Intracellular vesicles and translocates to cell surface membranes, including the sarcolemma and T-tubules following insulin, exercise, and ischemia (8, 14, 39, 42, 51). GLUT4 is thought to be primarily responsible for the increase in glucose uptake in response to insulin and ischemic stress (39, 44, 46, 51).

GLUT1 is also expressed in the heart, both in cardiac myocytes, where it has a role in basal glucose uptake (14, 41, 51), and in endothelial cells (9). GLUT1 undergoes modest translocation to the sarcolemma with insulin and ischemia (14, 51), as it does in adipocytes (50). GLUT1 is also regulated at the expression level during ischemia, in part through the action of the transcription factor HIF1-a (21). In addition to GLUT1 and GLUT4, novel glucose transporters such as GLUT8 (6, 10) and GLUT12 (37) are expressed in the heart, although their physiological roles are uncertain.

The ability to quantify cell surface GLUT4 is critical to understanding heart metabolism. In recent years, transgenic mice have provided important insights into the molecular mechanisms regulating the physiological response of the heart to ischemia, pressure overload, and diabetes (1). Although many techniques have been adapted to study mouse cardiac physiology (15) and metabolism (4, 35), the quantification of cell surface glucose transporters has proved challenging. Mouse hearts typically weigh only 150 mg, limiting the application of conventional membrane fractionation techniques that were developed for other species (13, 51). Immunohistochemical and immunofluorescence techniques (42, 51) require less tissue, but the results are difficult to quantify. In addition, glucose transport activity requires translocation, docking, and fusion of GLUT4 vesicles with the surface membrane (7, 22), and histological techniques often do not clearly discriminate between GLUT4 vesicles associated with surface membranes and active cell surface GLUT4.

Therefore, we sought to develop a new method to assess cell surface glucose transporters directly in the isolated perfused mouse heart. We used a cell surface impermeant biotinylated bis-glucose photolabeling compound 4,4'-O-[2-[2-[2-[2-[2-[2-[6-(biotinylamino)hexanoyl]aminio]ethoxy]ethoxy]ethoxy]ethoxy]-4-(1-azi-2,2,2-trifluoroethyl)benzoyl]aminio-1,3-propanediyl]bis-D-glucose (bio-LC-ATB-BGPA) (19). Bis-mannose and bis-glucose reagents have proven useful in assessing cell surface glucose transporters in isolated cells (14, 26), as well as in excised cardiac (27) and skeletal muscles (40). Thus, isolated mouse hearts were retrogradely perfused for physiological study prior to bio-LC-ATB-BGPA infusion through the aortic cannula.

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The hearts underwent UV irradiation to cross-link the phot-activated diazirine group (19) to glucose transport proteins. Photolabeled cell surface glucose transporters were isolated from cell membranes by using streptavidin-agarose and then were quantified by immunoblotting with specific GLUT antibodies.

MATERIALS AND METHODS

Animals. Wild-type C57BL/6 male mice (age 10–16 wk) were used for all experiments. Animals were housed in accordance with guidelines from the American Association for Laboratory Animal Care and fed a standard rodent chow diet with access to water ad libitum. All procedures were approved by the Yale University Animal Care and Use Committee.

Perfusion protocols. Hearts were isolated from anesthetized mice after the intraperitoneal injection of heparin sulfate (100 U) and pentobarbital sodium (100 mg/kg). Hearts were perfused in the Langendorff mode with Krebs-Henseleit buffer (KHB) containing (in mM) 118 NaCl, 4.75 KCl, 1.2 KH2PO4, 1.2 MgSO4, 25 NaHCO3, 7 glucoses, and 0.4 oleate bound to 1% BSA. Hearts were perfused with a constant coronary flow of 4 ml/min to eliminate potentially confounding vasodilatory effects of insulin on the delivery of the photo-label to the myocardium. To assess the effects of insulin, the perfusion protocol included an initial 30-min perfusion without insulin followed by an additional 35 min with or without 100 μU/ml (0.7 nM) of insulin.

