Wortmannin inhibits insulin-stimulated but not contraction-stimulated glucose transport activity in skeletal muscle

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Abstract In skeletal muscle, glucose transport is stimulated by insulin, contractions and hypoxia. In this study, we used the phosphatidylinositol 3-kinase (PI 3-kinase) inhibitor wortmannin to examine whether (i) PI 3-kinase activity is necessary for stimulation of glucose transport by insulin in muscle, and (ii) PI 3-kinase mediates a step in the pathway by which contractions/hypoxia stimulate glucose transport. Wortmannin completely blocked insulin- and insulin-like growth factor-1-stimulated glucose transport in muscle. In contrast, wortmannin had no effect on the stimulation of glucose transport by contractions or hypoxia, providing evidence that PI 3-kinase activity is not involved in the activation of glucose transport by these stimuli.

Key words: Muscle; Glucose transport; Insulin; Contraction; Phosphatidylinositol 3-kinase

1. Introduction

Glucose transport in skeletal muscle is stimulated via at least two distinct pathways. One pathway is activated by insulin and insulin mimetic agents, the other by contractions or hypoxia. That two pathways are involved is evidenced by the findings that the maximal effects of insulin and contractions [1,2] or hypoxia [3] on sugar transport are additive. The maximal effects of hypoxia and contractions are not additive [3], suggesting that these two stimuli activate glucose transport by the same mechanism. Further evidence for two distinct pathways is provided by the finding that muscles of obese Zucker rats are resistant to the action of insulin, but not of contractions, on glucose transport [4]. The two pathways appear to have one or more steps in common, as the effects of both insulin and contractions/hypoxia are inhibited by polymyxin [5] and sphingosine [6].

Binding of insulin to its receptor induces activation of the insulin receptor's intrinsic tyrosine kinase activity, which, in turn, causes phosphorylation of several intracellular substrates, including insulin receptor substrate-1 (IRS-1) [7]. In its tyrosine-phosphorylated form, IRS-1 acts as a docking protein for phosphatidylinositol 3-kinase (PI 3-kinase) [8]; association of PI 3-kinase with phosphorylated IRS-1 results in activation of the kinase [9,10]. Studies using wortmannin or LY294002, potent inhibitors of PI 3-kinase [11,12], have shown that activation of PI 3-kinase is essential for the stimulation of glucose transport in adipocytes [12–14]. In this context, the purpose of the present study was to determine, using the inhibitor wortmannin, whether PI 3-kinase activity is (i) necessary for stimulation of glucose transport by insulin in skeletal muscle, and (ii) involved in the stimulation of glucose transport by contractile activity and hypoxia.

2. Materials and methods

2.1. Materials

Purified porcine insulin (Iletin II) was purchased from Eli Lilly and Co. (Indianapolis, IN). Recombinant human insulin-like growth factor-1 (IGF-1) was obtained from United States Biochemical Corp. (Cleveland, OH). 2-Deoxy-D-[1,2-^{3}H]glucose was purchased from American Radiolabeled Chemicals (St. Louis MO) and [U-^{14}C]mannitol was obtained from New England Nuclear (Boston, MA). Wortmannin, and all other reagents were obtained from Sigma (St. Louis, MO).

2.2. Treatment of rats and muscle preparation

Male Wistar rats weighing 80–120 g were provided with Purina Rat Chow and water ad libitum. Food was restricted to ~3 g after 5:00 p.m. the evening before the experiment. Rats were anesthetized by an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body weight) followed by removal of the epitrochlearis muscles. The suitability of the epitrochlearis, a small, thin forelimb muscle consisting predominantly of type IIb fibers, for measurement of glucose transport in vitro has been demonstrated previously [1,15].

2.3. Muscle incubations

Muscles were incubated initially for 30 min at 30°C in a Dubnoff shaking incubator in 2 ml of oxygenated Krebs–Henseleit buffer (KHB) supplemented with 2 mM sodium pyruvate, 36 mM mannitol and 0.1% radioimmunoassay grade bovine serum albumin (BSA) in the presence or absence of the indicated concentrations of wortmannin. Wortmannin was added from a stock solution prepared in dimethyl sulfoxide (DMSO). Identical concentrations of DMSO were added to the control medium. The final concentration of DMSO in the medium never exceeded 0.1%. Muscles were then incubated for an additional 30 min in identical medium with or without 12 nM insulin or 40 min with or without 25 nM IGF-1.

