Ischemia Induces Translocation of the Insulin-Responsive Glucose Transporter GLUT4 to the Plasma Membrane of Cardiac Myocytes

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Background Acute myocardial ischemia is accompanied by an increase in glucose uptake and metabolism, which appears to be important in protecting myocardial cells from irreversible ischemic injury. Because insulin augments myocardial glucose uptake by inducing the translocation of glucose transporters from an intracellular compartment to the plasma membrane, we hypothesized that acute ischemia would trigger a similar translocation.

Methods and Results We used a subcellular fractionation method to separate intracellular membranes and plasma membranes from control, ischemic, and hypoxic Langendorff-isolated perfused rat hearts and determined the expression of the major myocardial glucose transporter, GLUT4, in these separated membrane fractions. We found that translocation of GLUT4 molecules occurred in ischemic, hypoxic, and insulin-treated hearts and in hearts that underwent ischemia plus insulin treatment. The percentages of GLUT4 molecules present on the plasma membrane in the different conditions were as follows: control, 18.0±2.8%; ischemia, 41.3±9.4%; hypoxia, 31.1±2.9%; insulin, 61.1±2.6%; and ischemia plus insulin, 66.8±5.7%. Among the statistically significant differences in these values were the difference between control and ischemia and the difference between ischemia alone and insulin plus ischemia.

Conclusions Ischemia causes substantial translocation of GLUT4 molecules to the plasma membrane of cardiac myocytes. A combination of insulin plus ischemia stimulates an even greater degree of GLUT4 translocation. GLUT4 translocation is likely to mediate at least part of the increased glucose uptake of ischemic myocardium and may be a mechanism for the cardioprotective effect of insulin during acute myocardial ischemia. (Circulation. 1994;89:793-798.)

Key Words • coronary disease • metabolism

Ins ischemia induces multiple changes in myocardial cell metabolism. Prominent among these changes is a marked increase in glucose uptake and use that occurs shortly after the onset of mild to moderate ischemia.1 Multiple clinical and experimental trials have shown that increased glucose uptake and metabolism during acute myocardial ischemia are associated with preserved diastolic and systolic myocardial function, decreased release of myocardial enzymes, improved recovery during reperfusion, and prevention of ischemic contracture.1-4 However, the mechanism by which ischemia stimulates glucose uptake remains unknown.

Over the past several years, cDNAs have been isolated for the proteins that transport glucose into myocardial cells.7-18 The predominant transporter in cardiac muscle is the insulin-responsive glucose transporter GLUT4.19 It appears that very few GLUT4 molecules are located on the plasma membrane of myocardial cells under basal conditions. However, when exposed to maximal insulin levels, approximately 40% of the myocardial GLUT4 molecules are translocated to the plasma membrane.20 This increase in plasma membrane GLUT4 molecules appears to be the major mechanism by which insulin acutely stimulates glucose uptake in insulin-sensitive tissues.21,22 To determine whether ischemia stimulates glucose uptake through a similar mechanism, we used a subcellular membrane fractionation method to separate rat myocardial plasma membranes from intracellular membranes. We have found that acute ischemia causes substantial translocation of GLUT4 molecules and that a combination of insulin plus ischemia stimulates an even greater degree of GLUT4 translocation.

Methods

Animal Models Sprague-Dawley rats (weight, 200 to 250 g) were used for all experiments. Control animals and animals used for ischemic and hypoxic studies were fasted overnight. Insulin-treated animals were not fasted and were given intraperitoneal injections of insulin (8 U/kg) plus D-glucose (1 g/kg) 30 minutes before they were killed. All animals were anesthetized with intraperitoneal injection of sodium pentobarbital. The hearts were removed and perfused via the Langendorff method. Aortic perfusion pressure was maintained at 60 mm Hg throughout. Control hearts were perfused for 30 minutes at 37°C with Krebs-Henseleit buffer containing 5 mmol/L glucose and gassed with 95% O2/5% CO2. Hypoxia hearts were perfused identically for 5 minutes, then changed to buffer with 95% air/5% CO2 for an additional 30 minutes. Ischemia hearts were perfused identically to the control hearts for 15 minutes, then had perfusion halted for 15 minutes. Insulin-stimulated hearts were perfused identically to the control hearts, but the perfusate also contained 100 nmol/L regular insulin. Insulin plus ischemia hearts were perfused identically to the insulin-stimulated hearts for 15 minutes, then had perfusion stopped...
for an additional 15 minutes. Only hearts that were still beating at the end of the experimental period were used for further assessment. At the end of the experimental period, the hearts were drained of excess perfusate and snap-frozen in liquid nitrogen.

