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EXPERIMENTAL STUDY

Renal effects of glucose transporter 4 in *N*ω-nitro-L-arginine/ /high salt-induced hypertensive rats

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Abstract: *Objectives:* The aim of study was to determine the renal effects of glucose transporter 4 (GLUT4) in a hypertensive nephropathy rat model.
Background: GLUT4 has been implicated in insulin resistance and hypertension in several animal models; however its role in hypertensive nephropathy still remains unclear.
Methods: Hypertensive nephropathy was induced by *N*ω-nitro-L-arginine (L-NNA), a nitric oxide (NO) synthase inhibitor, 100 mg/ml in drinking water and high salt (HS) diet (4 % NaCl), for 15 days in the presence of insulin, a GLUT 4 agonist (1 U/day) and indinavir, a GLUT4 inhibitor (80 mg/kg/day).
Results: Decreased basal renal medullary and cortical blood flow was enhanced in LNNA/HS/indinavir group ($p < 0.01$) but attenuated ($p < 0.05$) by insulin. Proteinuria was increased ($p < 0.01$) in LNNA/HS/indinavir group but attenuated ($p < 0.01$) by insulin. Insulin-treated rats decreased urine NO ($p < 0.01$) and urine Na^{2+} ($p < 0.01$) compared to other treated animals. In indinavir-treated animals, urine Na^{2+} was increased by benzamil, an epithelial sodium channel (ENaC) inhibitor ($p < 0.01$) and hydrochlorothiazide, a sodium/chloride co-transporter (NCC) inhibitor ($p < 0.05$).
Conclusion: GLUT4 exerts a renoprotective role which may be related to increase NO production. The antinatriuretic effects of GLUT4 appear to be due to enhancement of ion transport activity of ENaC and NCC at the renal tubules *(Fig. 9, Ref. 34)*. Text in PDF *www.elis.sk.*
Key words: GLUT4, hypertension, indinavir, nephropathy, renoprotective.

The interrelation between disturbances in glucose metabolism and hypertension has been known for a long time and subject to investigation in a multitude of trials and experimental studies (1–3). However, although much progress has been achieved during the last decade, the molecular mechanisms linking insulin resistance to hypertension, cardiac hypertrophy and development and/or progression of atherosclerosis are still unknown (4, 5).

Glucose transporter 4 (GLUT4), an insulin-dependent transmembrane glucose facilitative transporter is present at high levels in fat, skeletal muscle and cardiac muscle and is also expressed at lower levels in other tissues, such as the kidney were it is abundantly expressed in smooth muscle cells of the renal afferent vasculature, proximal tubules, convoluted segment of distal tubules, connected with juxtaglomerular apparatus (JGA) and in the epithelial cells of the thick ascending loop of Henle (6–8). GLUT4 expression has been shown to decrease in large arteries of hypertensive rats and mice (9–10) as well as that arterial reactivity in arteries from GLUT4-knockout mice was increased compared with vessels from wild types of animals (10), and was similar to arteries from hypertensive animals. Earlier reports showed that maintenance of GLUT4 expression reduced the hypertension-induced increased vascular reactivity and possibly prevented the development of hypertension itself (11). Subchronic inhibition of GLUT4 has been shown to alter angiotensin II-induced changes in systemic and renal haemodynamics by attenuating angiotensin-induced increase in medullary blood flow and glomerular filteration rate (12).

Chronic inhibition of NO synthesis by L-arginine analogues such as N-nitro-L-arginine (L-NNA) promotes progressive arterial hypertension associated with proteinuria and severe renal vascular, glomerular, and interstitial injury (13, 14). These events are exacerbated by the concomitant administration of a high-sodium diet (HS) (15, 16). Although GLUT4 is present in several segments of the nephron, there are no published reports on its role in hypertensive nephropathy, hence we hypothesized that GLUT4 has a protective role in hypertensive nephropathy and this effect is related to nitric oxide production. We tested this hypothesis in a hypertensive nephropathy model involving nitric oxide withdrawal and high salt (4 % NaCl) diet [high salt/N-nitro-L-arginine (L-NNA)] in rats and determined the possible mechanism(s) of GLUT4-induced tubular effects.

Materials and methods

Materials

*N*ω-nitro-L-arginine (L-NNA), benzamil, dimethylamiloride, furosemide and hydrochlorothiazide Sigma-Aldrich, St. Louis,

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MO, USA), Indinavir (Crixivan, Merck & Co.Whitehouse Station, NJ, USA), Bio-rad reagent (Bio-rad Laboratories, Inc, Hercules, CA).

