GLUT4 Facilitative Glucose Transporter Specifically and Differentially Contributes to Agonist-Induced Vascular Reactivity in Mouse Aorta

James L. Park, Robert D. Loberg, Damon Duquaine, Hongyu Zhang, Baljit K. Deo, Noelia Ardanaz, Jami Coyle, Kevin B. Atkins, MaryLee Schin, Maureen J. Charron, Arno K. Kumagai, Patrick J. Pagano, Frank C. Brosius III

Objective—We hypothesized that GLUT4 is a predominant facilitative glucose transporter in vascular smooth muscle cells (VSMCs), and GLUT4 is necessary for agonist-induced VSMC contraction.

Methods and Results—Glucose deprivation and indinavir, a GLUT4 antagonist, were used to assess the role of GLUT4 and non-GLUT4 transporters in vascular reactivity. In isolated endothelium-denuded mouse aorta, ~50% of basal glucose uptake was GLUT4-dependent. Norepinephrine-mediated contractions were dependent on both GLUT4 and non-GLUT4 transporters, serotonin (5-HT)-mediated contractions were mainly GLUT4-dependent, and prostaglandin (PG) F2α-mediated contractions were dependent on non-GLUT4 transporters, whereas indinavir had no effect in GLUT4 knockout vessels. We also observed a 46% decrease in GLUT4 expression in aortas from angiotensin II hypertensive mice. Indinavir caused a less profound attenuation of maximal 5-HT-mediated contraction in these vessels, corresponding to the lower GLUT4 levels in the hypertensive aortas. Finally, and somewhat surprisingly, chronic GLUT4 knockout was associated with increased vascular reactivity compared with that in wild-type animals, suggesting that chronic absence or reduction of GLUT4 expression in VSMCs leads to opposite effects observed with acute inhibition of GLUT4.

Conclusions—Thus, we conclude that GLUT4 is constitutively expressed in large arteries and likely participates in basal glucose uptake. In addition, GLUT4, as well as other non-GLUT4 facilitative glucose transporters, are necessary for agonist-induced contraction, but each transporter participates in VSMC contraction selectively, depending on the agonist, and changes in GLUT4 expression may account for some of the functional changes associated with vascular diseases like hypertension. (Arterioscler Thromb Vasc Biol. 2005;25:1596-1602.)

Key Words: glucose ■ GLUT4 ■ indinavir ■ vascular smooth muscle

Mammalian cells use glucose for the generation of ATP through oxidative and nonoxidative metabolism, but because the lipid bilayer of cellular membranes is impermeable to carbohydrates, the cell must rely on a system of hexose transporters to facilitate uptake of these sugars. To date, at least 13 facilitative glucose transporters have been cloned. The GLUT transporters are homologous glycosylated polypeptides with distinct substrate specificity, affinity, and tissue distribution. GLUT4 is considered the major insulin-responsive transporter expressed in fat and striated muscle tissues. In these tissues, the majority of GLUT4 molecules (~90%) are sequestered in intracellular vesicles in the absence of insulin or other stimuli such as muscle contraction. In the presence of these stimuli, the GLUT4-containing vesicles translocate to the plasma membrane where they participate in glucose uptake.

Vascular smooth muscle has been shown to express GLUT1 as well as GLUT4. However, the expression of other facilitative glucose transporters in vascular smooth muscle is unknown. Previous studies from our laboratory have demonstrated that in vascular diseases caused by diabetes and hypertension, GLUT4 expression is decreased, with a concomitant decrease in basal vascular glucose uptake. These data suggest that GLUT4 is an important glucose transporter in vascular smooth muscle cells (VSMCs). Previous studies also have demonstrated the necessity of glucose for normal agonist-induced VSMC contractions. However, there is scant information concerning the role of GLUT4 transporters in VSMC contraction and whether glucose transporters play a role in the compartmentation of glucose utilization. Therefore, the hypothesis tested in this study is that GLUT4 is a predominant glucose transporter in VSMCs.
and that GLUT4 is necessary for full agonist-induced VSMC contraction.

We tested this hypothesis by comparing norepinephrine (NE), serotonin (5-HT), and prostaglandin F2α (PGF2α)-mediated VSMC contractions in the absence of glucose, which would inhibit all glucose uptake, whether by GLUT4 or other transporters, or after specific inhibition of only GLUT4-mediated glucose uptake with indinavir, an HIV protease inhibitor. Indinavir has been demonstrated to non-competitively and specifically inhibit GLUT4 molecules.14,15 In this study, we have determined that indinavir inhibits GLUT4-mediated glucose uptake in an intact endothelium-denuded vascular preparation. In addition, we observed that glucose utilization has different effects on vascular reactivity, depending on whether uptake is via GLUT4 or non-GLUT4 transporters. In addition, we observed decreased GLUT4 expression in the aortas from angiotensin II hypertensive mice, and indinavir caused a less profound attenuation of maximal 5-HT–mediated contraction in accord with the lower GLUT4 expression in these vessels. These results suggest that glucose utilization for VSMC contraction may be compartmentalized at the level of glucose uptake through glucose transporters. Altered expression of these transporters in the vasculature may be a metabolic factor that affects VSMC function in vascular diseases such as hypertension.