**Subcellular Membrane Fractionation**

Membrane fractionation was performed by use of a modification of the method of Zaninetti et al.23 For the initial experiments, three hearts exposed to a single condition (control, ischemia, or hypoxia) were combined together. For subsequent experiments, the protocol was performed with individual hearts (other details of the fractionation protocol were identical). The hearts were thawed on ice, and each heart was trimmed of fat and washed in cold phosphate-buffered saline, pH 7.4. The heart was then transferred to an ice-cold Petri dish containing 4 mL of 10 mmol/L sodium bicarbonate and 5 mmol/L sodium azide, pH 7.0, and immediately minced with a razor blade. The resultant slurry was then homogenized with a Brinkmann Polytron tissue homogenizer at setting 5 for 20 seconds. The homogenate was transferred to a handheld 7-mL glass/glass homogenizer and subjected to 10 pestle strokes. A 100-mL aliquot of the homogenate was removed for later analysis (crude homogenate); the remainder was centrifuged at 12,000g for 20 minutes. The resultant supernatant was saved for preparing the intracellular membrane (MM) fraction, containing intracellular membranes, and the pellet was used for preparing the plasma membrane (PM) fraction. The pellet was resuspended in 4 mL of 10 mmol/L Tris-HCl, pH 7.4, and centrifuged at 200g for 20 minutes. The pellet was discarded, and supernatant was evenly divided into 0.8-mL portions, each of which was gently layered on top of a 20% (vol/vol) Percoll gradient in buffer C (255 mmol/L sucrose; 10 mmol/L Tris-HCl, pH 7.4, 2 mmol/L EDTA) in a 4-mL tube and centrifuged at 55 000g for 1 hour. The band at density 1.030 as determined by density marker beads (Pharmacia) was aspirated and pelleted by centrifugation at 170 000g for 1 hour and resuspended in 0.2 mL of buffer C as the PM fraction. The supernatant from the original 700-g centrifugation was centrifuged at 48 000g for 20 minutes. The membranes in the resultant supernatant were then pelleted by centrifugation at 170 000g for 1 hour and resuspended in 0.2 mL of buffer C as the MM fraction. Protein concentrations for each fraction were determined by Coomassie protein assay (Pierce).

Relative enrichments of the crude homogenates and PM and MM fractions were assayed by analysis of marker enzymes. NADPH-cytochrome c reductase is a mitochondrial enzyme that is enriched in the microsomal fraction,23 and Na+,K+-ATPase α1 and α2 subunits are enriched in the PM fraction. NADPH-cytochrome c reductase activity was determined by a modification of the protocol of Williams and Kamin.24,25 Relative levels of Na+,K+-ATPase α1 or α2 subunit were determined by immunoblotting using rabbit anti-rat polyclonal antiserum specific for each isotype (UBI). Antibodies to either subunit gave identical results in initial experiments. However, blots with the α1 antibody were more intensely labeled, so this antiserum was used exclusively in later experiments.

