Abstract: Cross-talk between insulin and the renin angiotensin system signaling system shows that angiotensin 2 (A2) negatively modulates insulin signaling by stimulating multiple serine phosphorylation events in the early stages of the insulin-signaling cascade; however, the biological actions of A2 on insulin sensitivity remain controversial. Preservation of glucose transporter 4 (GLUT4) expression during hypertension has been shown to prevent the increased vascular reactivity associated with hypertension. This study tested the hypothesis that GLUT4 contributes to the renal actions of A2. In the euvolemic anesthetized rat, acute infusion of the GLUT4 antagonist, indinavir (1 mg/kg/minute), enhanced an A2-induced increase in mean arterial blood pressure (MABP) \(P < 0.01\), but attenuated an A2-induced increase in medullary blood flow (MBF) and glomerular filtration rate \(P < 0.01\). Insulin, a GLUT4 activator (20 mU/kg/minute and 40 mU/kg/minute), decreased basal MABP and urine volume \(P < 0.05\), but it increased MBF, and these effects were reversed and blunted by indinavir. Subchronic indinavir treatment (80 mg/kg/day orally for 15 days) did not affect A2-induced changes in MABP, cortical blood flow, and MBF, but significantly decreased basal MBF \(P < 0.01\) and global kidney perfusion \(P < 0.05\). We concluded that acute but not subchronic inhibition of GLUT4 alters A2-induced changes in systemic and renal hemodynamics by attenuating A2-induced increase in MBF and glomerular filtration rate.

Keywords: angiotensin 2, indinavir, hemodynamics, GLUT4, medullary blood flow

Introduction

Glucose transporter 4 (GLUT4), the insulin-dependent, transmembrane glucose facilitative transport molecule, plays a decisive role in insulin-dependent cardiac glucose metabolism, myocardial and vascular stiffness,\(^1\) osmolarity, as well as compartmental water distribution and homeostasis.\(^3\) GLUT4 is present at high levels in fat, skeletal muscle, and cardiac muscle and is also expressed at lower levels in other tissues, including the kidneys. Recent studies have shown an intensive GLUT4 expression in the smooth muscle cells of the renal afferent vasculature, in the renal glomerulus of normal rat kidneys,\(^4\) in proximal tubules, in a convoluted segment of distal tubules connected with juxtaglomerular apparatus, and in the epithelial cells of the thick ascending loop of Henle.\(^5\)\(^6\) Cross-talk between insulin and the renin angiotensin signaling system has drawn great attention because hypertension and insulin resistance often coexist and are leading risk factors for cardiovascular diseases.\(^7\)

Several lines of evidence have shown that angiotensin 2 (A2) plays an important role in the development of hypertension, cardiovascular disease, and insulin resistance.\(^8\)\(^9\) Hence, agents that inhibit A2 actions, such as the A2 converting enzyme inhibitors...
and A2 receptors blockers not only reduce blood pressure, but also improve insulin sensitivity in hypertensive and insulin-resistant patients. Evidence has been provided that A2 interferes with insulin signaling in vascular cells mainly by insulin-induced tyrosine phosphorylation of insulin receptor substrate 1 (IRS-1), thus impairing the vasodilator effects of insulin mediated by the IRS-1/PI3-kinase/Akt/endothelial NO synthase pathway. Alternatively, A2 may decrease the IRS-1/PI3-kinase association via the activation or upregulation of a protein–tyrosine phosphatase, and this thereby negatively modulates insulin signaling of the early components of the insulin-signaling cascade. Several studies have shown that rat and murine models of hypertension are associated with a marked decrease in GLUT4 expression in arterial smooth muscle. This reduction in GLUT4 levels corresponds to enhanced arterial sensitivity to agonists such as 5-hydroxytryptamine (5-HT) and norepinephrine (NE). In addition, preservation of GLUT4 expression during hypertension has been shown to prevent the increased vascular reactivity associated with hypertension. On the other hand, the inhibition of GLUT4 with indinavir has been shown to decrease glomerular filtration rate (GFR), renal blood flow (RBF), and nitrous oxide (NO) excretion without having any significant effect on blood pressure, indicating a mutually exclusive effect by GLUT4 on systemic versus regional hemodynamics. Based on these observations, we evaluated the inhibition of GLUT4 on A2-induced changes in systemic and renal hemodynamics in an attempt to test the hypothesis that GLUT4 plays a role in the renal actions of A2.