### Methods

#### Cell Culture

A7r5 VSMCs or bovine retinal endothelial cells (BRECs) were grown in complete Dulbecco’s Modified Eagle Medium (Invitrogen Life Technologies, Carlsbad, Calif) with 10% fetal bovine serum and 100 U/mL penicillin-streptomycin at 37°C and 95% O2/5% CO2. BRECs were isolated, as described previously.16 Details of A7r5 VSMC and BREC culture are described in the online supplement (see http://atvb.ahajournals.org).

#### Glucose Transport Studies

Glucose transport was assessed by measuring [3H] 2-deoxyglucose (2-DOG) uptake. The [3H] 2-DOG assays were performed following the method of Tai et al.17 A more detailed description of the glucose transport studies is provided in the online supplement.

#### Confocal GLUT4 Immunofluorescence Imaging

A7r5 VSMCs plated on glass coverslips were treated with insulin (100 nmol/L) in the presence or absence of indinavir (25 μmol/L) for 20 minutes at 37°C. A more detailed description of GLUT4 immunofluorescence is described in the online supplement.

#### Immunoblotting for GLUT1 and GLUT4

Aortic or cellular detergent resistant membrane fractions or whole-cell lysates were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were blocked with Tris-buffered saline–Tween20 (TBS-T) and 5% milk and incubated at room temperature for 1 hour. After 3 washes in TBS-T, the appropriate horseradish peroxidase-linked secondary antibody was then added in TBS-T 5% milk overnight at 4°C. After 3 washes in TBS-T, the membranes were then washed 3 times in TBS-T, and enhanced chemiluminescence was used for autoradiography.

#### Animal Studies

Wild-type male C57BL/6J mice (8 to 10 weeks old; Jackson Laboratories, Bar Harbor, Me) or GLUT4 knockout (GLUT4KO) mice on a C57Bl/6J background were euthanized with sodium pentobarbital (50 mg/kg intraperitoneal), and the thoracic aortas were excised. The procedures used in our study were approved by the University of Michigan Committee on the Use and Care of Animals. The University of Michigan Unit for Laboratory Animal Medicine provided veterinary care. The University of Michigan is accredited by the American Association of Laboratory Animal Care. The animal care and use program conformed to the standards in The Guide for the Care and Use of Laboratory Animals, Department of Health, Education, and Welfare Publication number (National Institutes of Health) 86 to 23.

#### Separation of Detergent-Resistant Membrane Fractions

Detergent-resistant membrane (DRM) fractions from A7r5 VSMCs were isolated as described in detail in the online supplement and as previously described.18 DRM fractions from mouse aorta were isolated with minor modifications based on previous reports,19,20 as described in detail in the online supplement.

#### Vascular Reactivity Experiments

Vascular reactivity experiments were performed as described in detail in the online data supplement. Vascular reactivity was measured in a myograph system (Danish Myo Technology A/S, Aarhus, Denmark) based on the system previously described by Mulvany and Halpern.21 After vessels were equilibrated, the vessels were incubated with vehicle indinavir, or no glucose conditions (5.5 mmol/L mannitol in the physiological salt solution (PSS) instead of 5.5 mmol/L glucose) for 30 minutes. Cumulative concentrations of NE, 5-HT, or PGF2α were added to the bath to establish concentration–response curves. The concentrations of indinavir used in the 5-HT and PGF2α reactivity experiments were chosen based on the cell culture data and NE reactivity data demonstrating that specificity for GLUT4 antagonism was achieved at 25 μmol/L (see data in Results and online supplement).

#### Mouse Model of Angiotensin II-Mediated Hypertension

Angiotensin (Ang) II–mediated hypertension was induced in wild-type male C57BL/6J mice (12 to 14 weeks old) (Jackson Laboratories, Bar Harbor, Maine) as previously described22–24 and outlined in detail in the online supplement.

#### Chemicals

Indinavir was purified in crystalline form from pharmacy tablets, and was a gift from the laboratory of Dr David Fleisher (University of Michigan School of Pharmacy, Ann Arbor), and resuspended in phosphate-buffered saline (pH 1.0). The antibody against GLUT1 was a gift from Dr Christin Carter-Su (University of Michigan, Ann Arbor). The antibody against GLUT4 is an antipeptide antisera directed against the carboxy terminal portion of the rat GLUT4 molecule. Both antibodies are highly specific and have been extensively reported previously by our group.5,7 All other drugs were purchased from Sigma Chemical Co (St. Louis, Mo).

#### Data and Statistical Analysis

Agonist EC50 values were calculated with a nonlinear regression analysis with the algorithm [effect = maximum response/1 + (EC50/agonist concentration)] in the computer program GraphPad Prism (San Diego, Calif). Data were expressed as mean ± SEM and were analyzed using a Student’s t test or 1-way analysis of variance (ANOVA) followed by Bonferroni post hoc analysis. Data were considered significant at P<0.05.