Bio-LC-ATB-BGPA photolabeling. Following completion of heart perfusion, the aortic cannula was flushed with 1 ml of ice-cold glucose-free KHB followed by 1 ml of the same buffer containing various concentrations of bio-LC-ATB-BGPA. The infused bio-LC-ATB-BGPA was allowed 15 min to bind in the heart at 4°C. To improve exposure of the ventricular cavities to UV irradiation, the left ventricle (LV) and right ventricle (RV) cavities were opened sagitally. The bound bio-LC-ATB-BGPA was photochemically cross-linked to cell surface GLUTs on ice using a Rayonet photochemical reactor (340 nm, 3 min each on the epicardial and endocardial surfaces; Southern New England Ultraviolet, Branford, CT). Hearts were freeze-clamped and stored in liquid nitrogen until further analysis. In experiments designed to study the specificity of bio-LC-ATB-BGPA binding, α-glucose (0, 10, or 20 mM) was added to both the washout and bio-LC-ATB-BGPA buffers. In experiments assessing the homogeneity of bio-LC-ATB-BGPA delivery to different regions of the heart, the LV, RV, and intraventricular septum were dissected apart after completion of cross-linking prior to freezing the tissues.

Bio-LC-ATB-BGPA-labeled GLUT isolation. Approximately 50–100 mg of myocardial tissue were homogenized in 400 μl of HEPES-EDTA-sucrose buffer (20 mM HEPES, 5 mM Na-EDTA, 255 mM sucrose, 1 μg/l antipain, aprotinin, pepstatin, leupeptin, 100 μM AEBSF, 4-[2-aminoethyl]benzenesulfonyl fluoride hydrochloride), pH 7.2). Total cell membranes were isolated by ultracentrifugation (227,000 g for 50 min at 4°C) and resuspended in phosphate-buffered saline containing 2% Thesit. Insoluble cellular contents were removed by centrifugation at 20,000 g for 30 min at 4°C. The supernatant containing the membranes ("total membrane") was saved and its protein concentration measured by the Bradford method (Bio-Rad protein assay; Bio-Rad, Hercules, CA). To isolate the photolabeled GLUTs, 500 μl of total membrane protein were incubated with 100 μl of streptavidin bound to 6% agarose beads (Pierce, Rockford, IL) overnight at 4°C. The supernatant of the streptavidin-agarose isolation, which contained nonphotolabeled transporters, was saved as the "unlabeled fraction." The streptavidin-agarose isolated labeled fraction of GLUTs was washed extensively with phosphate-buffered saline containing decreasing concentrations of Thesit (1, 0.1, and 0%). The labeled GLUTs were then dissociated from the streptavidin by boiling in SDS-containing electrophoresis buffer for 30 min with the subsequent addition of 10% mercaptoethanol prior to SDS-PAGE.
membranes prepared by ultracentrifugation with that in the streptavidin-isolated “labeled” fraction and the post-streptavidin supernatant “unlabeled” fraction. Comparable aliquots from each fraction were subjected to SDS-PAGE and quantified by densitometric analysis of GLUT4 immunoblots. Total membrane GLUT4 was fully accounted for (99.6 ± 3.6%) by the sum of the labeled and unlabeled GLUT4 (Fig. 2B). To further assess the efficiency of the isolation procedures, the membrane proteins were also blotted with HRP-streptavidin. These blots revealed a biotin-containing protein band at a molecular mass of ~50 kDa, representing the bio-LC-ATB-BGPA-glucose transporter conjugate, in both the total membrane and streptavidin-isolated fractions, but no residual BGPA-GLUT4 in the streptavidin supernatant fraction (Fig. 2B). Taken together, these results provide evidence that the recovery of bio-LC-ATB-BGPA-photolabeled GLUT4 was efficient.

In addition, to exclude the possibility that the streptavidin isolation might nonspecifically recover other high-abundance cell surface membrane transport proteins, we immunoblotted the streptavidin isolates for Na\(^+\)-K\(^+\)-ATPase (\(\alpha_1\)-subunit). Na\(^+\)-K\(^+\)-ATPase was not detected in the streptavidin isolates, providing evidence for the specificity of the bio-LC-ATB-BGPA photolabeling and isolation methods (Fig. 2B).

Bio-LC-ATB-BGPA binding was generally performed without glucose in the labeling buffer in order to maximize the labeling of GLUT4. However, when D-glucose (0–20 mM) was added to the photolabel-containing buffer, bio-LC-ATB-BGPA binding to GLUT4 was inhibited in a dose-dependent manner and was essentially abolished by a 50-fold higher concentration of D-glucose (Fig. 2C). The competitive inhibi-
tion of bio-LC-ATB-BGPA photolabeling of glucose transporters by D-glucose further supports the specificity of this method in the intact heart.