2.4. Muscle stimulation

Epitrochlearis muscles were stimulated to contract in vitro using a Grass model S11 stimulator as described previously [16]. Prior to and during stimulation, muscles were incubated in the presence or absence of wortmannin for 30 min as described above. Tetanic contractions were produced by stimulating at 100 Hz with 0.2 ms pulses for 10 s at a rate of 1 contraction/min for 10 min.

2.5. Muscle hypoxia

For studies of the effect of wortmannin on the maximal effects of hypoxia on glucose transport activity, muscles were initially incubated for 30 min in KHB gassed with 95% O_2/5% CO_2 in the presence or absence of wortmannin, then transferred to medium gassed with 95% N_2/5% CO_2 for 90 min. The hypoxic KHB contained 8 mM glucose, 32 mM mannitol, and 0.1% BSA with or without wortmannin. Prior to measurement of glucose transport activity, muscles were transferred to oxygenated KHB containing 2 mM sodium pyruvate and 36 mM

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Abbreviations: PI 3-kinase, phosphatidylinositol 3-kinase; IRS-1, insulin receptor substrate-1; IGF-1, insulin-like growth factor-1; KHB, Krebs–Henseleit buffer; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; 2-DG, 2-deoxy-D-glucose.

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mannitol, with or without wortmannin, for 10 min to wash glucose out of the extracellular space.

2.6. Measurement of glucose transport activity

2-Deoxy-D-glucose (2-DG) transport was measured by a modification of a previously described method [17]. After the initial incubation periods or electrical stimulation, all muscles were incubated for 20 min in 1.5 ml KHB containing 4 mM 2-deoxy-D-[1,2-^{3}H]glucose (0.38 mCi/mmol), 36 mM [U-^{14}C]mannitol (9 μCi/mmol), and 0.1% BSA. Wortmannin and insulin or IGF-1 were added if they were present previously. The incubation temperature was maintained at 30°C and the gas phase was 95% O_2/5% CO_2. Muscles were processed by boiling, and the extracellular space and intracellular 2-DG concentration (μmol · ml intracellular water^{-1} · 20 min^{-1}) were determined as previously described [15,17]. Under these conditions, 2-DG uptake accurately reflects glucose transport activity [17].

2.7. Statistical analysis

All data are presented as means ± S.E. The concentration-dependent effects of wortmannin on insulin-stimulated glucose transport were analyzed using a one-way analysis of variance (ANOVA). Post hoc analysis was performed using the Newman–Keuls test. Differences in basal or stimulated glucose transport activity measured in the presence or absence of wortmannin were assessed using Student's unpaired two-tailed *t*-test.

3. Results

Glucose transport activity stimulated by a maximally effective concentration of insulin (12 nM) was inhibited by wortmannin in a dose-dependent manner (Fig. 1). Insulin increased 2-DG uptake ~5-fold; the presence of 0.1 μM wortmannin decreased insulin-stimulated transport by 82%, while 0.5 μM wortmannin completely inhibited the effect of insulin. A similar, complete inhibition of insulin-stimulated transport was observed with higher concentrations of wortmannin. The inhibitory potency of wortmannin appears to be similar in adipocytes and skeletal muscle, as previous reports have shown that 0.1 to 1.0 μM wortmannin inhibits insulin-stimulated glucose transport in rat adipocytes by 80 to 100% [13,14].

The effects of wortmannin on basal, insulin- or IGF-1-stimulated glucose uptake are shown in Fig. 2. Wortmannin (2 μM) decreased basal 2-DG uptake slightly, but this reduction was not statistically significant. 2-DG uptake stimulated by maximally effective concentrations of either insulin or IGF-1 was completely inhibited by 2 μM wortmannin, providing evidence for an essential role for PI 3-kinase activity in the activation of sugar transport in skeletal muscle by these two stimuli.