### Results

#### Characterization of GLUT4 Antagonism With Indinavir in Cell Culture

Indinavir at all concentrations up to 50 μmol/L failed to inhibit basal A7r5 VSMC glucose uptake (Figure IA, avail-
able online at http://atvb.ahajournals.org). In A7r5 VSMCs stimulated with 100 nmol/L insulin, glucose uptake was increased 2-fold, and both 25 μmol/L and 50 μmol/L indinavir prevented the insulin-stimulated increase in glucose uptake (Figure IB). Confocal immunofluorescence revealed diffuse punctate localization of GLUT4 in the cytoplasm of A7r5 VSMCs (Figure IC). After insulin stimulation, GLUT4 localization was evident at the plasma membrane, consistent with insulin-induced GLUT4 translocation (Figure IC). Indinavir (50 μmol/L) did not interfere with GLUT4 translocation, as indicated by the persistence of GLUT4 localization on the cell membrane (Figure IC). These findings corroborate previous reports in other cell systems that indinavir inhibits glucose flux via GLUT4 but does not affect GLUT4 translocation.14

Glucose uptakes in BRECs were performed to verify the specificity of indinavir. Immunoblot analysis of whole-cell lysates from BRECs indicated the presence of GLUT1 (Figure IIA, available online at http://atvb.ahajournals.org), but not GLUT4 (Figure IIB). Basal glucose uptake in BRECs was not significantly affected by the presence of indinavir during the 2-DOG uptake assay (Figure IIC). A minor decrease in glucose uptake in the presence of 50 μmol/L indinavir was observed, suggesting that a fraction of the inhibition of glucose uptake at the highest concentration of indinavir could be caused by inhibition of non-GLUT4 transporters, such as GLUT1. These data, combined with the A7r5 uptake data, indicate that 25 μmol/L indinavir specifically inhibited GLUT4-mediated glucose uptake in VSMCs.

GLUT1 and GLUT4 Distribution in Membrane Fractions From A7r5 Cells and Mouse Aorta
Immunoblotting for GLUT1 and GLUT4 in membrane fractions demonstrated the presence of both GLUT1 and GLUT4 in A7r5 cells and mouse aorta (data not shown), as we have previously shown.3 In A7r5 cells, GLUT1 was localized to detergent-resistant membrane fractions that also contained caveolin-1, whereas GLUT4 was not detected in these fractions. However, in whole mouse aorta, GLUT1 and GLUT4 were localized mostly to the nondetergent-resistant fractions, although the pattern of distribution for GLUT1 and GLUT4 differed somewhat. Neither GLUT4 nor GLUT1 localized in the fractions in which caveolin-1 was most prominent (Figure III, available online at http://atvb.ahajournals.org).

We also examined whether GLUT4 deficiency resulted in a compensatory increase in GLUT1 expression. We did not observe any changes in GLUT1 expression in whole aortic homogenates from male GLUT4KO mice (data not shown), but a moderate increase in aortic GLUT1 expression was observed in female GLUT4KO mice (Figure IV, available online at http://atvb.ahajournals.org).

Basal Glucose Uptake With GLUT4 Inhibition in Endothelium-Denuded Mouse Aorta
Basal glucose uptake in wild-type mouse aorta was measured to correlate glucose uptake with vascular function in the presence and absence of GLUT4 antagonism (Figure 1). In contrast to results in A7r5 cells, GLUT4 antagonism with indinavir (25 μmol/L and 50 μmol/L) inhibited basal glucose uptake in aorta by 40% to 50%. The lowest concentration of indinavir (5 μmol/L) did not affect basal glucose uptake in the mouse aorta. These data suggest that GLUT4 mediates nearly half of basal glucose uptake in mouse aorta and corroborate findings in aortas from hypertensive animals in which reduced GLUT4 expression accompanied a substantial reduction in basal glucose uptake.5

Vascular Reactivity in the Presence of Indinavir or Absence of Glucose
NE
NE caused a concentration-dependent VSMC contraction in endothelium-denuded aortas (Figure 2). GLUT4 antagonism with indinavir did not significantly affect the NE EC50 (Table I, available online at http://atvb.ahajournals.org), but GLUT4 antagonism with indinavir significantly attenuated maximum contraction to NE in a concentration-dependent manner. After glucose deprivation, the NE EC50 was significantly shifted to the right (Table II, available online at http://atvb.ahajournals.org), and NE-mediated maximum contraction was further attenuated, compared with indinavir, as illustrated in Figure IIA. NE-mediated VSMC contraction in aortas from GLUT4KO mice treated with vehicle or indinavir were not different (Figure IIB), but glucose deprivation caused a significant rightward shift of the EC50 (Table II) and de-

Figure 1. Basal 2-deoxyglucose (2-DOG) uptake in endothelium-denuded mouse aorta is inhibited by indinavir. *P<0.05 compared with vehicle.

Figure 2. Effect of GLUT4 inhibition or glucose deprivation on norepinephrine (NE)-mediated vascular smooth muscle cell (VSMC) contractions in endothelium-denuded mouse aortic rings. A, NE-mediated VSMC contractions in endothelium-denuded mouse aortic rings from wild-type mice. B, NE-mediated VSMC contractions in endothelium-denuded mouse aortic rings from GLUT4KO mice. Data are expressed as a percentage of the contraction elicited by 100 mmol/L KCl. *P<0.05 compared with vehicle.
creased contraction in the aortas from GLUT4KO mice (Figure IIB).

5-HT
5-HT caused a concentration-dependent VSMC contraction in endothelium-denuded aortas (Figure 3; Figure V, available online at http://atvb.ahajournals.org). GLUT4 antagonism with indinavir did not significantly affect the 5-HT EC₅₀ (Table II), but indinavir caused a significant attenuation in the maximum contraction to 5-HT in endothelium-denuded aortas from wild-type mice (Figure 3A), and did so in a concentration-dependent manner (Figure V). Glucose deprivation did not cause a shift in the EC₅₀ (Table II), but it significantly attenuated maximum contraction to 5-HT. This attenuation by glucose deprivation was identical to that caused by 25 μmol/L indinavir (Figure 3A). Neither GLUT4 antagonism with indinavir nor glucose deprivation affected vascular reactivity to 5-HT in the endothelium-denuded aortas from GLUT4KO mice, confirming the specificity of the indinavir effects. These data strongly suggest that 5-HT-induced vascular reactivity is enhanced by uptake of glucose through GLUT4 (Figure 3B) but not by glucose taken-up by non-GLUT4 transporters.