**Stimulation of GLUT translocation by insulin: quantification of cell surface GLUT by bio-LC-ATB-BGPA GLUT4 photolabeling.** Insulin is the prototypical stimulus for heart GLUT4 translocation to the cell surface (12, 14, 23, 39, 42, 49). Therefore, we assessed whether bio-LC-ATB-BGPA photolabeling could effectively detect changes in cell surface GLUTs after insulin stimulation in the isolated perfused mouse heart. In these experiments, bio-LC-ATB-BGPA-labeled cell surface GLUT4 was compared in control and insulin-stimulated hearts (Fig. 3A). Hearts perfused with physiological concentrations of insulin (100 μU/ml), had a three- to fourfold greater cell surface GLUT4 compared with control hearts ($P < 0.01$; Fig. 3B). During the same perfusions, glucose uptake measured by the production of $^{3}$H$_{2}$O from D-[2-$^{3}$H]glucose increased comparably (3.5-fold, $P < 0.001$) in insulin-stimulated hearts (Fig. 3C).

**Heart GLUT1 cell surface labeling with bio-LC-ATB-BGPA.** GLUT1 is expressed in cardiomyocytes (13, 14, 51) and endothelial cells (9). GLUT1 is generally considered to be primarily involved in basal heart glucose transport but also undergoes translocation to plasma membranes in response to insulin (14, 51). We also analyzed the cell surface labeling of GLUT1 in intact hearts after the infusion of bio-LC-ATB-BGPA. GLUT1 was present on cell surface membranes in control mouse hearts, and the content of labeled GLUT1 increased modestly (1.5-fold, $P = 0.05$) in response to insulin (Fig. 4A). To examine the relative amounts of cell surface GLUT4 and GLUT1, an additional strategy was developed in which cell membranes from photolabeled hearts were immunoprecipitated with either GLUT4 or GLUT1 antibodies, submitted to SDS-PAGE, and then probed with HRP-streptavidin. Assuming equal efficiency of GLUT1 and GLUT4 labeling using this approach, GLUT4 accounted for 43 ± 6% of total labeled GLUTs after insulin stimulation (Fig. 4B).

**DISCUSSION**

New methods to quantify cell surface GLUT4 content in the mouse heart are needed to advance our understanding of myocardial glucose utilization in physiological and pathological states, such as ischemic preconditioning (32, 48), ischemia-reperfusion (38), ischemia-resistance (3), and heart failure (5). Established methods are available in the isolated perfused mouse heart to measure rates of glucose uptake and phosphorylation with [2-3H]glucose by tissue analysis (3, 5) and $^{31}$P NMR spectroscopy (46). However, these approaches do not address the cellular mechanisms responsible for changes in glucose uptake and also require that deoxyglucose and native glucose be comparably transported and phosphorylated (18, 25, 31). The photolabeling technique complements these methods and provides a direct and easily quantifiable measure of cell surface GLUT4, which may prove useful in studying glucose transport regulation in the isolated perfused mouse heart.

The isolated heart has advantages over excised heart muscles (27) for the study of glucose transport regulation, because it is an intact, contracting, and more physiological preparation. We devised an infusion technique, which produced homogenous photolabeling of cell surface GLUT4 throughout different regions of the perfused heart. This might also prove important in terms of studying pathological processes that involve specific regions of the heart. Successful implementation of this approach required technical considerations that warrant emphasis. Effective perfusion of both the left and right coronary arteries required that the aortic cannula be placed above the aortic valve and the coronary artery ostia. This was facilitated by cutting the aorta just prior to the innominate artery during cardiac excision to allow an adequate aortic length for cannulation. Heparin administration to the mouse prior to heart
excision was needed to prevent coronary microthrombi that might otherwise interfere with the uniform delivery of the infused photolabel. Finally, meticulous attention was required to avoid the introduction of air bubbles into the aortic cannula when the heart was removed from the perfusion apparatus and connected to the syringe containing the photolabel.

The concentrations of bio-LC-ATB-BGPA that saturated GLUT4 labeling in our experiments (400 μM) are very similar to those observed in isolated rat adipocytes (300–500 μM) (20). GLUT4 labeling was not only dose dependent but also was competitively inhibited by the addition of glucose to the photolabeling buffer. The specificity of the technique was further supported by the absence of a high abundance cell surface protein (Na⁺-K⁺-ATPase-α1) in the streptavidin isolates. In addition, it would appear unlikely that there was significant labeling of intracellular GLUT4 because of the cell-impermeant nature of bio-LC-ATB-BGPA compound (19) and the finding that only 15% of GLUT4 was present on the cell surface in the absence of insulin, which is quite similar to previous reports based on membrane fractionation in rat and canine hearts (47, 51).