Animal model and experimental protocol

Female Sprague-Dawley rats (350–400 g body weight; Harlan Sprague Dawley, Houston, TX) were maintained on standard rat food (Purina Chow; Purina, St Louis, MO) and allowed *ad libitum* access to water and food until the beginning of the experiments. Experiments were conducted in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals. Protocols for the study were approved by the Institutional Animal Care and Use Committee.

Animals were divided into four groups (n = 6–7 rats per group).

Group 1; tap water for 15 days.

Group 2; N^{ω}-nitro-L-arginine (L-NNA), a nitric oxide synthase inhibitor, 100 mg/ml in drinking water and high salt diet (4 % NaCl), Harlan Teklad Custom Research Diets, (Madison, USA) for 15 days.

Group 3; N^{ω}-nitro-L-arginine (L-NNA), 100 mg/ml in drinking water, high salt diet (4 % NaCl) and indinavir, a GLUT4 inhibitor (80 mg/kg/day, oral gavage) for 15 days (17).

Group 4; N^{ω}-nitro-L-arginine (L-NNA), 100 mg/ml in drinking water, high salt diet (4 % NaCl) and insulin, a GLUT 4 agonist (1 U/day) (18) for 15 days. This dose of insulin was found to produce moderate hypoglycaemia and the animals survived without sucrose supplementation in drinking water.

Animals were treated for 15 days and during this treatment period, they were placed in metabolic cages on days 0, 1, 7 and 15 for 24 h collection of urine. At the end of the 15.day treatment, animals were weighed and anaesthetised with thiobutabarbital (Inactin), 100 mg/kg ip and placed on a heated platform to maintain body temperature at 37 °C. Thereafter, tracheostomy was performed for spontaneous ventilation and the left carotid artery was cannulated for mean arterial blood pressure measurements. A left laparatomy was done and a surface probe (PF 407) or an optical fibre LD probe (PF 402) laser Doppler was used to measure cortical blood flow (CBF) and medullary blood flow (MBF), respectively. Thereafter the heart and kidney were removed and weighed separately. Organ (heart and kidney) weight index was then determined by dividing the weight of the organ by the weight of the animal. Urinary Na^+ excretion ($U_{Na}V$) was determined by flame photometry (Genway FP7, Jenway Ltd, Essex, UK) while urine volume (UV) was determined gravimetrically.

Determination of NO using Griess assay

Nitric oxide (NO) was determined colorimetrically in urine samples using the Griess assay. Griess reagent was freshly prepared by mixing Solutions A and B in a 1:1 ratio. Solution A contains 1 g sulphanilamide dissolved in 5 ml phosphoric acid (H_3PO_4) and 95 ml distilled water while Solution B contains 100 mg N-(1-naphthyl) ethylenediamine (NEDD) dissolved in 100 ml distilled water. Standard concentrations of $NaNO_2$ at 1, 2, 5, 10 and 20 µM/ml were prepared in distilled water for plotting the standard curve by adding equal volumes of the Griess reagent and the varying standard concentration. For the samples, 0.5 ml Griess reagent and 0.5 ml of urine samples were mixed together and transferred into a curvette. A blank was done for each sample (0.5 ml of Griess reagent and 0.5 ml of distilled water). After measuring absorbance at 540 nm by a spectrophotometer (Spectronic, Genysis 5, Spectronic Instrument Inc, Rochester; USA) and determining the NO concentrations in the samples, the values obtained from the blank were subtracted from each sample to obtain the actual concentration of NO in the urine.

Determination of protein

The BIORAD assay is a colorimetric assay for protein determination. Briefly, a 1 in 5 dilution of the BIORAD reagent (Bio-rad Laboratories, Inc, Hercules, CA) was made and varying standard concentrations (0.125, 0.25, 0.5, 1 and 2 mg/ml) was prepared from Bovine Serum Albumin (BSA). A 1 in 5 dilution of urine samples was made and thereafter 990 µl of diluted BIORAD reagent was added to 10 µl of each urine sample and standard concentration before absorbance was determined at 595 nm with a spectrophotometer (Spectronic, Genysis 5, Spectronic Instrument Inc, Rochester, USA).

