Effects of wortmannin on insulin- and ischemia-induced stimulation of GLUT4 translocation and FDG uptake in perfused rat hearts

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Abstract

Objective: Myocardial glucose transport is enhanced by hormonal and other stimuli such as ischemia and hypoxia which induce glucose transporter 4 (GLUT4) translocation. Whether insulin and ischemia share a common signaling mechanism is not yet known. This study investigated whether phosphatidylinositol 3-kinase PI3K, a signaling intermediate of the insulin-responsive pathway, also participates in the ischemia-induced stimulation of glucose.

Methods: Isolated Langendorff-perfused Sprague-Dawley rat hearts were subjected to 100 nmol/l insulin or 15 min of no-flow ischemia with/without 1 μmol/l wortmannin, an inhibitor of PI3K. After perfusion, relative subcellular glucose transporter GLUT4 distribution was assessed by membrane fractionation and immunoblotting and compared to controls. Uptake kinetics of the glucose analog [18F]fluoro-deoxyglucose (FDG) were also studied during perfusion of rat hearts.

Results: GLUT4 translocation to the plasma membrane (PM) was increased by insulin 1.8-fold and by ischemia 2.4-fold (P < 0.05). FDG uptake was increased by insulin 6.0-fold and by ischemia 6.2-fold (P < 0.05). Wortmannin 1 μmol/l inhibited insulin-mediated translocation of GLUT4 and increase in FDG uptake completely. However, it did not show any effect on ischemia-stimulated GLUT4 translocation or on ischemia-induced increase in FDG utilization. A significant correlation was found between relative GLUT4 translocation and FDG uptake in hearts of the insulin series (r = 0.9, P < 0.05) and of the ischemia series (r = 0.8, P < 0.05).

Conclusions: Our results demonstrate that wortmannin did not inhibit ischemia-induced stimulation of myocardial glucose transport, supporting the hypothesis of different signaling pathways for ischemia and insulin.

Keywords: Ischemic heart disease; GLUT4 translocation; Intracellular signaling; Phosphatidylinositol 3-kinase; Wortmannin; Rat, Sprague-Dawley

1. Introduction

Glucose plays an important role in the energy metabolism of the heart. Its utilization depends on a complex interplay of plasma substrate levels, humoral factors and energy demand. Uptake of glucose into the myocyte is stimulated by hormones including insulin or insulin-like growth factor IGF-I, which enhance glycolytic flux by increasing glucose transport and glycolytic enzyme activities [1–4]. During myocardial ischemia and hypoxia, glycolysis becomes the predominant metabolic pathway. Previous studies in our laboratory have shown that the increased glucose uptake under these conditions involves translocation of the insulin-responsive glucose transporter GLUT4 from intracellular compartments to the plasma membrane [5].

Recently, several investigators have partially characterized the insulin-induced signaling pathway leading to GLUT4 translocation. After insulin binds to its receptor, the activated receptor tyrosine kinase leads to the phosphorylation of several intracellular substrates such as insulin-receptor substrate-1 (IRS-1), which binds to phosphatidylinositol-3 kinase (PI3K). The association of IRS-1 with PI3K increases the kinase activity of the 110 kDa catalytic subunit, which phosphorylates phosphatidylinositol and its derivatives [6–8]. Although the exact mechanism is not yet defined, PI3K appears to play an important role in insulin-responsive stimulation of glucose transporter translocation [9–11]. This has been shown in several cell lines and in
isolated skeletal muscle using a potent inhibitor of the enzyme, wortmannin [12], which binds to the 110 kDa catalytic subunit of PI3K.

In contrast to the insulin pathway, very little is known about mechanisms of stimuli such as ischemia that also induce translocation of GLUT4. Recent results in skeletal muscle tissue indicate an additive effect of insulin and hypoxia or contraction on glucose transport but not of hypoxia and contraction [13–18], suggesting the existence of at least two different signaling pathways. Wheeler et al. [19] also described similar findings in the heart; however, other investigators did not observe a significant additive effect on glucose transport in isolated hearts or in cardiomyocytes induced by combined treatment with insulin and ischemia, hypoxia or insulin and contraction, respectively [5,20].

To gain more insight into the ischemia-induced stimulation of myocardial glucose transport, and to evaluate the possible role of PI3K in the ischemia-mediated signaling pathway, the present study investigated the effect of wortmannin on insulin- and ischemia-mediated GLUT4 translocation. Using the isolated perfused rat heart, the effect of insulin and ischemia with or without wortmannin on $^{18}$F-fluoro-deoxyglucose (FDG) kinetics was also investigated.