PGF₂α
PGF₂α caused a concentration-dependent VSMC contraction in endothelium-denuded aortas (Figure 4). GLUT4 antagonism with indinavir did not affect PGF₂α-mediated VSMC contractions, but glucose deprivation for 30 minutes before the concentration response performed in the EC₅₀ (Table II), as well as a decreased maximum contraction in the PGF₂α concentration response performed in aortas from wild-type mice (Figure 4A). As expected, PGF₂α-induced VSMC contractions in aortas from GLUT4KO mice were not affected in the presence of GLUT4 antagonism with indinavir (Figure 4B). However, glucose deprivation caused a rightward shift in the EC₅₀ (Table II) and a decrease in the maximum contraction mediated by PGF₂α in the GLUT4KO mice, although the changes were somewhat less substantial than those observed in the wild-type aortas.

Effects of GLUT4 Knockout on Vascular Reactivity
Although acute inhibition of GLUT4-mediated glucose transport resulted in reduced vascular reactivity, a different scenario was found in the GLUT4KO aortas. As already noted, there was no effect of indinavir on reactivity in the aortas from GLUT4KO animals, which validates the specificity of indinavir. However, in separate head-to-head experiments, the maximum contractions with NE (Figure 5A) and 5-HT (Figure 5B) but not PGF₂α (not shown) were augmented in aortas from GLUT4KO mice. There were no changes in blood pressures between wild-type and GLUT4KO mice (Figure VI, available online at http://atvb.ahajournals.org), nor was there a significant shift in the EC₅₀ values, although these were somewhat variable (Table I and Table II). These data suggest lack of GLUT4 gene expression leads to enhanced vascular reactivity, which is opposite to the effect of acute inhibition of GLUT4 with indinavir.

GLUT4 Expression and Vascular Reactivity in Ang II Hypertension
Systolic blood pressure in normotensive mice was 106±3 mm Hg (n=11), whereas systolic blood pressure was 137±6 mm Hg in mice infused with Ang II for 7 days (n=11; P<0.05 compared with normotensive). Immunoblotting for GLUT4 in whole aortic homogenates showed that GLUT4 expression was reduced by ~46% in the aortas from hypertensive mice (Figure 6A), similar to previous findings in aortas from hypertensive rats. 5-HT caused a concentration-dependent VSMC contraction in endothelium-denuded mouse aortic rings. A, 5-HT–mediated VSMC contractions in endothelium-denuded mouse aortic rings from wild-type mice. B, 5-HT–mediated VSMC contractions in endothelium-denuded mouse aortic rings from GLUT4KO mice. Data are expressed as a percentage of the contraction elicited by 100 mmol/L KCl. *P<0.05 compared with vehicle.

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Dependent contraction in endothelium-denuded aortas from normotensive and hypertensive mice (Figure 6B and 6C). GLUT4 antagonism with indinavir, however, caused a significant attenuation in the maximum contraction to 5-HT in aortas from normotensive mice (~42% reduction in maximum contraction), similar to what was shown in Figure 3. Contractions to 5-HT were significantly potentiated in the aortas from the hypertensive mice similar to findings with the GLUT4 KO mice (Figure 6B and 6C). In accord with lower GLUT4 levels in the hypertensive aortas, indinavir caused a less profound attenuation of maximal 5-HT–mediated contraction in these vessels.

Discussion

We demonstrated the specificity of the GLUT4 antagonist, indinavir, in cultured cells and observed that inhibition of GLUT4 in endothelium-denuded mouse aortas reduced basal glucose uptake by approximately half, supporting the conclusion that GLUT4 is a major glucose transporter that participates in basal, as well as in insulin-stimulated, glucose uptake in VSMCs in vivo. We also found that GLUT4 and other glucose transporters specifically and differentially contribute to VSMC contraction. Moreover, this differential contribution to contraction varies depending on the contractile agonist. We also observed that GLUT4 expression in vessels from hypertensive animals was diminished, and that indinavir caused a less profound attenuation of maximal 5-HT–mediated contraction in these vessels. We have found that chronic knockout of the GLUT4 gene results in augmented arterial reactivity to the same agonists that elicited attenuated reactivity in response to indinavir. Similar increases in reactivity were found in arteries from Ang II hypertensive mice that had decreased VSMC GLUT4 expression. Thus, the present study verifies that vascular smooth muscle contraction is dependent on glucose, but more importantly, it demonstrates that glucose uptake through different glucose transporter isoforms has specific agonist-dependent functional effects in arterial preparations. In addition, our data suggest that changes in GLUT4 expression may have a physiological impact on VSMC contractility in hypertension. In contrast, chronic absence or reduction of GLUT4 expression in VSMCs leads to opposite effects to those of acute inhibition of GLUT4, supporting the likelihood of compensatory changes in expression of other glucose transporters or cellular signaling in the more chronic models.