Determination of renal ion transport

Using the indirect method of evaluating *in vivo* ion transport sensitivity as described (19), two sets of experiments were carried out. Animals were divided into two groups (n = 6 per group) and treated with vehicle (0.05 M citric acid, 0.25 ml) or indinavir (80 mg/kg/day) by oral gavage for 15 days. At the end of the 15-day treatment, animals were randomly allocated to groups and treated intraperitoneally with the following;

a) 0.3 ml normal saline, i.p.,

b) furosemide, a sodium-potassium-2chloride cotransporter (NKCC2) inhibitor (12.5 mg/kg, i.p.) (20),

c) benzamil, an epithelial sodium channel (ENaC) inhibitor (0.7 mg/kg, i.p.) (19),

d) dimethylamiloride, a sodium/hydrogen exchanger (NHE) inhibitor (0.7 mg/kg, i.p.) (21),

e) hydrochlorothiazide, a sodium/chloride co-transporter (NCC) inhibitor (3.75 mg/kg, i.p.) (19).

All groups were administered simultaneously with 0.9 % NaCl at 2.5 ml/100 g rat i.p. (acute sodium loading). Animals were then placed in metabolic cages and urine was collected after a 6-hour period and assayed for urinary excretion of Na^+ ($U_{Na}V$) and urine volume (UV).

Statistical analysis

All data were expressed as mean ± standard error of mean (SEM). Differences between groups were assessed by two-way analysis of variance with Bonferroni post hoc tests for multiple comparisons. In all cases, $p < 0.05$ was considered statistically significant.

Results

There was no significant difference in kidney weight (KW) index or heart weight (HW) index between the treated groups (Figs 1 and 2). However, there was a significant increase in CBF and

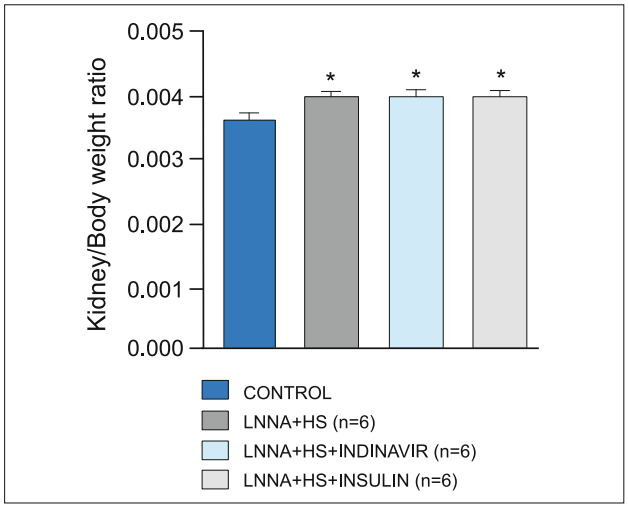

Fig. 1. Kidney weight index in LNNA/HS (4 % NaCl diet)-treated rats receiving vehicle (0.05 M citric acid), indinavir (80 mg/kg, p.o.) or insulin (1 U/day) for 15 days (* p < 0.05 vs control). Control animals were placed on tap water.

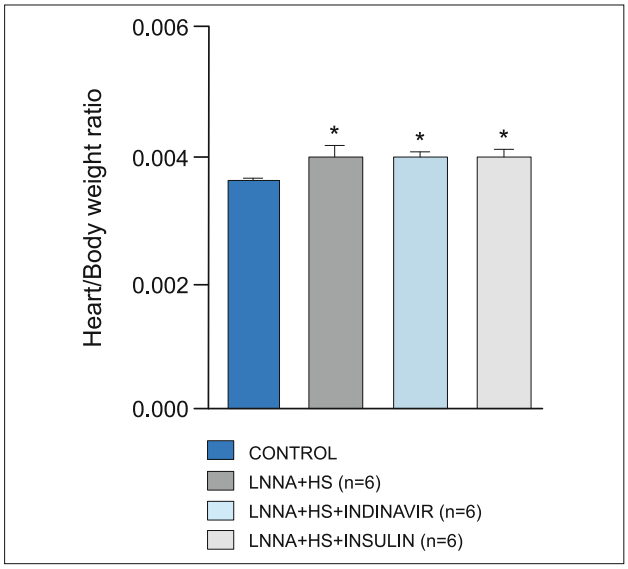

Fig. 2. Heart weight index in LNNA/HS (4 % NaCl diet)-treated rats receiving vehicle (0.05 M citric acid), indinavir (80 mg/kg, p.o.) or insulin (1 U/day) for 15 days (p < 0.05 vs control). Control animals were placed on tap water.**

MBF in animals treated with insulin compared to LNNA/HS and LNNA/HS/indinavir groups (Fig. 3).