2. Materials and methods

2.1. Materials

Wortmannin (Sigma, Deisenhofen, FRG) was dissolved as a 10 mmol/l stock solution in dimethylsulfoxide (DMSO) and kept frozen at −20°C. Just before each experiment wortmannin was diluted to the appropriate concentration in perfusion buffer. DMSO concentration in the perfusate did not exceed 0.002%.

Rabbit anti-rat polyclonal antiserum against the carboxy-terminus of GLUT4 was kindly provided by Dr. Maureen Charron (Albert Einstein College of Medicine, Bronx, New York, USA). Polyclonal rabbit anti-rabbit antibody against Na⁺,K⁺-subunit α1 was purchased from Biomol (Hamburg, FRG), iodine-125 labeled goat anti-rabbit IgG from ICN (Meckenheim, FRG). Percoll and density marker beads were purchased from Pharmacia (Freiburg, FRG), coloured protein molecular weight marker were from Amersham (Braunschweig, FRG). 2-[$^{18}$F]FDG was synthesized via nucleophilic substitution using a modified Jülich method [21] with a radiochemical purity of >98%.

2.2. Methods

2.2.1. Animal model

The isolated, perfused rat heart was used to investigate cellular GLUT4 distribution by subcellular membrane fractionation technique and glucose utilization by FDG tissue tracer kinetics.

Male Sprague-Dawley rats, weighing 200–250 g, were used for all experiments. Rats were fed standard rat diet and given water ad libitum. One day before the experiment all rats were fasted overnight. Rats were anesthetized i.p. with pentobarbital (15 mg/100 g). Beating hearts were rapidly excised, rinsed in ice-cold Krebs-Henseleit bicarbonate buffer and mounted on an aortic cannula, fixed between two bismuth-germanate crystal (BGO) detectors for monitoring of radioactivity [22]. The hearts were perfused according to Langendorff with non-recirculating Krebs-Henseleit bicarbonate buffer (constant input function), containing 10 mmol/l glucose, approx. 500 μCi/l FDG, 100 mmol/l insulin (Hoechst, FRG) and 1 μmol/l wortmannin as described in the protocol. The buffer was gassed with 95% O₂ and 5% CO₂ and kept at 37°C. Aortic perfusion pressure was maintained at 60 mmHg. The perfusion apparatus consisted of multiple circuits connected with 3-way valves to allow switching between perfusion medium.

2.2.2. Perfusion protocol (Table 1)

All hearts were allowed to stabilize for 10 min (equilibration period), followed by 15 min monitoring of basal FDG uptake without additives. Six groups of hearts were investigated: The control group C, n = 8, insulin group (Ins, n = 4), insulin and wortmannin group (Ins + W, n = 4), wortmannin group (W, n = 3), ischemia group (I, n = 5) and ischemia and wortmannin group (I + W, n = 5). C hearts were perfused with FDG without further additives. Ins hearts were perfused with 100 mmol/l insulin added after baseline measurements. Ins + W hearts were perfused with both insulin and 1 μmol/l wortmannin after a 5 min pretreatment with wortmannin alone. W hearts were perfused with only wortmannin added. I and I + W hearts were subjected to 15 min of a no-flow ischemia, followed by 15 min of reperfusion. Prior to the no-flow ischemia and during reperfusion I + W hearts were perfused with wortmannin (Table 1). The concentration of 1 μmol/l wortmannin was chosen based on previous experiments with various concentrations of wortmannin which demonstrated complete inhibition of insulin-induced stimulation of FDG uptake [23]. All hearts in the study survived the perfusion with 1 μmol/l wortmannin. Duration of study of hearts in the ischemic group was longer than hearts in other groups due to the no-flow ischemia period, during which the hearts were not perfused. Control hearts of the insulin series were perfused for only 25 min to determine GLUT4 status at the end of baseline FDG uptake. However, duration of perfusion and the perfusion itself appear not to have any effects on the translocation of GLUT4 (for details, see Section 4).

At the end of the perfusion study, rat hearts were immediately placed in ice-chilled buffer, then gently blotted on paper towels to drain excess perfusate, weighed,
quick-frozen in liquid nitrogen and stored at \(-70^\circ C\). In order to determine whether the 15 min of reperfusion after ischemia in groups I and I+W would affect subcellular glucose transporter distribution compared to the status immediately after ischemia, 3 hearts in each group were frozen immediately after ischemia without reperfusion. This ensured that the measured translocation effects were due to experimental interventions and not affected by reperfusion.