**Immunoblot Analysis**

GLUT4 levels were semiquantified by immunoblotting. Aliquots of 50 μg of the MM and PM fractions and crude homogenate from either fasted and insulin-treated hearts or control, ischemia, hypoxia, and insulin plus ischemia hearts were electrophoresed on individual 10% SDS-polyacrylamide gels. Immunoblotting and GLUT4 determinations were performed as previously described.26 The contents of the gels were transferred electrophoretically to nitrocellulose membranes, which were then stained briefly with Ponceau S to determine uniformity of electrophoretic transfer. The membranes were washed in Tris-buffered saline (TBS) (20 mmol/L Tris-HCl, pH 7.5; 150 mmol/L NaCl; 1% Nonidet-P-40 [Sigma]) and blocked in 5% dry milk in TBS. The membranes were then incubated with a 1:800 dilution of a rabbit polyclonal antiserum directed against a peptide encoding the 16 carboxy-terminal amino acids of rat GLUT426 (gift of Dr Maureen Charron). The filters were then washed three times in TBS, blocked again, and incubated with 125I-donkey anti-rabbit IgG (ICN). Finally, the filters were washed three times in TBS, dried, and exposed to film. Semiquantification of the 125I signal was determined either by direct scintillation counting of the excised GLUT4 membrane bands or by videosensitometry using the National Institutes of Health IMAGE 1.43 program. For densitometric analysis, several exposures of each blot were performed on prefleshed Xomat-AR film, and only those films were used for which the resultant autoradiographic signals were within the linear response range of the film.

**Data Analysis**

GLUT4 data were expressed as the relative number of GLUT4 molecules in 50 mg protein from the various fractions from paired sets of control, ischemia, and hypoxia hearts. For all groups, the data were also expressed as the percentage of total GLUT4 protein in either of the two membrane fractions as follows:

\[ \text{PM GLUT4} = \frac{\text{OD PM GLUT4} \times \% \text{of Total Protein Recovery in PM Fraction}}{\text{OD PM GLUT4} + \% \text{of Total Protein Recovery in PM Fraction}} \]

\[ \text{MM GLUT4} = \frac{\text{OD MM GLUT4} \times \% \text{of Total Protein Recovery in MM Fraction}}{\text{OD PM GLUT4} + \% \text{of Total Protein Recovery in MM Fraction}} \]

where OD is the relative densitometric value of GLUT4 in 50 mg of membranes and % of total protein recovery in MM (or PM) fraction is the amount of protein recovered in final PM or MM fraction divided by the amount of protein in the original crude homogenate. Expression of the GLUT4 data in this way allows comparison with immunohistochemical findings from other studies in which GLUT4 translocation has been analyzed.20 The equations also correct for variabilities in the percentage of total cardiac protein recovered in each fraction. Statistical analysis of GLUT4 translocation was performed by ANOVA, and differences between groups were determined by the Fisher least significant difference test. Differences were considered significant at 95% confidence intervals.

**Results**

MM fractions derived from the subcellular fractionation method were substantially enriched in NADPH-cytochrome c reductase activity, and the PM fractions were substantially enriched in the α subunits of the Na+,K+-ATPase (Table). None of the experimental treatments changed the relative enrichments of the marker enzymes in either of the fractions. Along with substantial marker enrichment, the fractionation protocol resulted in a recovery of approximately 60% of the total GLUT4 molecules in the MM and PM fractions (Table). Total GLUT4 recovery was not affected by the various experimental conditions.

Compared with control animals, maximal insulin stimulation produced a 1.9-fold increase in the number of GLUT4 molecules detected in the myocardial PM fraction and a 62% decrease in the number of MM GLUT4 molecules. When these data were corrected for variations in protein recovery and expressed as the percentage of total GLUT4 molecules found in the PM fraction, insulin increased PM GLUT4 expression from 18.2% to 61.1 ± 2.6%, indicating translocation of
approximately 43% of total GLUT4 molecules from an intracellular compartment to the plasma membrane (Figs 1 and 2). Comparable to insulin stimulation, hypoxia and ischemia caused 2.4-fold and 3.1-fold increases in PM GLUT4 molecules and 47% and 40% decreases in MM GLUT4 numbers. Expressed as percentages of total GLUT4 molecules, hypoxia increased PM GLUT4 to 31.1±2.3% and ischemia increased PM GLUT4 to 41.3±9.3%, indicating substantial translocation of GLUT4 molecules by acute hypoxia and ischemia (Figs 2 and 3). Ischemia plus insulin increased PM GLUT4 molecules to 66.8±5.7%, which was significantly greater than that caused by ischemia alone and was slightly greater than that caused by insulin alone (Figs 1 and 2).