Materials and methods

Drugs and chemicals

Insulin from bovine pancreas, bovine serum albumin, inulin, sulfanilamide, N-((1-naphthyl)ethylenediamine, phosphoric acid, trichloroacetic acid, sodium nitrite, A2, sulfamic acid (Sigma-Aldrich, St Louis, MO, USA), dextrose (EM Science, Gibbstown, NJ, USA), citric acid and sodium citrate (Thermo Fisher Scientific, Waltham, MA, USA), indinavir (Crixivan; Merck and Co, Inc, Whitehouse Station, NJ, USA), and they were allowed ad libitum access to water and food prior to the experiments.

Acute studies

Surgical preparation

Animals were anesthetized with thiobutabarbital (Inactin®; Sigma-Aldrich), 100 mg/kg intraperitoneally and placed on a heated platform to maintain body temperature at 37°C. Both tail veins were cannulated with a 25-gauge butterfly needle (Vacutainer; Becton and Dickson, Franklin Lakes, NJ, USA) for the infusion or administration of drugs. The urethra was tied, and the urinary bladder was exposed by an abdominal incision. A cannula, polyethylene tubing, (PE-205) was inserted to drain and collect urine at 30-minute intervals. Dead space was minimized by a ligature that functionally excluded a large part of the bladder. A tracheotomy using PE-250 was performed for spontaneous ventilation, and a cannula (PE-50) was placed in the left carotid artery to monitor blood pressure. Mean arterial blood pressure (MABP) was measured with a pressure transducer (model BLPR2; World Precision Instruments, Inc, Sarasota, FL, USA) to a signal manifold (Transbridge, model TBM-4; World Precision Instruments, Inc) and recorded on a data acquisition system (model DI720; DATAQ Instruments, Inc, Akron, OH, USA). The left kidney was exposed by an abdominal incision, and intra-RBF was measured simultaneously by laser-Doppler flowmeter (system 5000, version 1.20; PeriFlux, Perimed AB, Stockholm, Sweden) via a surface probe (model PF 407) to measure cortical blood flow (CBF) or using an optical fiber laser-Doppler probe (model PF 402) fixed to a micromanipulator and placed in the medulla (5 mm below the kidney surface) to measure medullary blood flow (MBF). CBF and MBF were recorded as perfusion units (PU). An intravenous bolus dose of inulin (20 mg/kg) in normal saline was administered through the tail vein followed by a maintenance infusion (20 mg/hour) in normal saline throughout the experiment to maintain euvoelemia. Clearance of inulin was determined to evaluate GFR using the anthrone method. Rats were allowed to equilibrate for approximately 45 minutes, or until urine flow from the bladder was steady. After this postsurgical equilibration period, a 30-minute control clearance period was obtained, and urine was collected at the end of the clearance period. Arterial blood (0.2 mL) was collected from the carotid artery into 3.2% sodium citrate during the middle of a 30-minute urine collection period and immediately centrifuged at 6000 x g for 1 minute using a microcentrifuge (Model RS-102; APR&D Inc, Center, Minnesota, USA) to obtain plasma. An equal amount of normal saline was infused for volume replacement.