Proteinuria increased on days 7 and 15 in LNNA/HS/indinavir group (81.52 ± 4.4; 214.15 ± 28.22 µg/24 h, $p < 0.01$) and decreased in the LNNA/HS/insulin group (24.74 ± 4.37; 41.56 ± 13.70 µg/24 h, $p < 0.05$) compared to LNNA/HS-treated group of animals (54.43 ± 8.31; 81.28 ± 7.17 µg/24 h) (Fig. 4). On day 15, urine output was increased in the LNNA/HS/indinavir group significantly ($p < 0.05$) compared to LNNA/HS group (Fig. 5).

Sodium excretion was significantly ($p < 0.05$) increased in the LNNA/HS/indinavir group (5302.33 ± 334.8 mM/24 h) compared to the LNNA/HS group (3758.38 ± 343.2 mM/24 h) on day 15. However, in the LNNA/HS/insulin group, Na excretion was

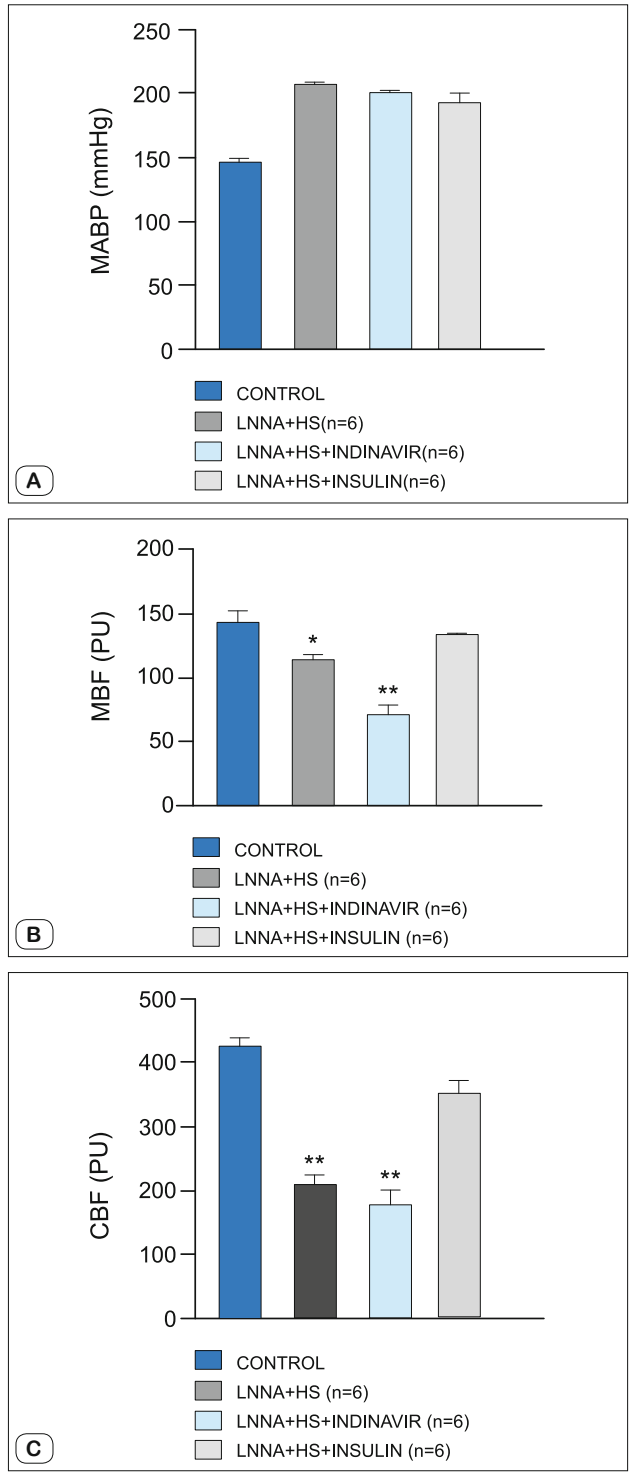

Fig. 3. Baseline values for systemic (a) and renal (b and c) hemodynamics in LNNA/HS (4 % NaCl diet)-treated rats receiving vehicle (0.05 M citric acid), indinavir (80 mg/kg/day) or insulin (1 U/day) for 15 days (* p < 0.05, ** p < 0.01 vs control). Control animals were placed on tap water.