The availability of pharmacological inhibitors of glucose transporters is limited. Indinavir is the only GLUT4-specific inhibitor identified to date.14 We now have demonstrated indinavir has similar specificity in cultured VSMCs. We found that indinavir (25 μmol/L and 50 μmol/L) inhibited all of the insulin-stimulated increases in glucose uptake in A7r5 VSMCs but did not inhibit basal uptake. Thus, indinavir inhibited uptake only through GLUT4 transporters in these cells. Similarly, indinavir had a minimal effect on uptake by endothelial cells that do not express GLUT4.25 Although previous reports have documented the expression of GLUT4 in VSMCs5–11,26 and suggested that GLUT4 plays an important role in vascular function,5 the current study documents, for the first time to our knowledge, GLUT4 translocation and mediation of glucose uptake in cultured VSMCs.

In contrast to the results in cultured VSMCs, we found that basal glucose uptake in aortas was inhibited by indinavir, demonstrating that ~50% of basal glucose uptake in VSMCs in vivo is mediated by GLUT4. These data extend our previous observations in rat arteries,5 suggesting that GLUT4 trafficking is substantially different in vascular smooth muscle than in striated muscle and adipose tissue. In striated muscle and adipose tissue under basal conditions, virtually all the GLUT4 molecules are in an intracellular compartment and basal glucose uptake is mediated by another GLUT isoform, such as GLUT1. Whereas there is some possibility that GLUT4 molecules were induced to translocate to the plasma membrane of aortic VSMCs during tissue harvesting, this seems unlikely because similar translocation does not occur with much harsher procedures used to isolate adipocytes or skeletal muscle cells in which GLUT4 remains in intracellular compartments.27

The vasopressors, NE, 5-HT, and PGF2α, have been shown to be potent agonists in endothelium-denuded mouse aorta,28 but the relationship between glucose metabolism and VSMC
contraction previously has been characterized only for NE-mediated contractions.12,13 Zhang and Paul found that normal NE-dependent contraction in porcine carotid arteries was glucose-dependent and glucose was found to be necessary for normal regulation of intracellular Ca2+ stores.13 In these studies, the effects of glucose were examined by measuring VSMC contractility in the absence of glucose. Adams and Dillon demonstrated that secondary NE-stimulated VSMC contraction after glucose withdrawal (for 2 to 10 hours) was significantly diminished.12 We also observed that mouse aorta incubated for 30 minutes in the absence of glucose displayed significantly attenuated NE-mediated VSMC contraction. GLUT4 antagonism also resulted in attenuated NE-mediated VSMC contraction, but to a lesser extent than that seen with complete glucose withdrawal. Our data corroborate those of Adams and Dillon, but we have further characterized the relationship to glucose uptake through GLUT4 by pharmacologically antagonizing GLUT4. The current study is the first to show that 5-HT–mediated and PGF2α–mediated VSMC contractions are also dependent on glucose uptake via facilitative transporters. However, in contrast to the contractions induced by NE, 5-HT–mediated contractions are completely dependent on glucose taken-up by GLUT4 and are not affected by glucose uptake via non-GLUT4 transporters. Conversely, PGF2α–induced contractions are dependent on non-GLUT4 transporter-mediated uptake alone and not affected by glucose uptake via GLUT4.

This study provides evidence that modulation of GLUT4-mediated glucose uptake has an impact on agonist-induced VSMC contraction, especially in response to NE and 5-HT. Interestingly, contractile responses to these agonists are affected oppositely when GLUT4 is inhibited acutely versus when GLUT4 transporter expression is chronically eliminated or reduced. We observed that NE and 5-HT contractility is attenuated after acute pharmacological inhibition of GLUT4, but contractility to NE and 5-HT is potentiated in the GLUT4 knockout mice (Figure 5), in which GLUT4-mediated uptake is prevented chronically. Although these results were less dramatic and somewhat more variable for 5-HT (Figure 5 versus Figure 3), they were clearly evident in the studies that were performed head-to-head (Figure 5), which are more reliable. These findings suggest that compensatory changes in glucose uptake or cellular signaling in response to chronic ablation of GLUT4-mediated glucose uptake reverse the effects of acute inhibition of GLUT4-mediated uptake. Alternately, there could be direct effects of the GLUT4 transporter on contractility that are independent of the transport function of GLUT4. At present, there are no data that support either of these possibilities.

In both hypertension and diabetes, GLUT4 expression is significantly decreased in vascular smooth muscle.5,11 In this article, we demonstrate that GLUT4 expression is decreased in the aorta in the established mouse model of Ang II-mediated hypertension,22–24 that this model of hypertension is associated with potentiated 5-HT contraction, and that inhibition of GLUT4 does not affect VSMC contractility in the aortas from the hypertensive animals as greatly as it occurs in the aortas from the normotensive animals (Figure 6). These observations corroborate and extend our previous findings in aortas from DOCA-salt hypertensive rats5 and demonstrate a pathophysiological correlation between decreased GLUT4 expression and potentiated VSMC contractility associated with hypertension. The finding that GLUT4 knockout enhances VSMC contractility similar to that observed in hypertension underlines the potential primacy of GLUT4 reduction in the altered contractile responses found in the hypertensive aortas. Therefore, it will be important to dissect the long-term effects of GLUT4 expression and function in vascular responses in hypertension and diabetes.