2.2.3. Fractionation of plasma and intracellular membranes

Relative quantification of GLUT4 protein in subcellular compartments was achieved by a modified membrane preparation method according to Zaninetti et al. [24] and Sun et al. [5]. Experiments were performed in two separate series: In one series, the wortmannin effect on insulin treatment was investigated and compared to controls (groups C, Ins, Ins+W). In a subsequent protocol, the wortmannin effect on ischemia-treated hearts was investigated and compared to a new set of controls (groups C, W, I, I+W). By using separate sets of controls in each series, systematic variations in the assay, which may affect GLUT4 results, as well as dietary and differences in the metabolic state of the rats could be taken into account.

During a single preparation procedure, hearts of groups C, Ins, Ins+W or hearts of groups C, W, I, I+W were fractionated in parallel to exclude differences due to preparation.

Hearts were thawed on ice and minced with a razor blade in bicarbonate buffer (10 mmol/l NaHCO\(_3\), 5 mmol/l NaCl, pH = 7.0). Further homogenization was performed with an Ultra-Turrax-homogenizer (10,000 rpm, 2 \times 10 s), then a glass-glass homogenizer (10 strokes). A 100 \(\mu\)l aliquot of the resulting ‘crude homogenate’ (CH) was removed for further protein analysis and the remainder centrifuged (8000 \(\times\) g, 20 min). The supernatant was used for enrichment of intracellular membranes and therefore centrifuged at 44000 \(\times\) g (20 min). The pellet was discarded and the supernatant further centrifuged (200000 \(\times\) g, 1 h). The resulting pellet rich in intracellular membranes (MM) was resuspended in 300 \(\mu\)l of sucrose buffer (255 mmol/l sucrose, 10 mmol/l Tris-HCl, pH = 7.4, 2 mmol/l EGTA). To obtain the fraction rich in plasma membranes (PM), the 8000 \(\times\) g pellet was resuspended in a 10 mmol/l Tris-HCl buffer (pH = 7.4) and centrifuged at 200 \(\times\) g (20 min). The pellet was discarded and the resulting supernatant centrifuged at 44000 \(\times\) g (20 min). The 44000 \(\times\) g pellet was resuspended in 2 ml Tris-HCl buffer and layered on top of a 20% Percoll gradient in sucrose buffer. During 2 h of centrifugation at 55000 \(\times\) g in a Beckman SW 40 swinging bucket rotor, a plasma-membrane-rich fraction concentrates at a density of 1.030 g/ml as determined by density marker beads. This fraction was diluted with Tris-HCl buffer, pelleted at 200000 \(\times\) g for 1 h and resuspended in 300 \(\mu\)l sucrose buffer.

2.2.4. Immunoblotting procedure

For glucose transporter detection and quantification, 50 \(\mu\)g aliquots of MM, PM and CH of each heart together with a molecular weight marker were loaded on a discontinuous 10% SDS-polyacrylamide gel and electrophoretically separated according to the method of Laemmli [25]. With a modified tank transfer principle [26] separated proteins were blotted onto nitrocellulose membranes. The integrity of the transfer was evaluated by Ponceau S staining. After destaining in TBS (20 mmol/l Tris-HCl, pH = 7.5, 150 mmol/l NaCl, 1% Nonidet P-40), the nitrocellulose filter was blocked in 5% dry milk in TBS. A dilution of 1:800 for GLUT4 antiserum was prepared for further protein incubation. After washing and blocking, nitrocelluloses were incubated with \(^{125}\)I goat anti-rabbit IgG in 10% rat serum. Radioactive nitrocellulose membranes were washed in TBS, dried and exposed to a film or a phosphorus screen. The antibody directed against GLUT4 detected a band with an apparent molecular mass of 46 kDa.

2.2.5. Other assays

The BCA Protein Assay (Pierce) was used for determination of protein concentrations. Bovine serum albumin was used as standard.
To determine the relative enrichment of plasma membranes the corresponding marker enzyme, Na$^+$.K$^+$-ATPase, was detected via immunoblotting as described above with a specific polyclonal antibody against subunit $\alpha_1$ (1:500 in blocking buffer). Subunit isoform $\alpha_1$ was chosen on account of results which demonstrated no change in amount or subcellular distribution after treatment with insulin [27].

2.3. Data analysis

2.3.1. FDG data

Tissue radioactivity was monitored by a pair of BGO detectors interfaced with a computer. Coincidence, single and random events were sampled every second. Time–activity curves were generated by subtraction of random events from coincidence events, decay corrected, and normalized to the radioactivity of perfusate [22,28]. FDG uptake data were expressed as ml perfusate/g heart tissue/min or presented as the ratio of post-intervention FDG uptake to baseline FDG uptake.