Table 1
Effect of wortmannin on basal, contraction- and hypoxia-stimulated 2-DG transport in skeletal muscle

Treatment	2-DG Transport (μmol · ml^{-1} · 20 min^{-1})
Basal	0.56 ± 0.08 (6)
Wortmannin (2 μM)	0.45 ± 0.04 (6)
Contraction	2.75 ± 0.38 (7)
Contraction + wortmannin (2 μM)	2.46 ± 0.35 (7)
Hypoxia	2.55 ± 0.30 (11)
Hypoxia + wortmannin (2 μM)	2.32 ± 0.27 (11)

Values are means ± S.E. for the number of muscles indicated in the parentheses. Epitrochlearis muscles were incubated for 30 min with or without 2 μM wortmannin. Muscles were then stimulated to contract (10 min) or incubated in hypoxic medium (90 min), in the presence or absence of wortmannin, prior to measurement of 2-DG transport as described in section 2.

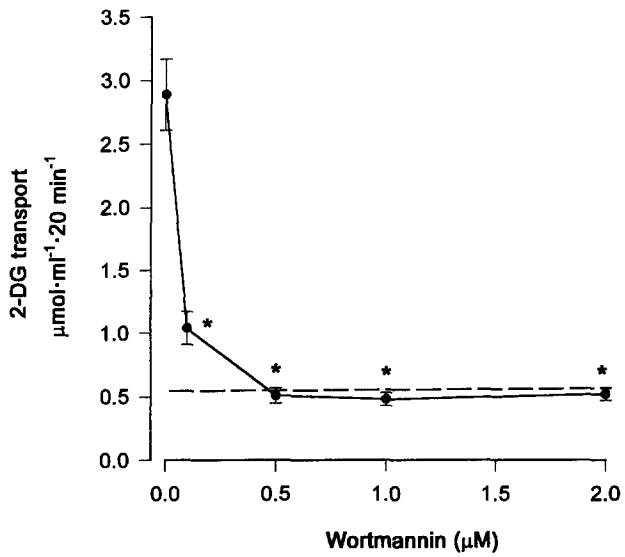

Fig. 1. Effect of wortmannin concentration on maximally insulin-stimulated 2-DG transport rate. Epitrochlearis muscles were incubated for 30 min at 30°C with the indicated concentrations of wortmannin, followed by a 30 min incubation in identical medium supplemented with 12 nM insulin. The rate of 2-DG transport was then measured as described in section 2. The broken line represents transport rate in the absence of insulin or wortmannin. Each point represents mean ± S.E. for 8–14 muscles. *$P < 0.01$ vs. insulin alone.

In contrast to the complete inhibition of insulin- or IGF-1-stimulated 2-DG uptake, the stimulation of glucose transport activity by hypoxia or muscle contractions was insensitive to the action of wortmannin (Table 1). This finding provides evidence that PI 3-kinase activity is not involved in the signaling pathway by which contractile activity and hypoxia activate glucose transport in skeletal muscle.

4. Discussion

There is increasing evidence to suggest that both the insulin receptor and IRS-1 are required for most insulin-mediated metabolic and mitogenic events [18–20]. An important point of divergence for the various insulin-mediated signaling pathways appears to be at the level of IRS-1, as activation of pp70 S6 kinase has been shown to require signaling to PI 3-kinase through IRS-1, while the IRS-1-dependent activation of mitogen activated protein kinase and pp90 S6 kinase appears to be PI 3-kinase independent [12]. A requirement for PI 3-kinase activity in the insulin-stimulation of Glut4 translocation [12,21] and glucose transport [12–14,21] has been demonstrated using two structurally distinct PI 3-kinase inhibitors, wortmannin and LY294002, but it was not known whether PI 3-kinase activity is required for the activation of glucose transport in muscle. Increased phosphorylation of IRS-1 and increased association of IRS-1 and PI 3-kinase have been demonstrated in skeletal muscle following insulin stimulation [22,23]. These observations, coupled with the complete inhibition of insulin-stimulated muscle glucose transport activity by wortmannin demonstrated in this study, indicate that the early signaling events by which insulin stimulates glucose transport in skeletal muscle are similar to those previously characterized in