**Discussion**

Glucose uptake by cardiac myocytes is mediated by a facilitative diffusion glucose transport process in which glucose moves down its concentration gradient into cells. Over the past several years, the proteins that transport glucose across the plasma membrane have been identified by molecular cloning. At present, six related proteins have been identified that can conduct glucose across biological membranes. The members of this family differ in terms of their affinity for glucose and other hexoses, their regulation by hormonal and other factors, and their distribution in tissue. In accordance with previous functional data, it has been determined that the major glucose transporter in heart is an insulin-responsive transporter known as GLUT4. GLUT4 is expressed primarily in fat and in skeletal and cardiac muscle. Under fasting conditions, in each of these tissues, the majority of GLUT4 molecules reside in an intracellular membrane compartment and therefore do not participate in cellular glucose uptake. However, when the cells are stimulated by insulin, GLUT4 molecules are rapidly translocated to the plasma membrane and augment cellular glucose uptake up to 20-fold. After insulin levels drop, the transporters are recycled to the intracellular compartment. Stimuli such as exercise, hypoxia, and other hormones have also been shown to cause translocation of GLUT4 molecules in fat or muscle. Because ischemia is known to cause a rapid increase in myocardial glucose extraction from the blood, the present study investigated the hypothesis that acute myocardial ischemia induces GLUT4 translocation to the plasma membrane of cardiac myocytes.

To test our hypothesis, we used a modified subcellular membrane fractionation technique for rat myocardium that yields reliable separation of intracellular membranes from plasma membranes independent of experimental conditions. The method produces enrichments of marker enzymes that are comparable to those obtained with other reported myocardial subcellular fractionation methods. Furthermore, this method yields a recovery of approximately 60% of myocardial

![Image](https://example.com/image.png)

**Fig 1.** GLUT4 immunoblots of 50 μg of crude homogenates (CH) and plasma membrane (PM) and microsomal membrane (MM) fractions from insulin-treated and insulin plus ischemia rat hearts, demonstrating substantial GLUT4 expression in PM fractions. Positions of molecular weight markers are indicated in kilodaltons to the left of the blots.

**Fig 2.** Bar graph showing summary of plasma membrane GLUT4 expression (mean±SEM) in hearts from control, ischemia, hypoxia, insulin-stimulated, and insulin plus ischemia hearts. The number of animals in each group is indicated in parentheses below the condition. *Conditions in which plasma membrane (PM) GLUT4 expression was significantly greater than control; #conditions in which PM GLUT4 expression was significantly greater than ischemia.
GLUT4 molecules in the two final fractions. This relatively quantitative GLUT4 recovery, which has not previously been documented in earlier membrane fractionation studies, ensures that translocation results will not be affected spuriously by losses of GLUT4 in discarded fractions. Our results show that the major cardiac glucose transporter, GLUT4, is translocated to the plasma membrane after myocardial cells are subjected to an episode of acute ischemia or hypoxia, although the magnitude of this translocation is less than that stimulated by pharmacological doses of insulin. To the best of our knowledge, this is the first report of the effects of myocardial ischemia on glucose transporter translocation. The conditions or stimuli that are now known to induce translocation of glucose transporters in heart include insulin, catecholamines, increased workload, hypoxia, and ischemia.