2.3.2. Membrane protein data

Documentation and quantification of protein bands, lying in the linear range of the screen, was achieved by a Phosphor Imager (Molecular Dynamics). Total protein recoveries in PM and MM, GLUT4 recoveries and percentages of subcellular GLUT4 distribution were calculated according to Sun et al. [5].

2.3.3. Statistical analysis

All data are expressed in mean $\pm$ standard deviation (s.d.), except coronary flow data (mean $\pm$ standard error, s.e.m.). Statistical significance ($P < 0.05$ with Bonferroni correction) was evaluated by paired or unpaired Student’s t-test. Differences among experimental groups were evaluated using unpaired Student’s t-test. Differences between post-intervention data compared to baseline data from the same heart were determined using paired Student’s t-test.

2.4. Statement

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication. No. 85-23, revised 1985).

3. Results

3.1. Subcellular membrane fractionation

3.1.1. Insulin series

Total protein recoveries of groups C, Ins and Ins + W were $1.7 \pm 0.8\%$ for PM and $2.0 \pm 0.2\%$ for MM compared to CH. There was no significant difference in total protein recovery among these groups (Table 2, $P > 0.05$). GLUT4 recoveries in the plasma membrane- and intra-cellular membrane-enriched fractions were $20.5 \pm 7.7\%$ compared to the amount of GLUT4 in CH and did not differ among groups (Table 2, $P > 0.05$). Enrichment of Na$^+$.K$^+$-ATPase subunit $\alpha_1$ was $6.8 \pm 2.8$-fold in groups C, Ins and Ins + W. Differences in enrichment between groups were not significant (Table 2, $P > 0.05$).

3.1.2. Ischemia series

Total protein recoveries in PM and MM compared to CH of groups C$_2$, W, I and I + W were $1.0 \pm 0.3$ and $2.4 \pm 0.2\%$, respectively. Mean GLUT4 recovery was $21.4 \pm 3.3\%$ in PM and MM vs. CH. Both total protein and GLUT4 recovery did not show a statistically significant difference (Table 3, $P > 0.05$). Marker enzyme Na$^+$.K$^+$-ATPase enrichment was $10.5 \pm 4.8$-fold in groups C$_2$, W, I and I + W and was not significantly different between groups (Table 3, $P > 0.05$).

3.2. Influence of wortmannin on insulin- or ischemia-induced translocation of GLUT4 from intracellular membrane compartments to the plasma membrane

3.2.1. Insulin series

Results of the cellular distribution of GLUT4 in control, insulin-, and insulin + wortmannin-treated rat hearts are shown in Fig. 1a. Addition of insulin to the perfusate induced a 1.8-fold significant increase in GLUT4 proteins on PM compared to control C$_1$ hearts ($P < 0.05$). GLUT4 on MM decreased concomitantly. Treatment of hearts with

| Table 2 |
| Membrane preparation recoveries of the rat heart study with insulin |

<table>
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<tr>
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<th>Control 1</th>
<th>Insulin</th>
<th>Insulin + wort.</th>
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<td>($n = 4$)</td>
<td>($n = 4$)</td>
<td>($n = 4$)</td>
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<tr>
<td>Total protein</td>
<td>PM: 1.9 $\pm$ 0.9</td>
<td>PM: 1.6 $\pm$ 0.6</td>
<td>PM: 1.7 $\pm$ 0.8</td>
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<tr>
<td>Recovery (%) of CH</td>
<td>MM: 1.9 $\pm$ 0.1</td>
<td>MM: 2.1 $\pm$ 0.2</td>
<td>MM: 2.1 $\pm$ 0.3</td>
</tr>
<tr>
<td>GLUT4 recovery (%) in MM + PM</td>
<td>20.0 $\pm$ 7.4</td>
<td>18.9 $\pm$ 8.6</td>
<td>22.5 $\pm$ 7.2</td>
</tr>
<tr>
<td>Na$^+$.K$^+$-ATPase $x$-fold (PM vs. MM)</td>
<td>6.8 $\pm$ 2.8</td>
<td>7.1 $\pm$ 3.1</td>
<td>6.8 $\pm$ 2.5</td>
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</table>

Total protein recovery in PM and intracellular vesicles (MM) is shown as a percentage of total protein compared to the crude homogenate (CH = 100%). GLUT4 recovery in PM and MM is shown as a percentage of total GLUT4 compared to CH (100%). Na$^+$.K$^+$-ATPase is expressed as the $x$-fold amount of enrichment in PM vs. MM. Data shown are means $\pm$ s.d. of 4 independent experiments.
both insulin and 1 \( \mu \text{mol/l} \) wortmannin not only inhibited the insulin-mediated increase in GLUT4 on PM, but also significantly decreased GLUT4 molecules on PM below GLUT4 levels of C hearts. Representative Western-blotting results are shown in Fig. 3a.