Although vascular smooth muscle was originally determined to derive almost all of its ATP from glucose, it now seems clear that other substrates, especially acetate, are important for VSMC metabolism in vivo. Because our experiments were not performed in solutions containing these additional substrates, we may have overestimated the effects of glucose and glucose transporters on reactivity. Recent studies suggest that acetate utilization, when acetate is at physiological concentrations, provides a major input to the trichloroacetic acid cycle, whereas glucose input into the trichloroacetic acid cycle accounts for ≈30% to 60% of the total.29 Together, glucose and acetate account for ≈80% of the substrates oxidized by vascular smooth muscle,30 whereas glycogen oxidation contributes minimally to substrate oxidation,29 and exogenous glucose also contributes to glycolysis apart from the trichloroacetic acid cycle because vascular smooth muscle undergoes aerobic glycolysis normally in vivo. Thus, although acetate could have a modulatory effect on the glucose-dependent contractility differences we have identified, there is little question that glucose uptake and metabolism have substantial effects on VSMC function, and that the effects we have demonstrated are not likely to be eliminated by provision of other substrates. Moreover, it seems critical to perform initial studies with single substrates so that specific effects can be identified.

Our results of increased reactivity with glucose uptake by GLUT4 and non-GLUT4 transporters are somewhat in contrast to what has been shown previously by Kahn et al. The previous studies showed that 5-HT–induced contraction and Ca2+ flux was acutely inhibited, instead of potentiated, by increased glucose uptake in cultured VSMCs.8 These previous studies used insulin (1 nmol/L) to mediate increased glucose uptake, which has multiple signaling effects that could alter responses to glucose and may explain the difference between our current findings and those from the previous study. Moreover, it is likely that glucose uptake effects are quite different in cultured VSMCs used in the previous study. As we demonstrate in the current report, regulation of glucose uptake and glucose transporter localization is very different in cultured VSMCs than in arteries in vivo.

In summary, the current study supports the hypotheses that GLUT4 is an important basal glucose transporter in VSMCs, and that non-GLUT4 transporter and GLUT4-mediated glucose uptake are both necessary for full agonist-induced VSMC contraction. However, glucose uptake via non-GLUT4 transporters affects agonist mediated contractions in a qualitatively different manner than does glucose taken up by GLUT4. Mechanisms that explain this surprising compartmental effect of glucose uptake and metabolism are currently being explored.
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The GLUT4 Facilitative Glucose Transporter Specifically and Differentially Contributes to Agonist-Induced Vascular Reactivity in Mouse Aorta

James L. Park, Robert D. Loberg, Damon Duquaine, Hongyu Zhang, Baljit K. Deo, Noelia Ardanaz, Jami Coyle, Kevin B. Atkins, MaryLee Schin, Maureen J. Charron, Arno K. Kumagai, Patrick J. Pagano, and Frank C. Brosius, III

METHODS

Cell Culture

*A7r5 rat vascular smooth muscle cells*

A7r5 VSMCs (ATCC #CRL-1444) were grown in complete Dulbecco’s Modified Eagle Medium (DMEM; Invitrogen Life Technologies, Carlsbad, CA) with 10% fetal bovine serum (FBS) and 100 units/mL penicillin-streptomycin at 37°C and 95% O2/5% CO2. Cells were used for 2-DOG uptake assays when they reached 80% confluence. Both basal and insulin-stimulated glucose uptakes were measured in the A7r5 cells to assess indinavir’s specificity to inhibit GLUT4-mediated uptake, as described above. For insulin-stimulated uptake, A7r5 cells were stimulated with 100 nmol/L insulin 20 minutes prior to incubation with 2-DOG. Detergent-resistant membrane preparations also were prepared from cultured A7r5 cells for immunoblot analysis of GLUT1 and GLUT4. A7r5 cells also were grown for confocal imaging for insulin-stimulated GLUT4 translocation.

*Primary bovine retinal endothelial cells (BRECs)*

 Cultures of primary BRECs were established from fresh calf eyes and grown on fibronectin-coated flasks in DMEM with 10%FBS, 100 µg/mL endothelial growth supplement, 88 µg/mL heparin, and antibiotic-antimycotic solution under 5% CO2. Passages 2-10 were used for all experiments. Cells were used for 2-DOG uptake assays
when cells were 80% confluent. Basal 2-DOG uptake was measured to test whether indinavir inhibits glucose uptake in a cell-type that expresses GLUT1 and not GLUT4.

**Glucose Transport Studies**

Glucose transport was assessed using $[^3H]$ 2-deoxyglucose (2-DOG) in A7r5 VSMCs, primary bovine retinal endothelial cells, or cleaned sections of endothelium-denuded mouse aortas, opened longitudinally (approximately 6-7 mm in length). Cells or aortic sections were deprived of glucose for 30 minutes in Krebs-Ringer Phosphate (KRP) buffer + 1% BSA at 37°C in the presence of vehicle or indinavir (5 µmol/L, 25 µmol/L, or 50 µmol/L) ± 20nmol/L cytochalasin B. The cells or aortic sections were removed from the KRP buffer+ 1% BSA and incubated in 0.5 µCi/mL $[^3H]$ 2-DOG (cells) or 5 µCi/mL $[^3H]$ 2-DOG (aortic sections) solution with vehicle or indinavir for 5 minutes. All samples were subsequently washed quickly 3 times with ice-cold KRP buffer containing phloretin (55 µg/mL) to quench 2-DOG uptake. Cells were incubated for 30 minutes in the presence of lysis buffer (125 mmol/L Tris-HCl, pH 6.8, 10% SDS, 100mM phenylmethanesulfonylfluoride (PMSF) and protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN)). Aortic sections were ground with 50 strokes of a glass mini dounce homogenizer (Radnoti Glass Technology, Inc., Monrovia, CA) in the presence of the same lysis buffer. A portion of each sample was used to determine protein concentration by bicinchoninic acid assay (Pierce, Rockford, IL), and an aliquot of the sample was scintillation counted. $[^3H]$ 2-DOG uptake was expressed as picomoles cytochalasin B-inhibitable 2-DOG uptake per mg protein per minute.
Confocal GLUT4 Immunofluorescence Imaging