Partly because of the difficulties inherent in subcellular membrane fractionation of cardiac tissue, there have been only a few previous studies of the translocation of myocardial glucose transporters. Watanabe et al. were the first to develop a subcellular fractionation method that documented translocation of rat myocardial glucose transporters by insulin. Wheeler, using a modification of the Watanabe protocol, subsequently showed that hypoxia caused translocation of myocardial glucose transporters. Both of these studies were performed before the molecular cloning of GLUT4 and its identification as the major myocardial glucose transporter. Therefore, in Wheeler’s study, the degree of translocation was evaluated by comparing three properties of the plasma and microsomal membrane fractions: reconstituted glucose transport activity; binding of the ligand cytochalasin B, which recognizes all glucose transporter isoforms to some extent; and immunoblotting with an antibody that recognizes GLUT1, a transporter that is expressed only at low levels in myocardium. In both studies, insulin caused a 60% to 100% increase in PM glucose transporters and a 16% to 38% decrease in MM fractions (called “high-speed pellet” fractions). Hypoxia caused similar changes and induced a 20% to 70% increase in PM glucose transporters and a 20% to 30% decrease in glucose transporters in the microsomal fraction. Using the same membrane fractionation procedure, Rattigan et al. documented GLUT4 translocation after catecholamine stimulation, with a 7% increase in GLUT4 molecules in the fractions enriched in plasma membranes, and a 34% to 38% decrease in GLUT4 molecules in the fraction enriched in microsomal membranes. Zaninetti et al. used a much different fractionation method yielding PM protein recoveries approximately 5 times greater than those from the Watanabe and Wheeler protocols. Using cytochalasin B binding determinations, they also documented a twofold increase in PM glucose transporters with insulin. More recently, Slot et al. studied the extent of GLUT4 translocation in rat hearts induced by a combination of maximal insulin and exercise stimulation using immunohistochemical localization of GLUT4. In contrast to results found with subcellular fractionation, these investigators demonstrated minimal (<1%) PM expression of GLUT4 under basal, fasting conditions and showed that insulin plus exercise induced translocation of approximately 42% of the GLUT4 molecules to the PM. By thus demonstrating a much greater degree of glucose transporter translocation than shown by the other studies, these findings called into question the ability of subcellular fractionation techniques to adequately separate plasma membranes from other intracellular membranes.

With minor modifications of the membrane fractionation protocol of Zaninetti et al., we found that approximately 18% of GLUT4 molecules were expressed in the PM fraction in fasted animals and that insulin caused translocation of another 43% of the total GLUT4 molecules. The differences between our results and those of the earlier fractionation studies are most likely a result of our use of a different fractionation procedure that allowed us to capture approximately 60% of GLUT4 molecules within our final two fractions and produced relatively small amounts of cross-contamination. Our results, however, also differ to some extent from those of Zaninetti et al. These differences may be a result of our use of semiquantitative immunoblotting with a specific GLUT4 antibody rather than the more difficult and less specific cytochalasin B binding assays. Another potential factor is that the cytochalasin B binding experiments in the previous studies would have detected GLUT1 molecules as well as GLUT4. However, GLUT1 appears to be expressed at much lower levels than GLUT4 in rat myocardium. Therefore, this difference in technique is unlikely to be of significant importance to the higher basal PM GLUT4 expression and diminished translocation found in the earlier studies. Of all the fractionation studies, our results are most consistent with the immunolocalization findings of Slot et al. The higher PM GLUT4 expression found under basal conditions in our study is most likely a result of contamination of the PM fraction with intracellular membranes, as evidenced by enrichment of a mitochondrial enzyme, NADPH-cytochrome c reductase, in the PM fraction. The relative contamination was not substantial, however, and although both basal and insulin-stimulated PM GLUT4 expression were somewhat overrepresented with this method, the absolute degree of translocation was nearly identical to that detected by the immunolocalization study. Therefore, myocardial membrane fractionation accompanied by specific immunoblotting appears to produce reliable data on subcellular glucose transporter localization and can be used as a complement to immunohistochemical studies for this purpose. Furthermore, this method possesses several distinct advantages over immunohistochemical studies.
For example, quantification of specific proteins is easier and more reproducible, and multiple samples can be more reliably assayed and compared.