### Table 3

<table>
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<th>Membrane preparation recoveries in rat heart experiments with ischemia</th>
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<tr>
<td>Control 2 ((n = 4))</td>
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<tr>
<td>Total protein recovery (% of CH)</td>
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<tr>
<td>GLUT4 recovery (%) in MM + PM</td>
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<tr>
<td>Na(^+),K(^+)-ATPase (PM vs. MM)</td>
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Total protein recovery in plasma membranes (PM) and intracellular vesicles (MM) is shown as a percentage of total protein compared to the crude homogenate (CH = 100%). Percentage of GLUT4 recovery in PM and MM was calculated and compared to CH as described in Ref. [5]. Na\(^+\),K\(^+\)-ATPase is expressed as the \( x \)-fold enrichment in PM vs. MM. Data shown are means \( \pm \) s.d. of 3–5 independent experiments.

#### 3.2.2. Ischemia series

Results in Fig. 2a show that in control hearts of the ischemia experiments \((C_2)\), 14.7 \( \pm \) 2.4\% of total GLUT4 was found in the plasma-membrane-rich fraction. Perfusion with 1 \( \mu \text{mol/l} \) wortmannin alone did not significantly change basal GLUT4 distribution \((P = 0.347\) versus \(C_2\)). Subjecting rat hearts to 15 min of no-flow ischemia re-

![Fig. 1. Effect of wortmannin on insulin-induced (a) translocation of GLUT4 and (b) stimulation of FDG uptake in isolated perfused rat hearts.](image1)

![Fig. 2. Influence of wortmannin on ischemia-stimulated (a) GLUT4 translocation and (b) increase in FDG uptake.](image2)
Control 1 Control 2 Wortmannin Insulin Insulin + wortmannin Ischemia Ischemia + wortmannin
Baseline FDG uptake (ml / g / min)
Control 1 (n = 4) Control 2 (n = 4) Wortmannin (n = 3) Insulin (n = 4) Insulin + wortmannin (n = 4) Ischemia (n = 3) Ischemia + wortmannin (n = 5)
0.0244 ± 0.0048 0.0225 ± 0.0162 0.0570 ± 0.0495 0.0212 ± 0.0142 0.0250 ± 0.0057 0.0159 ± 0.0075 0.0127 ± 0.0044
Coronary flow (ml / min / g)
Baseline 12.4 ± 0.6 9.9 ± 0.4 11.1 ± 0.2 10.0 ± 0.7 9.9 ± 0.7 9.2 ± 0.6 9.1 ± 0.7
Post 9.2 ± 1.2 11.3 ± 0.2 9.8 ± 0.5 9.4 ± 0.8 10.6 ± 0.5 11.6 ± 1.4

Baseline FDG uptake shown as ml perfusate/g tissue/min.
Values are means ± s.d. Coronary flow values of Langendorff-perfused rat hearts in ml perfusate/min/g tissue during baseline FDG uptake (baseline) and during or after treatment with insulin, ischemia with/without wortmannin (post). Values are means ± s.e.m. Control 1 = controls of the insulin series, Control 2 = controls of the ischemia series.

3.3. Effects of wortmannin on insulin- and ischemia-stimulated FDG uptake in the isolated perfused rat heart

Mean baseline FDG uptake of all groups (C, Ins, Ins + W, W, I, W + I) was 0.0244 ± 0.0241 ml/g/min and did not significantly differ among experimental groups (Table 4).

Results calculated as a ratio of post-FDG uptake versus baseline FDG uptake are shown in Fig. 1b and Fig. 2b. In control hearts a small increase in baseline FDG utilization (1.49 ± 0.22-fold) during 50 min of perfusion was observed (P < 0.05). Insulin 100 nmol/l significantly increased FDG uptake in rat hearts 6.0 ± 2.9-fold as compared to baseline values (P < 0.05, Fig. 1b). Perfusion of insulin-treated hearts with 1 μmol/l wortmannin completely inhibited the insulin-induced increase in FDG uptake (1.37 ± 0.75-fold vs. 1.49 ± 0.22-fold of controls). Perfusion with wortmannin alone did not significantly change basal FDG uptake (1.04 ± 0.86-fold versus baseline).