The biotin-containing compound bio-LC-ATB-BGPA has significant technical advantages over tritiated photolabeling reagents (14, 20) for assessing cell surface GLUT4 in the heart. The use of the biotin compound avoids the radioactivity containment challenges that would be associated with the use of tritiated compounds in intact hearts. It also allows for cell surface GLUT4 quantification without the need to excise the photolabeled glucose transporters from SDS-PAGE gels for scintillation counting. The highly efficient recovery of cell surface labeled GLUT4 with streptavidin-agarose can be attributed to both the high affinity of the biotin-streptavidin interaction and the long side-chain design of bio-LC-ATB-BGPA that makes the biotin moiety readily accessible (19). Once cell surface labeled GLUT4 is separated from other GLUT4 protein, immunoblotting techniques are intrinsically highly specific for GLUT transporters. Therefore, the use of the biotinylated compound provides an effective measure of cell surface GLUT4 in the perfused heart.

GLUT4 translocation to the cell surface is the principal component of the cardiomyocyte response to insulin (12, 14, 39). Thus, as proof of principle, we assessed the ability of the BGPA infusion method to measure changes in cell surface GLUT4 content in response to a physiological concentration of insulin (100 μU/ml). Insulin increased cell surface GLUT4 content three- to fourfold, which was consistent with the increase in glucose uptake observed in this and previous studies of perfused mouse hearts (3). The magnitude of insulin stimulation in contracting hearts is less than that observed in isolated noncontracting cardiac myocytes (3), highlighting the importance of studying the heart under these more physiological conditions. One limitation of this method is that it does not differentiate between cell surface GLUT4 in sarcolemma and T-tubule membranes. However, given the size of bio-LC-ATB-BGPA compound, it is unlikely to be excluded from the T-tubule space, and tritiated-BMPA is known to label T-tubules in skeletal muscle (11).

The high efficiency of GLUT4 labeling observed in the perfused hearts confirms that bio-LC-ATB-BGPA delivered via aortic perfusion was readily able to reach cardiomyocytes, since the heart contains little noncardiomyocyte GLUT4. Aortic smooth muscle cells express GLUT4 (25, 34), but GLUT4 does not appear to be present in heart blood vessels (9, 49). Although infusion of photolabel leads to initial contact with capillary endothelium, endothelial cells do not express GLUT4 transporters (9, 45).

The present results also indicate that the heart contains a significant amount of cell surface GLUT1, consistent with prior reports (13, 14, 51). GLUT1 is highly expressed in endothelial cells (34, 36), and a recent immunogold electron microscopy study demonstrated greater GLUT1 expression in endothelial cells than in cardiomyocytes in the adult rat heart (9). The photolabel experiments indicate that insulin modestly increases cell surface GLUT1 in the heart but do not elucidate...
which cell type might be insulin responsive. Insulin is known to increase cell surface GLUT1 in isolated cardiomyocytes (14), but glucose uptake in endothelial cells is typically not responsive to insulin at physiological concentrations except when cultured in the presence of very high glucose concentrations (17). The observation that the degree of insulin stimulation of heart glucose uptake paralleled the increase in cell surface GLUT4 content supports the contention that GLUT4 is primarily responsible for the insulin effect, as suggested by earlier studies in the cardiac-specific GLUT4 knockout mouse (44). This observation is also consistent with the possibility that a significant amount of GLUT1 in the heart functions as an endothelial transporter (9) and that endothelial transport is not rate limiting for insulin stimulation of total heart glucose uptake. GLUT1 contributes to cardiomyocyte glucose transport (14), and our results also do not exclude the possibility that insulin activates GLUT1 activity in cardiomyocytes.

Additional glucose transporters, such as GLUT8 (10) and GLUT12 (37), are also expressed in the heart, although their roles in mediating glucose transport remain uncertain. The photolabeling technique potentially could be used to assess the cell surface content of these novel glucose transporters. Although we have been unable to detect these transporters on the cell surface under control or insulin-stimulated conditions using the bio-LC-ATB-BGPA approach (E. Miller, K. Moley, S. Rogers, L. Young, unpublished data), it is possible that these novel glucose transporters may undergo translocation to the cell surface under pathological conditions or with other stimuli.

In conclusion, photolabeling with bio-LC-ATB-BGPA is a novel, efficient, and quantifiable method that can be used to assess cell surface GLUT4 in the isolated perfused mouse heart. The infusion method may help to further elucidate the molecular mechanisms controlling GLUT4 translocation and the role of GLUT4 in the mouse heart under physiological and pathophysiological conditions. Although we assessed cell surface GLUT4 in the heart, the approach is potentially also more broadly applicable to the investigation of additional cell surface transporters in the heart and other perfused organs.

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REFERENCES

Innovative Methodology