A7r5 VSMCs plated on glass coverslips were treated with insulin (100 nmol/L) in the presence or absence of indinavir (25 µmol/L) for 20 min at 37°C. Cells were washed once with chilled 1x PBS for 5 min then fixed and permeabilized with methanol:acetone (1:1 ratio) for 5 min at room temperature. After the cells were permeabilized, they were washed three times with 1x PBS. PBS with 10% filtered goat serum was then added to the cells for 15 min and washed off with 1x PBS (three times). Labeling of GLUT4 was accomplished by exposing the cells to GLUT4 antibody (1:2000) in 1x PBS + 10% filtered goat serum for 1 hour at room temperature. After primary antibody labeling, cells were washed twice with 1x PBS, and the anti-rabbit Cy3 secondary antibody (Jackson Labs) was added in 1x PBS + 10% filtered goat serum for 1 hour at room temperature. Cells were washed further three times with 1x PBS and mounted on glass slides (Fisher) using the Prolong Antifade mounting kit (Molecular Probes).

GLUT4 detection in A7r5 VSMCs was accomplished using confocal microscopy. Confocal imaging was performed with a Noran OZ laser scanning confocal microscope equipped with a 60× oil Nikon objective. To determine cellular localization of GLUT4, a 15-µm slit was applied to maximum spatial resolution available, using the 60× objective. Digital images were converted to TIFF files for analysis and presentation using Adobe Photoshop™.
Separation of Detergent-Resistant Membrane Fractions

*A7r5 VSMCs*

Five 100mm dishes of A7r5 VSMCs were washed with cold PBS and lysed in a buffer containing 250 mM sodium chloride, 5 mM EDTA, 1 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM sodium pyrophosphate, and protease inhibitor mixture tablet (Roche Molecular Biochemicals) with 1% Triton X-100. 0.67 ml of cell lysis was mixed with 1.33 of 60% Optiprep solution (Nycomed Pharma AS) to obtain a final density of 40%. The samples were overlaid with 1.5 ml of 30% Optiprep and then 1.5 ml of 5% Optiprep solution. Optiprep dilutions were obtained by diluting 60% stock Optiprep with Tricine buffer containing 20 mM Tricine, 0.25 mM sucrose, and 1 mM EDTA. The gradient was centrifuged at 37,000 rpm using a Beckman SW 41Ti rotor for 3 h at 4 °C. The Triton X-100-insoluble lipid layer was identifiable at the interface between the 5 and 30% Optiprep solutions. 0.5-ml fractions from 1 (top) to 9 (bottom) were sequentially collected.

*Mouse Aorta*

Aortas from two mice were rapidly excised under general anesthesia. The aortas were carefully trimmed to remove the fat and connective tissue and washed twice in ice cold PBS and once in 25 mM 2-Morpholinethanessulfonic acid, 150 mM NaCl, pH6.5 (MBS). The aortas were flash frozen in liquid nitrogen, minced and resuspended in 1.0 ml of 1% Triton X-100 in MBS supplemented with a protease inhibitor mix (Boehringer Mannheim) and incubated at 4°C for 30 min. These suspensions were homogenized with 10 strokes of a Dounce homogenizer, and centrifuged 800 x g for 5 minutes. A 0.67 ml
aliquot of the resulting homogenate was added to 1.33 ml of 60% (wt/vol) Optiprep (Nycomed Pharma AS) in MBS to obtain a final density of 40%. This mixture was overlayed successively with 1.5 ml of 30% and 1.5 ml of 5% Optiprep in MBS. After centrifugation at 248,000 × g in a Beckman SW40 rotor for 18 h, 0.5-ml fractions were collected from the top of the gradient [designated fractions number 1 (top) through 10 (bottom)] and immediately supplemented with protease inhibitors.

**Vascular Reactivity Experiments**

Mouse aortas were cleaned and cut into 2-mm length rings. Endothelium was removed by gently rubbing the lumen of the aortic ring with 4-0 silk suture. For experiments shown in Figure 7 and Figure III of the Online Supplement, endothelial denudation was accomplished with a 30-gauge needle. This appeared to result in a lower total reactivity to agonists. The denuded aortic rings were mounted in a myograph system (Danish Myo Technology A/S, Aarhus, Denmark). Vessels were bathed with warmed (37°C), aerated (95% O2/5% CO2) physiological salt solution (PSS, mmol/L: NaCl 130, KCl 4.7, KHPO4 1.18, MgSO4 1.17, CaCl2 1.6, NaHCO3 14.9, dextrose 5.5, CaNa2 EDTA 0.03). Rings were set at 700mg passive tension and equilibrated for 1 hour, washing every 20 minutes. Prior to performing concentration response curves, vessels were contracted by adding a stock KCl solution to the bath to achieve a final concentration of 60 mmol/L KCl in the bath. The vessels were washed and contracted again with 60 mmol/L KCl. After washing out the KCl contraction, the vessels were contracted with serotonin (5-HT; 1 µmol/L) and subsequently treated with acetylcholine (10 µmol/L) to test for absence of endothelium. All arteries were then contracted with KCl by adding a stock KCl solution
to achieve a final concentration of 100 mmol/L in the bath, allowed to plateau and washed. Thereafter, the vessels were incubated with vehicle (PBS, pH 1.0), indinavir (5 µmol/L, 25 µmol/L or 50 µmol/L) or no glucose conditions (5.5 mmol/L mannitol in the PSS instead of 5.5 mmol/L glucose) for 30 minutes. Cumulative concentrations of norepinephrine (NE), serotonin (5-HT), or prostaglandin F$_2$$\alpha$ (PGF$_2$$\alpha$) were added to the bath to establish concentration-response curves. Contractions to vasopressor were expressed as a percent of the 100 mmol/L KCl contraction.