Similar to insulin 15 min of no-flow ischemia significantly stimulated myocardial FDG uptake 6.2 ± 4.1-fold (P < 0.05), but addition of 1 μmol/l wortmannin did not alter the ischemia-induced stimulation of FDG utilization (6.8 ± 2.36-fold in group I + W vs. 6.2 ± 4.1-fold in group I) (Fig. 2b).

3.4. Coronary flow

Table 4 shows coronary flow data recorded during non-recirculating Langendorff perfusion. In all groups there were no statistically significant differences in coronary flow values between baseline and post-FDG uptake. However, baseline coronary flow in the wortmannin group was higher than that in the other groups, but did not change significantly during subsequent wortmannin treatment. Coronary flow in the ischemia group increased immediately after ischemia during reperfusion, then returned to near-baseline values. However, the difference compared to baseline was not statistically significant (P = 0.06 by paired t-test). After normalization of FDG uptake with coronary flow data (extraction), the same linear relationship between extraction of FDG and GLUT4 protein on PM could be shown as was demonstrated for FDG uptake and GLUT4 translocation (see below) (insulin hearts, r = 0.9; ischemia hearts, r = 0.8; data not shown). Thus, small apparent changes in flow in some cases had no significant effect on FDG uptake.
3.5. Correlation between FDG uptake and GLUT4 translocation

In 10 rat hearts of the insulin series and 8 rat hearts of the ischemia series, both FDG uptake and GLUT4 translocation status were investigated in the same heart at the end of the 15 min post-intervention FDG perfusion. As shown in Fig. 4a,b, changes in myocardial FDG utilization under different conditions significantly correlated with changes in the subcellular distribution of GLUT4 (insulin hearts: $r = 0.9$, ischemia hearts: $r = 0.8$, both $P < 0.05$).

4. Discussion

The results of this study show that wortmannin, a PI3K inhibitor, in contrast to its inhibitory effects on insulin-induced stimulation of GLUT4 translocation and FDG uptake, did not alter GLUT4 translocation and increased FDG uptake induced by 15 min of no-flow ischemia. In the perfused rat heart model it was demonstrated that the approximate 2.4-fold stimulation of GLUT4 translocation and 6-fold increase in FDG uptake following ischemia was not affected by 1 μmol/l wortmannin, confirming reports from glucose utilization studies during hypoxia in skeletal muscle [29,30]. At this concentration, however, wortmannin completely blocked GLUT4 translocation and the increase in FDG uptake stimulated by insulin. Other studies also reported inhibition of insulin-induced glucose transport by wortmannin in rat epitrochlearis and hemidiaphragma muscle, L6 skeletal muscle cells and 3T3 L1 fibroblasts [29–31,10]. Based on these data, the PI3K-dependent signaling system, which plays a role in insulin-responsive pathways, does not appear to participate in the ischemia-responsive stimulation of GLUT4 translocation to PM in the heart.

4.1. Glucose metabolism in ischemia

Myocardial energy metabolism depends on the oxidation of various substrates [32–34]. Substrate availability, hormonal milieu and energy demand govern the relative distribution of each substrate to the myocardial high-energy phosphate production. Under ischemic conditions, glucose becomes an important source for both anaerobic and aerobic energy production. Under ischemic conditions, glucose becomes an important source for both anaerobic and aerobic energy production [34,35]. Previous studies have shown that extraction of glucose and glucose analogue FDG increases during and immediately after ischemia in a variety of experimental models [34,36]. Clinical studies using metabolic imaging with positron emission tomography (PET) support the notion that ischemically compromised myocardium displays increased uptake of glucose acutely after ischemia [37] as well as in the chronic state (hibernating/stunned myocardium) [38,39]. Regulation of glucose metabolism in the myocardium involves key glycolytic enzymes such as hexokinase, phosphofructokinase and pyruvate dehydrogenase as well as the modification of glucose transport across the cell membrane. The transport proteins, GLUT4 and GLUT1, are known to play an important role in the heart [5,24,19]. In response to insulin, translocation of GLUT4 is mainly responsible for the increased glucose utilization, while GLUT1 proteins provide glucose transport principally independently of hormonal control. Acute hypoxia and ischemia have been shown to enhance glucose transport primarily by translocation of GLUT4, while extended periods of ischemia may increase expression of GLUT1 as suggested in animal as well as in clinical studies [40–42].
This study addressed for the first time the regulatory processes of GLUT4 translocation in myocardial ischemia with respect to PI3K and compared it to the insulin-mediated mechanisms. The independence of GLUT4 translocation from the PI3K signaling pathway in acute ischemia supports the hypothesis that alternate pathways are responsible for the increase in myocardial glucose transport.