Our data suggest that much of the increase in glucose metabolism of acutely ischemic myocardial cells is caused by the translocation of GLUT4 molecules. An increase in plasma membrane transporters leads to more rapid glucose uptake, and because myocardial glucose uptake and phosphorylation normally appear to be rate limiting for glucose metabolism, the number of transporters on the cell surface at least partly determines the rate of myocardial glucose metabolism. Indeed, conditions or stimuli that acutely augment glucose uptake and metabolism in skeletal muscle and in other tissues do so through the translocation of glucose transporters.27

It is interesting that global ischemia stimulates GLUT4 translocation less than does maximal insulin treatment. Insulin alone increased PM GLUT4 molecules from 18% to 41%, whereas insulin treatment alone and ischemia plus insulin treatment resulted in the plasma membrane expression of 61% and 67% of total myocardial GLUT4 molecules, respectively. The reason for the difference in the degree of GLUT4 translocation induced by either ischemia or insulin is not known. It is conceivable that separate intracellular pools of GLUT4 molecules exist, one of which is responsive to insulin and the other responsive to ischemia. This conclusion seems somewhat unlikely, since insulin plus ischemia failed to increase plasma membrane GLUT4 expression in an additive manner. It is also conceivable that the severe global ischemia produced in our model could have been associated with the accumulation of toxic metabolites (eg, lactate) that may have suppressed maximal GLUT4 translocation. Indeed, clinical and experimental studies have found that severely ischemic myocardium loses its ability to extract increased amounts of glucose from the blood.1,26 However, toxic suppression of translocation seems unlikely to have occurred in our ischemic model, since hypoxia, which produced a degree of myocardial cell GLUT4 translocation similar to that of severe ischemia, does not cause the buildup of toxic metabolites and consistently increases myocardial glucose uptake and utilization.1,26 Therefore, decreased glucose metabolism in severely ischemic myocardium is probably caused by depressed glycolytic enzyme activity and not by decreased transporter expression. It seems most likely that the difference between the degree of translocation induced by ischemia and hypoxia on the one hand and insulin on the other is a result of the stimulation of different intracellular signaling pathways. The elucidation of these complex pathways is now being pursued energetically by a number of investigators.

Despite these unanswered questions, there appears to be a significant clinical correlate to the finding that insulin causes a greater degree of GLUT4 translocation than ischemia alone. Numerous clinical studies have shown that administration of insulin, alone or in combination with glucose and potassium, improves cardiac function and results in less extensive myocardial injury in patients with ischemia, infarction, or coronary bypass.3,37-41 We propose that insulin infusion is protective in these conditions because it stimulates additional translocation of GLUT4 molecules to the plasma membrane of ischemic cardiac myocytes, thus enhancing the protective increase in glucose metabolism. Our biochemical findings may therefore give added impetus to clinical efforts to enhance glucose uptake and utilization by myocardium at risk.

We studied the effects of ischemia on the translocation of the GLUT4 isoform only, and it is possible that myocardial ischemia also causes translocation of the other cardiac transporter, GLUT1, to the plasma membrane. Under acute conditions, such as those of the present study, GLUT1 translocation is unlikely to contribute substantially to myocardial glucose uptake, because GLUT1 appears to be expressed at much lower levels than GLUT4 in heart.19 However, under more chronic ischemic or hypoxic conditions, GLUT1 expression could be induced to physiologically significant levels. Indeed, it was shown recently that chronic hypobaric hypoxia resulted in a modest increase in GLUT1, but not GLUT4, mRNA and protein expression in rat hearts.42 Interestingly, GLUT4 expression actually decreased in the right ventricle of hypobaric hypoxic rats.43 Thus, many alterations in glucose transporter expression may accompany chronic hypoxic and ischemic conditions. It would be intriguing to determine the exact role that glucose transporter expression plays in mediating myocardial glucose metabolism under these chronic conditions and to determine whether modulating this response alters myocardial viability. Molecular reagents and techniques with which these important issues may be addressed are now available.

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