**Mouse Model of AngiotensinII-Mediated Hypertension**

Alzet osmotic minipumps (Alza Corporation, Palo Alto, CA) containing either vehicle (0.01 N acetic acid in saline solution) or angiotensin II (0.75 mg/kg/day) were implanted subcutaneously under sterile conditions. Seven days later, systolic blood pressure was measured in trained, awake mice using a noninvasive computerized tail-cuff system (BP-2000, Visitech, Apex, NC). Mice were placed in temperature-controlled chambers (37°C) and blood pressure was recorded in 3 cycles of 10 measurements. Cycles with standard deviations higher than 20 were discarded. The animals were killed and the thoracic aortas were removed for vascular reactivity experiments.
Figure I. Effect of GLUT4 inhibition by indinavir on basal and insulin-stimulated glucose uptake in A7r5 VSMCs. A) Basal glucose uptake in A7r5 VSMCs in the absence and presence of indinavir. B) Insulin (100 nmol/L)-stimulated glucose uptake in A7r5 VSMCs in the absence and presence of indinavir. C) Confocal GLUT4 immunofluorescence of A7r5 VSMCs. 1) Unstimulated A7r5 VSMCs in the absence of indinavir. 2) Insulin (100 nmol/L)-stimulated A7r5 VSMCs in the absence of indinavir. 3) Insulin (100 nmol/L)-stimulated A7r5 VSMCs in the presence of 50 µmol/L indinavir. Arrows indicate staining for GLUT4. † = p<0.05 compared to basal, and * = p<0.05 compared to insulin (100 nmol/L).
**Figure II.** Effect of indinavir on basal glucose uptake in primary culture of bovine retinal endothelial cells (BRECs). A) GLUT1 immunoblot of whole cell BREC lysates (20 µg) (lanes 1-3). B) GLUT4 immunoblot of whole cell BREC lysates (20µg) (lanes 2-4) or whole heart lysates (lane 1), as a positive control. C) Basal glucose uptake in BRECs in the absence and presence of indinavir. * = p<0.05 by ANOVA compared to basal.
Figure III. Immunoblot detection of GLUT1 and GLUT4 in different detergent-resistant microdomains of A) cultured A7r5 cells or B) mouse aorta.
Figure IV. GLUT1 immunoblot of aortic homogenates (20 µg) from female wild-type or GLUT4KO mice.
Figure V. Effect of GLUT4 inhibition with indinavir or glucose deprivation on serotonin (5-HT)-mediated VSMC contractions in endothelium-denuded mouse aortic rings. Conditions for these experiments were slightly different than for those in other figures, so total contractile force generation was lower in all groups. Data are expressed as a percentage of the contraction elicited by 100 mmol/L KCl. * = p<0.05 compared to vehicle.
**Figure VI.** Systolic blood pressures measured by tail cuff in wild-type and GLUT4KO mice. Blood pressures were not different between the 2 groups of mice (n=6; p>0.05 by Student $t$ test).
Table 1. Potency of norepinephrine, as indicated by EC$_{50}$ values, in endothelium-denuded wild-type mouse aorta after aortas were incubated with vehicle, 5µM, 25 µM, 50 µM indinavir, or in no glucose conditions. Number of animals is indicated in parentheses.

* Indicates p<0.05 compared to Vehicle.

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<thead>
<tr>
<th>Agonist</th>
<th>Group</th>
<th>-log EC$_{50}$ [M]</th>
</tr>
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<tbody>
<tr>
<td>Norepinephrine</td>
<td>Vehicle</td>
<td>8.36 ± 0.07 (5)</td>
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<tr>
<td></td>
<td>5 µM Indinavir</td>
<td>8.33 ± 0.05 (5)</td>
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<tr>
<td></td>
<td>25 µM Indinavir</td>
<td>8.27 ± 0.04 (5)</td>
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<tr>
<td></td>
<td>50 µM Indinavir</td>
<td>8.26 ± 0.07 (5)</td>
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<tr>
<td></td>
<td>No Glucose</td>
<td>7.50 ± 0.12 (5)*</td>
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Table 2. Potency of different vasopressors, as indicated by EC$_{50}$ values, in endothelium-denuded mouse aorta from wild-type or GLUT4 knockout mice after aortas were incubated with vehicle, 25 µM indinavir, or in no glucose conditions. Number of animals is indicated in parentheses. * Indicates p<0.05 compared to Vehicle, and † indicates p<0.05 compared to 25 µM indinavir.