4.2. Glucose transport proteins and FDG kinetics

The assessment of subcellular distribution of transport proteins and comparison with FDG kinetics allows the correlation of membrane transport capacity and substrate flux. However, due to the above discussed complex interaction of substrates, FDG tissue kinetics represent the utilization of only one substrate in overall energy metabolism. Therefore, the variation in myocardial FDG uptake during baseline (Table 4) and after stimulation with insulin and ischemia (Fig. 1b and Fig. 2b) were not surprising despite the perfusion with glucose as the only substrate and the standardization of metabolic conditions prior to the study. These differences in metabolism among individuals emphasized the need for comparison of FDG uptake after intervention with its own baseline.

The results also show that FDG uptake increased 1.49-fold over time in untreated rat hearts (Fig. 1b). Since glucose was the only substrate in the perfusate and endogenous breakdown of triglycerides and glycogen may decrease over time, the rate of exogenous glucose utilization may increase. These changes, however, were small compared to the large increases induced by insulin or by 15 min of no-flow ischemia but emphasized the necessity for such controls. Parallel experiments investigating the extent of GLUT4 translocation in untreated control rat hearts without perfusion and in hearts with 50 min perfusion did not show a significant difference (non-perfused hearts 15.3 ± 1.4% vs. perfused hearts 14.1 ± 4.3% GLUT4 on PM). Thus, the perfusion appeared not to have affected GLUT4 status.

Measurement of subcellular glucose transporter distribution was used to determine the participation of PI3K in the signaling pathway of insulin- and ischemia-induced GLUT4 translocation to exclude the possibility of wortmannin affecting deoxyglucose phosphorylation. The results of subcellular GLUT4 distribution in insulin- and ischemia-stimulated myocardial tissue after treatment with 1 μmol/l wortmannin correlated with our findings of FDG-uptake measurements. Wortmannin appeared to have had no effect on ischemia-stimulated GLUT4 translocation (Fig. 2a and Fig. 3b). However, with the same wortmannin concentration GLUT4 translocation induced by insulin was completely inhibited (Fig. 1a and Fig. 3a). This is confirmed by others who have shown inhibition of insulin-stimulated GLUT4 translocation with the same or smaller concentrations of wortmannin in 3T3 L1 cells [10], skeletal muscle [17], adipocytes [3], oocytes [43] or in Chinese hamster

ovary cells transfected with GLUT4myc [11]. The observed inhibition of translocation below control levels was unexpected. It is probably due to the somewhat variable baseline values in some hearts, as also observed in FDG uptake data. In hearts with low baseline FDG uptake, wortmannin did not reduce GLUT4 on PM below that of controls.

Previous results in skeletal muscle treated with a combination of wortmannin and insulin, hypoxia or electrically stimulated contraction showed an inhibitory effect of wortmannin on glucose transport only in the case of insulin stimulation but not in combination with hypoxia and contraction [17,29,30]. In contrast, another study recently observed a 95% inhibition of contraction-stimulated glucose uptake in skeletal muscle with 10 μmol/l wortmannin, whereas glucose uptake due to hypoxia was unaffected by wortmannin [44]. These findings and the observed additive effect of insulin and hypoxia or contraction [13–18] further suggest the existence of at least 2 or 3 different signaling pathways. A common pathway may be shared by insulin and IGF-I, while another pathway may exist for stimuli such as ischemia/hypoxia, and probably a third pathway for contraction. Investigations with sphingosin and polymyxin B, which inhibit glucose transport induced by insulin, hypoxia and contraction suggest that all pathways share some common steps [29], probably the last few steps prior to GLUT4 vesicle trafficking. At present, the primary signals activated by ischemia or hypoxia are unknown. However, protein kinase C (PKC) may be involved in these cases. Recently, it was shown that PKC is activated by phorbol 12-myristate 13-acetate (PMA) and PMA stimulates GLUT4 translocation without affecting PI3K [45]. However, the role of PKC in ischemia-induced stimulation of GLUT4 translocation remains to be investigated.