Animals

Experiments were conducted on male Sprague Dawley rats (300–350 g body weight) (Harlan Sprague Dawley, Houston, TX, USA) in accordance with the Care and Use of Laboratory Animals guidelines of the National Institutes of Health. Protocols for the study were approved by the Institutional Animal Care and Use Committee. Animals were maintained on standard rat food (Purina Chow; Purina, St Louis, MO, USA), and they were allowed ad libitum access to water and food prior to the experiments.
Studies of indinavir effect on angiotensin 2-mediated systemic and renal hemodynamics

Rats were infused through the tail vein with the GLUT4 antagonist, indinavir (1 mg/kg/minute), or vehicle (1 mL/hour) (dilute H$_2$SO$_4$, pH 4) at a constant rate throughout the experiment. Ten minutes after the commencement of infusion, graded doses of A2 (300 ng/kg/minute and 1000 ng/kg/minute) each lasting for 30 minutes were infused concurrently. A tracheotomy (PE-250) was performed for spontaneous ventilation, and a cannula (PE-50) was placed in the left carotid artery to monitor blood pressure. MABP, CBF, and MBF were measured as described above, and CBF and MBF were recorded as PU. Urine was collected every 30 minutes, while blood was collected at the midpoint of every 30-minute urine collection, and GFR was determined by inulin clearance using the anthrone method. Urine volume (UV) was determined gravimetrically.

Studies of indinavir effect on insulin (GLUT4 agonist)-mediated systemic and renal hemodynamics

Rats were infused with graded doses of insulin (20 mU/kg/minute and 40 mU/kg/minute), or vehicle (0.5% bovine serum albumin in normal saline, 1 mL/hour), each dose lasting 30 minutes. Ten minutes after the beginning of insulin infusion, a 50% dextrose solution (in saline) was infused at a variable rate to maintain baseline blood glucose level (98 ± 0.1 mg/dL) as monitored by frequent arterial blood glucose determinations (Glucometer Prestige; Walgreen Co, Deerfield, IL, USA) performed at 10-minute intervals using blood from the tail. In another group of rats, indinavir (1 mg/kg/minute) was infused 10 minutes prior to a concurrent infusion of graded doses of insulin (20 mU/kg/minute and 40 mU/kg/minute). Both drugs were infused at a constant rate throughout the experiment. MABP, CBF, and MBF were measured simultaneously. Urine and blood were collected as stated above for GFR estimation. UV was determined gravimetrically.

Subchronic studies

Male Sprague Dawley rats were treated with indinavir (80 mg/kg/day) or vehicle (0.05 M citric acid solution) by oral gavage for 15 days. This dose regimen was shown to significantly reduce urinary NO, GFR, and RBF in rats. At the end of treatment, animals were placed in metabolic cages for 24-hour urine collection. Animals were then anesthetized with thiobutabarbital (Inactin), 100 mg/kg intraperitoneally. An incision, approximately 5 cm long, was made on the left side of the abdomen and was kept open using a clamp to expose the left kidney. A laser scanner (Moor Instruments Inc, Axminster, UK) was positioned directly over the left kidney for the measurement of whole kidney perfusion. Two repetitive scans were performed and the mean value for each kidney was determined. Renal perfusion was expressed as PU per unit area of kidney.

Immediately after determination of whole kidney perfusion, the tail veins were cannulated for infusion or drug administration, and tracheostomy was performed for spontaneous ventilation. The urethra was tied and the urinary bladder catheterized for urine collection. A cannula was placed in the left carotid artery to measure MABP, and a left laparatomy was performed to monitor the CBF and MBF.

Graded doses of A2 (300 ng/kg/minute and 1000 ng/kg/minute), each lasting for 30 minutes, were infused concurrently. MABP, CBF, and MBF were measured simultaneously. Urine was collected every 30 minutes, while blood was collected at the midpoint of every 30-minute urine collection to determine GFR by inulin clearance. UV was also determined.

Urine and plasma inulin measurements

Urine and plasma inulin concentrations were measured using the standard colorimetric techniques, and GFR was calculated as the ratio of urine and plasma inulin concentrations multiplied by urine flow.

Statistical analysis

All data were expressed as mean ± standard error of the mean. Changes in systemic and renal hemodynamics were expressed as absolute values and absolute changes from baseline. The effects of A2 and indinavir were analyzed using a two-way analysis of variance followed by Tukey’s multiple comparison test when appropriate.

