.26 (15)*
Insulin + 50 μmol/L DMSO	4.26 ± 0.42 (8)*
Insulin + 50 μmol/L U73343	3.93 ± 0.26 (9)*
Insulin + U73122	
+10 μmol/L	3.57 ± 0.25 (14)*†
+20 μmol/L	3.34 ± 0.19 (6)*†
+50 μmol/L	3.19 ± 0.34 (12)*†

NOTE. Data are presented as mean ± SE for the number of observations in parentheses. The glucose concentration in all samples was 4 mmol/L.

*Significantly different than basal 3-*O*-methylglucose transport, $P < .05$.

†Significantly different than insulin 3-*O*-methylglucose transport, $P < .05$.

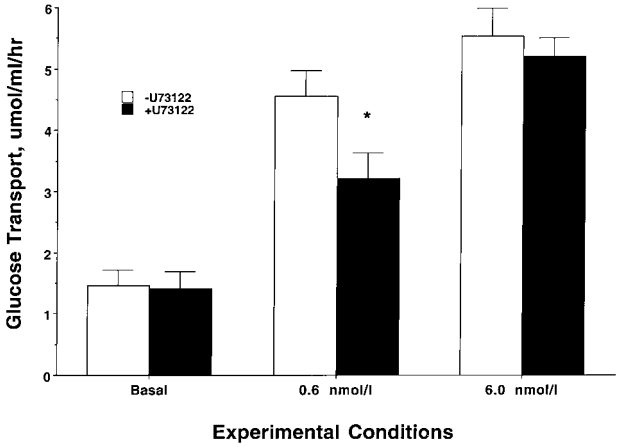

Fig 1. The effects of 50 μmol/L U73122 on basal (4 mmol/L glucose only), insulin (0.6 nmol/L + 4 mmol/L glucose), and supraphysiologic (6.0 nmol/L + 4 mmol/L glucose) insulin-stimulated 3-*O*-methylglucose transport in slow twitch rat skeletal muscle. Data are presented as mean ± SE for 6 to 17 observations/group. *Significantly different than the corresponding non-U73122 condition, $P < .05$.

RESULTS

To determine an optimal inhibitory effect of U73122, a dose response approach was used. A total of 10 μmol/L U73122 significantly attenuated glucose transport by approximately 17%, while 20 and 50 μmol/L U73122 inhibited glucose transport by approximately 23% and 26%, respectively (Table 1). Increasing the concentration of U73122 to 150 μmol/L led to an approximate 38% attenuation in glucose uptake. Although it represented a slightly greater inhibition, there was no significant difference between glucose transport at 50 and 150 μmol/L U73122. Therefore, all subsequent experiments used 50 μmol/L U73122. The carrier media (dimethyl sulfoxide [DMSO]) and the inactive coneger of U73122 were also tested at 50 μmol/L to ensure that there were no nonspecific inhibitory effects due to the chemical composition of the inhibitor or the medium in which it was delivered. DMSO and U73343 did not alter insulin-stimulated 3-*O*-methylglucose transport (Table 1). Moreover, glucose transport in the presence of 50 μmol/L U73122 was significantly less than glucose transport in the presence of an equal molar concentration of DMSO. Increasing the insulin concentration to 6.0 nmol/L ameliorated the inhibitory effects of 50 μmol/L U73122 (Fig 1).

DISCUSSION

This is the first investigation to use a PLC inhibitor to assess the role of PLC in glucose transport in intact skeletal muscle. Herein we demonstrate that U73122 leads to a concentration-dependent inhibition of insulin-stimulated 3-*O*-methylglucose transport. At low concentrations, U73122 has been reported to be a specific inhibitor of PLC. Kayali et al[5] noted that the insulin-induced activation of protein kinase B (AKT) and P70 S6 kinase were not inhibited by U73122. Furthermore several laboratories, including our own, have found that the inactive coneger of U73122 does not significantly reduce glucose transport.[4,5] The results obtained using higher concentrations of

U73122 should be interpreted with caution due to potential nonspecific inhibitory effects of the compound.

Our results are in agreement with previously published reports demonstrating that PLC inhibition leads to an attenuation in insulin-stimulated glucose transport in rat adipocytes.[4,5] However, the degree of inhibition in skeletal muscle is less pronounced than that reported in fat cells. Differences in the degree of involvement of GLUT 1 in insulin-stimulated glucose transport may explain this discrepancy. Insulin-induced glucose transport in 3T3–L1 adipocytes is dependent on the movement of GLUT 4 and to a lesser extent GLUT 1 to the cell membrane, whereas skeletal muscle is a predominantly GLUT 4-dependent process.[14,15] Eichorn et al[16] recently reported that the attenuation of insulin-stimulated glucose transport in the presence of U73122 in 3T3-L1 adipocytes was due, in part, to an inhibition of GLUT 1 translocation. Because this process is not involved to a similar degree in skeletal muscle, it could explain the blunted attenuation of insulin-stimulated glucose transport in skeletal muscle in the presence of U73122 compared with 3T3-L1 adipocytes. Alternatively, it could be suggested that skeletal muscle is less sensitive to the inhibitory effects of U73122 than adipocytes. However, without the measurement of PLC or its intermediate components in the presence or absence of U73122, this is merely speculation.

Although the degree of inhibition in glucose transport was less robust than that reported in adipocytes, we found that U73122 inhibited glucose transport at all concentrations tested. The inhibition in glucose transport could potentially be the result of either a decrease in GLUT 4 translocation or a decrease in transporter activity. Several investigations have shown that the decrement in insulin-stimulated glucose transport in the presence of U73122 is matched by a similar decrease in GLUT 4 translocation, suggesting that transporter movement, not activity, is affected by U73122.[4,5] Although GLUT 4 translocation was not determined in the present investigation, it would seem reasonable to assume that a similar mechanism may be operating in skeletal muscle based on previous observations in adipocytes.[4,5]

In adipocytes, U73122 has been shown to attenuate insulin-stimulated glucose transport. In this investigation, we report similar results in quantitatively the most important insulin responsive tissue, skeletal muscle. The fact that the inhibitory effects of the U73122 were ameliorated in the presence of a maximal insulin stimulus suggests that the PLC signal mechanism may influence insulin action. The ability to increase skeletal muscle insulin sensitivity, while having no effect on responsiveness,[17,18] has been demonstrated as part of the post-exercise effects of muscle contraction and raises the possibility that insulin sensitivity may be mediated, in part, by a PLC-dependent process.

In summary, this is the first investigation to explore the effects of U73122 on insulin-stimulated glucose transport in skeletal muscle. We found that insulin sensitivity, not responsiveness, was affected by U73122, implying that a PLC-dependent process may be involved in the control of insulin sensitivity. Future research is needed to clarify the effects of U73122 on insulin-induced GLUT 4 translocation.

ACKNOWLEDGMENT

We would like to thank Brian Barnes for his technical expertise in assisting with the animal surgeries.

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