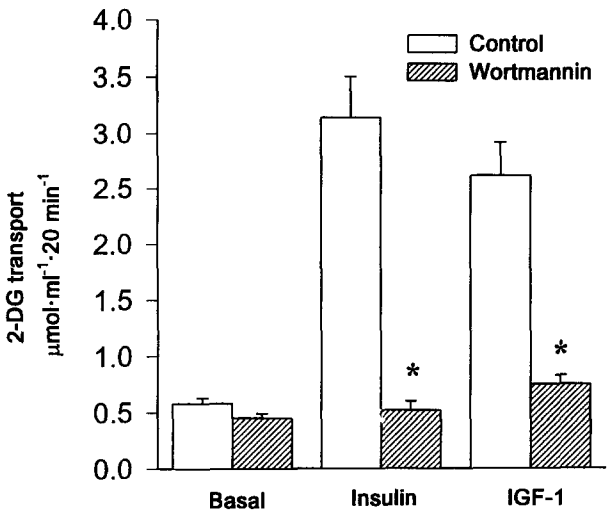

Fig. 2. Effect of wortmannin (2 μM) on insulin- and IGF-1-stimulated 2-DG transport. Epitrochlearis muscles were incubated for 30 min at 30°C with 2 μM wortmannin, followed by a 30 min incubation in identical medium with or without 12 nM insulin, or a 40 min incubation with or without 25 nM IGF-1. Glucose transport activity was assayed as described in section 2. Each bar represents mean ± S.E. for 6–11 muscles. *$P < 0.001$ vs. control.

adipocytes. Our findings provide evidence for an absolute requirement for PI 3-kinase activity in the insulin stimulation of glucose transport activity in skeletal muscle.

Insulin and IGF-1 stimulate glucose transport activity in skeletal muscle through separate receptors, each containing protein tyrosine kinase activity [24]. Although the receptors are distinct, the maximal effects of insulin and IGF-1 on glucose uptake [24,25] or Glut4 translocation [25] in isolated skeletal muscle are not additive, suggesting a common intracellular signaling mechanism for the activation of glucose transport. This concept is supported by the recent finding that both insulin and IGF-1 stimulate increased tyrosine phosphorylation of IRS-1, and increased IRS-1-associated PI 3-kinase activity in 32D myeloid progenitor cells coexpressing the insulin receptor and IRS-1 [20]. Our observations are consistent with the interpretation that the pathways for stimulation of glucose transport by insulin and IGF-1 in skeletal muscle converge at IRS-1, and that PI 3-kinase is involved in IGF-1 signaling.

Studies on isolated rat skeletal muscle preparations have demonstrated that the maximal effects of insulin and contractile activity [1,2] or insulin and hypoxia [3] on glucose transport activity are completely additive, findings that have been interpreted as evidence for a signaling pathway for insulin that is distinct from that for hypoxia or contractions. The lack of an inhibitory effect of wortmannin on contraction- or hypoxia-stimulated glucose uptake in the current study is consistent with this 'two pathway' concept. The finding that contraction- or hypoxia-stimulated glucose uptake were wortmannin-insensitive provides evidence that PI 3-kinase is not involved in the signaling cascade by which these stimuli activate muscle glucose transport.

Activation of glucose transport activity by insulin, hypoxia, and contractile activity are all inhibited by polymyxin B [5] and sphingosine [6], indicating that the two pathways possess one or more common steps. The finding that the PI 3-kinase inhibitor wortmannin completely inhibited insulin-stimulated, but had no effect on contraction- or hypoxia-stimulated, glucose transport activity indicates that any common elements of the signaling pathways leading to Glut4 translocation in skeletal muscle are downstream from PI 3-kinase.

In conclusion, the ability of wortmannin to inhibit 2-DG uptake stimulated by insulin and IGF-1, but not by contractions or hypoxia, provides further evidence that two distinct pathways exist for the activation of skeletal muscle glucose transport and that PI 3-kinase activity is not involved in the signaling pathway by which contractions or hypoxia stimulate glucose transport. In addition, our findings provide evidence for an essential role for PI 3-kinase activity in the activation of glucose transport by insulin or IGF-1 in skeletal muscle.

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