Results

Effect of acute inhibition of GLUT4 on systemic and renal hemodynamics induced by angiotensin 2 infusion

In Figure 1, acute indinavir infusion (1 mg/kg/minute) significantly enhanced A2-induced increase in MABP (P < 0.05), but only at the 1000 ng/kg/minute dose, and it had the tendency to attenuate the A2-induced increase in MBF, but it did not affect CBF. As illustrated in Figure 2, A2 produced an unexpected increase in UV, while simultaneous indinavir administration did not produce any significant change in UV. The increase in UV elicited by A2 may be due to pressure natriuresis. In Figure 3, A2 produced an increase in GFR at a dose of 1000 ng/kg/minute, and this effect was attenuated by
acute indinavir administration ($P < 0.05$). These data imply that GLUT4 may attenuate A2-induced increase in GFR.

**Effect of subchronic indinavir administration on basal systemic and renal hemodynamics**

The role of GLUT4 on basal systemic and renal hemodynamics after subchronic administration of indinavir was evaluated. Figure 4 illustrates that the subchronic inhibition of GLUT4 did not change basal MABP or CBF, but significantly reduced MBF ($113 \pm 8 \, \text{PU}$) compared to control ($176 \pm 19 \, \text{PU}, \ P < 0.01$). These data suggest that GLUT4 is involved in the maintenance of basal renal medullary perfusion.

**Effect of angiotensin 2 infusion on systemic and renal hemodynamics after subchronic indinavir administration**

The effect of the subchronic inhibition of GLUT4 on A2-mediated effects on systemic and renal hemodynamics was evaluated in animals treated with indinavir (80 mg/kg/day orally for 15 days). Figure 5 illustrates that...
the subchronic administration of indinavir did not affect the changes in MABP, CBF, and MBF induced by acute A2 administration, nor did it cause changes in urine output and GFR (Figures 6 and 7), but there was a reduction in global kidney perfusion ($P < 0.05$) (Figure 8). These data imply that GLUT4 did not contribute significantly to A2-mediated effects that were determined in these experiments, but they may be increasing overall intra-RBF.

Effects of acute activation of GLUT4 on systemic and renal hemodynamics in the absence or presence of acute indinavir administration

In order to evaluate the effects of acute activation of GLUT4 on basal systemic and renal hemodynamics in the absence or presence of a GLUT4 antagonist, animals were treated with insulin, a GLUT4 activator, at doses of 20 mU/kg/minute and 40 mU/kg/minute in the absence or presence of acute indinavir administration. Figure 9 shows that insulin decreased basal MABP and increased MBF in a dose-related fashion with minimal effects on CBF. The insulin-induced decrease in MABP and subsequent increase in MBF were reversed and blunted, respectively, by acute infusion of indinavir. Figure 10 illustrates that, as expected, insulin infusion reduced UV in a dose-related manner, and this effect was attenuated by indinavir ($P < 0.05$). However, Figure 11 illustrates that insulin produced no significant change in GFR. These data suggest that GLUT4 activation increases MBF, but it does not translate into an increase in

![Figure 2](image-url) **Figure 2** Effect of GLUT4 inhibition by indinavir on the effects of acute Ang 2 infusion on urine output. **Abbreviations:** GLUT4, glucose transporter 4; ANG 2, angiotensin 2; n, number.

![Figure 3](image-url) **Figure 3** Effect of GLUT4 inhibition by indinavir on the effects of acute Ang 2 infusion on GFR. **Note:** $^{*} P < 0.05$ versus control. **Abbreviations:** GLUT4, glucose transporter 4; GFR, glomerular filtration rate; Ang 2, angiotensin 2; n, number.
urine output, thus implying that the stimulation of GLUT4 in the kidney may be acting through independent vascular and tubular mechanisms.