4.3. Limitations of the study

Recently, the specificity of wortmannin, a fungal secondary metabolite and an efficient inhibitor of PI3K in cell culture or isolated skeletal muscle models [12,46], has been questioned. In addition to wortmannin’s inhibitory effect on myosin light chain kinase and phospholipase D, it has also been shown to block phosphatidylinositol 4-kinase. However, in all cases reported, the enzyme sensitivity to wortmannin inhibition was at least 10- to 1000-fold less than that of PI3K [12,47]. Based on these data, it is reasonable to assume that the concentration used in our studies specifically inhibited PI3K. It is important to note that treatment with wortmannin alone did not affect coronary flow values (post-intervention flow compared to baseline flow) or FDG kinetics as an index of exogenous glucose utilization.

Relative estimation of glucose transporter localization by the subcellular membrane fractionation technique has been used. The high amount of myofibrils closely connected to the sarcolemma complicates the separation of the
sarcolemma membrane, and therefore cross-contamination with intracellular vesicles containing glucose transporters cannot be excluded completely and may account for some underestimation of glucose transporter translocation [48]. However, enrichment of the plasma membrane marker, Na⁺,K⁺-ATPase, in the plasma membrane was similar to or better than values reported in previous publications [5,24]. Although the Na⁺,K⁺-ATPase was not significantly different in the hearts of each series, there was a difference in enrichment of this marker enzyme between the series with insulin and the later prepared series with ischemia, most probably due to a small variation during the membrane preparation. However, no correlation was found between enrichment of Na⁺,K⁺-ATPase subunit α1 and percentage of GLUT4 on PM (data not shown). Thus, the different enrichment of Na⁺,K⁺-ATPase could not explain the differences in amount of GLUT4 on PM between the two groups of control hearts. The slightly increase in plasmalemmal GLUT4 in C₁ may be due to metabolic differences or minor technical variations. Comparison of FDG uptake values showed no significant differences between the two control groups, indicating technical variations during membrane preparation as a probable reason for the differences measured. Therefore, analysis of controls and intervention studies in the same series of experiments are necessary.

Hariharan et al. [49] has reported limitations of FDG in the determination of absolute rates of myocardial glucose utilization compared to tritiated glucose, because of a different affinity of hexokinase for glucose and the glucose analog. FDG. FDG kinetics were used in the presented study as a functional index for glucose transport and not for measuring absolute myocardial glucose utilization. The 6-fold increase in FDG uptake after ischemia demonstrated in this study correlates closely with previous observations from other groups: In skeletal muscle tissue a 6- and 4-fold increase in FDG uptake after ischemia demonstrated for measuring absolute myocardial glucose utilization. The study as a functional index for glucose transport and not an analog, FDG. FDG kinetics were used in the presented different affinity of hexokinase for glucose and the glucose utilization compared to tritiated glucose, because of a determination of absolute rates of myocardial glucose in the composition of the perfusion medium lower insulin.

This discrepancy in our results may be due to differences or minor technical variations. Comparison of FDG uptake values showed no significant differences between the two control groups, indicating technical variations during membrane preparation as a probable reason for the differences measured. Therefore, analysis of controls and intervention studies in the same series of experiments are necessary.

In addition, it has been shown that catecholamines are released during ischemia [50]. It is possible that the myocardial release of catecholamines may have an important role in the ischemia-induced stimulation of FDG uptake and GLUT4 translocation. However, this question was not addressed in the experimental protocol of this study and requires further investigation. The role of catecholamines on glucose transport and utilization is controversially discussed in the literature. Rattigan et al. [51] recently reported on the effect of catecholamines on glucose transport and GLUT4 translocation. In perfused isolated rat heart they showed that epinephrine and the β-agonist, isoproterenol, both stimulated 2-deoxyglucose uptake and lead to GLUT4 translocation. Interestingly, the combination of the catecholamines and insulin showed no additive effect compared to the effect of insulin alone, suggesting a similar signaling pathway for α-adrenoreceptor stimulation and insulin. These results differ somewhat from the results of Fischer et al. [52] which also showed that catecholamines stimulate cardiomyocyte glucose transport and GLUT1, GLUT4 translocation through α-adrenergic receptors. This study suggested a mechanism distinct from that mediated by insulin, because the effects were additive. Recently, Shimizu et al. [53] also showed in brown adipocytes that noradrenaline stimulation of glucose transport is additive to that of insulin. However, in their study GLUT1 and GLUT4 translocation did not contribute to noradrenaline-stimulated glucose transport. Therefore, the effect of catecholamines on glucose transport remains controversial and is being investigated in various laboratories.

In conclusion, the results of the present study provide the first direct evidence that PI3K activity does not play a role in the myocardial ischemia-responsive signaling mechanism, in contrast to its participation in the insulin-responsive signaling mechanism. The data suggest a different signaling pathway induced by ischemia from that induced by insulin, which needs to be investigated in future studies.

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