**Discussion**

Several studies have shown that A2 inhibits insulin-stimulated glucose transport and GLUT4 translocation in multiple organs and tissues, including the intact rat heart and the rat aortic smooth muscle cells. Conversely, treatment with an angiotensin receptor type 1 (AT₁) blocker improved tyrosine phosphorylation of IRS-1, insulin receptor IRS-1/PI3-K association, and GLUT4 translocation to the plasma membrane. In the present study, acute inhibition of GLUT4 with indinavir enhanced an A2-induced increase in MABP, but attenuated A2-induced increase in MBF and GFR. These observations are consistent with studies that report that the abrogation of GLUT4 expression by genetic knockout is associated with both increased total force generation and enhanced sensitivity to agonists such as 5-HT and NE. In addition, the preservation of GLUT4 expression during hypertension would prevent the associated increase in vascular reactivity.
A2 has been shown to increase renal MBF via stimulation of AT type 2 (AT\(_2\)) receptors, and AT\(_1\), is associated with increased NO production, leading to vasodilation and an increase in medullary flow. Insulin-stimulated translocation of GLUT4 is associated with an increase in NO production by a pathway involving IRS-1/P13-kinase/Akt/endothelial NO synthase. Therefore, inhibition of GLUT4 and the ensuing decrease in NO production/medullary blood flow probably accounts for the decreased A2-induced increase in MBF.

A2 conserves salt and water through a combination of the hemodynamic and tubular epithelial cell sodium chloride and water transport mechanisms. Thus, A2 elicited a direct effect on the proximal tubule apical sodium/hydrogen exchanger.

Figure 5 Effect of acute Ang 2 infusion on systemic and renal hemodynamics in the presence of subchronic inhibition of GLUT4 with indinavir (80 mg/kg/day for 15 days).

Effect of acute Ang 2 infusion on systemic (A) and renal (B and C) hemodynamics in the presence of subchronic inhibition of GLUT4 with indinavir (80 mg/kg/day for 15 days).

Note: Vehicle for indinavir was 0.05 M citric acid (0.3 mL/day).

Abbreviations: Ang 2, angiotensin 2; GLUT4, glucose transporter 4; MABF, mean arterial blood flow; n, number; CBF, cortical blood flow; MBF, medullary blood flow.
and on the basolateral sodium-bicarbonate cotransporter, stimulating proximal tubular Na\(^+\) and water reabsorption at low doses, but inhibiting it at higher doses. A2 can also stimulate transport activity in distal nephron segments, and thereby directly stimulates sodium reabsorption in distal nephron segments via the proximal tubule apical sodium/hydrogen exchanger, Na\(^+\)Cl\(^-\) cotransporter, epithelial sodium channel, potassium channel, and H\(^+\)–ATPase transport mechanisms. A2 produced an unexpected increase in UV, while simultaneous indinavir infusion did not produce any significant change in UV. The increase in UV elicited by high dose of A2 may be due to pressure natriuresis. A2 also exerts several effects that may alter the GFR by constricting the afferent arteriole and the mesangial cells to decrease GFR, or by constricting the efferent arterioles to increase GFR, the net effect depending on the physiological state. In this study, A2 produced an expected decrease in urine output at low doses, probably through an AT\(_1\) receptor-mediated decrease in RBF and/or stimulation of sodium transport in the proximal tubule and distal segments of nephron; however, it paradoxically increased urine flow at high dose due to pressure natriuresis. The attenuation of A2-induced
reduction in urine output following GLUT4 inhibition may occur through a mechanism that probably involves a direct interaction between the inhibition of GLUT4 and A2 receptors at sites of A2-induced sodium reabsorption in the nephron. A decrease in glucose transport following GLUT4 inhibition is expected to increase GFR as a result of decrease in intracellular adenosine triphosphate (ATP), and subsequent activation of K\textsubscript{ATP} channels leading to vasodilation.\cite{4,20} In the present study, GLUT4 inhibition-induced attenuation of the unexpected increase in GFR induced by the high dose of A2 could be explained by the known A2 inhibition of K\textsubscript{ATP} channels.\cite{30,31} A previous study reports that chronic indinavir treatment induces moderate hyperbilirubinemia and attenuates A2-dependent hypertension in mice,\cite{12} and this may be through improved renal hemodynamics and GFR.\cite{33} The increase in bilirubin levels in A2-treated mice may result in a greater decrease in vascular superoxide (O\textsubscript{2}) levels and an increase in NO bioavailability. Plausible as this is, it varies from data obtained in the present study, probably because the low dose of indinavir used (80 mg/kg/day) in the present study is lower than the high dose of 500 mg/kg/day that produced hyperbilirubinemia in previous studies.\cite{16,32}

After subchronic inhibition of GLUT4, there were no changes in basal MABP or CBF, but there was a significant reduction in MBF. The reduction in basal MBF was similar to the results with acute GLUT4 inhibition. The lack of change in both systemic and renal hemodynamics after subchronic inhibition suggests that GLUT4 may not interact with A2 when activated chronically. Alternatively, there could be compensatory changes in glucose uptake or cellular signaling in response to the chronic inhibition of GLUT4-mediated glucose uptake as compared to the effects of acute inhibition of GLUT4-mediated uptake. At present, there are no data that support either of these possibilities, but NE and 5-HT-induced contractility was attenuated after acute inhibition of GLUT4, while contractility to NE and 5-HT was potentiated in the GLUT4 knockout mice,\cite{14} suggesting that the chronic absence or reduction of GLUT4 expression in vascular smooth muscle cells (VSMCs) leads to opposite effects.
Recent studies have shown that insulin acts as a pressor as well as a depressor, depending on experimental circumstance and physiological state.\textsuperscript{34,35} In evaluating the effects of insulin in the absence or presence of indinavir on systemic and renal hemodynamics, insulin decreased basal MABP, and this effect was reversed by indinavir, thus confirming that GLUT4 was involved in arterial blood pressure regulation.\textsuperscript{13–15} The blunting of an insulin-induced increase in MBF by indinavir again confirms that the activation of GLUT4 will increase renal MBF, possibly through an increase in NO production. The dose-related reduction in UV by insulin obtained in the present study is in agreement with a previous report,\textsuperscript{36} where the stimulation

\textbf{Figure 9} Effect of acute activation of GLUT4 with insulin (20 mU/kg/minute and 40 mU/kg/minute) on systemic and renal hemodynamics in the presence or absence of acute indinavir administration. Effect of acute activation of GLUT4 with insulin (20 mU/kg/minute and 40 mU/kg/minute) on systemic (A) and renal (B and C) hemodynamics in the presence or absence of acute indinavir administration.

\textit{Note:} *\(P < 0.05\) versus control.

\textbf{Abbreviations:} GLUT4, glucose transporter 4; MABF, mean arterial blood flow; n, number; CBF, cortical blood flow; MBF, medullary blood flow.
of GLUT4 by insulin has been shown to enhance sodium and water retention in the renal tubules. This effect was blunted by GLUT4 inhibition, suggesting that GLUT4 activation may be exerting an indirect tubular effect. Though insulin is capable of stimulating mechanisms that promote both antinatriuresis and natriuresis, under the conditions of these experiments, the net effect appears to be that of increased sodium reabsorption and decreased urine output. Insulin infusion did not produce any significant changes in GFR; however, indinavir appears to increase GFR when infused concurrently with insulin in the present study, and this result may be explained by decreased glucose transport. GLUT4 inhibition leads to diminished ATP generation, subsequently resulting in increased activity of $K_{ATP}$ and relaxation of the VSMC. This leads to increased GFR, as observed in the present study, when indinavir is infused concurrently with insulin. This is in agreement with a previous study, which showed that the decreased expression of GLUT4 in VSMCs of afferent renal microvasculature could lead directly to vasodilation and increased glomerular filtration. In conclusion, this study demonstrates that GLUT4 plays a role in A2-induced changes in systemic and renal hemodynamics.
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Disclosure
The authors report no conflicts of interest in this work.

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GLUT4 and angiotensin-induced hemodynamics