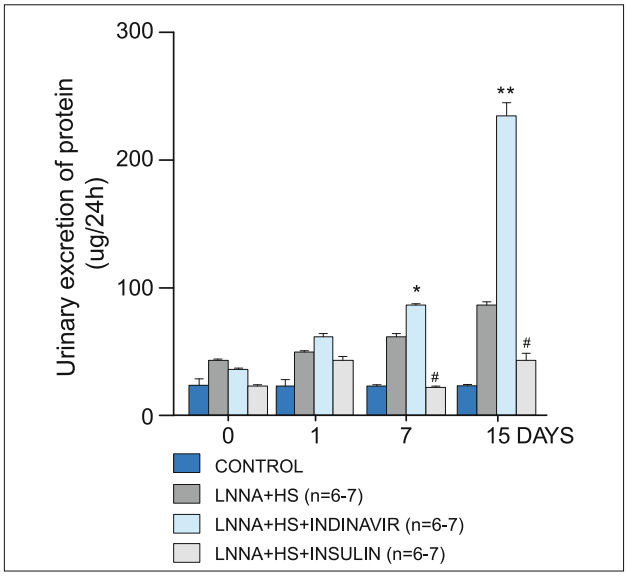

Fig. 4. Urinary protein excretion in LNNA/HS (4 % NaCl diet)-treated rats receiving vehicle (0.05 M citric acid), indinavir (80 mg/kg/day) or insulin (1 U/day) for 15 days (* p < 0.05, ** p < 0.01 vs LNNA/HS; # p < 0.05 vs LNNA/HS). Control animals were placed on tap water.

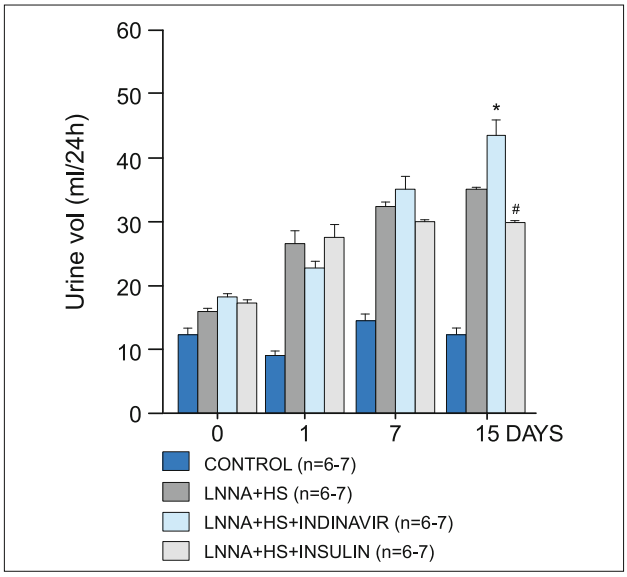

Fig. 5. Urine volume in LNNA/HS (4 % NaCl diet)-treated rats receiving vehicle (0.05 M citric acid), indinavir (80 mg/kg/day) or insulin (1 U/day) for 15 days (* p < 0.05 vs LNNA/HS; # p < 0.01 vs LNNA/HS/indinavir). Control animals were placed on tap water.

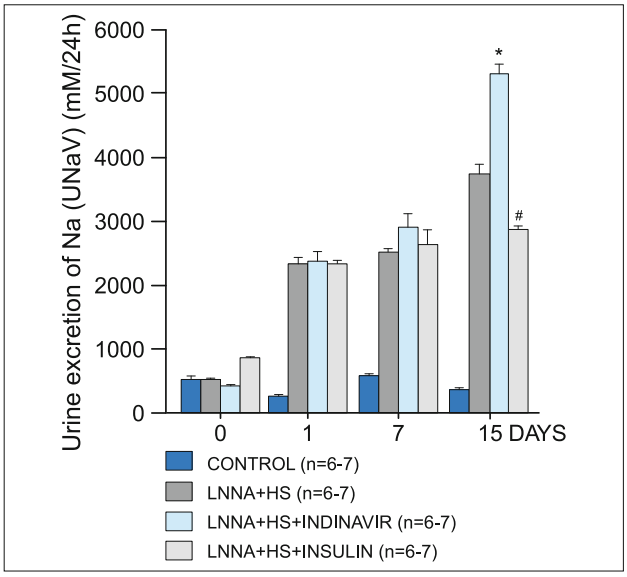

Fig. 6. Urinary Na excretion in LNNA/HS (4 % NaCl diet)-treated rats receiving vehicle (0.05 M citric acid), indinavir (80 mg/kg/day) or insulin (1 U/day) for 15 days (* p < 0.05 vs LNNA/HS; # p < 0.01 vs LNNA/HS/indinavir). Control animals were placed on tap water.

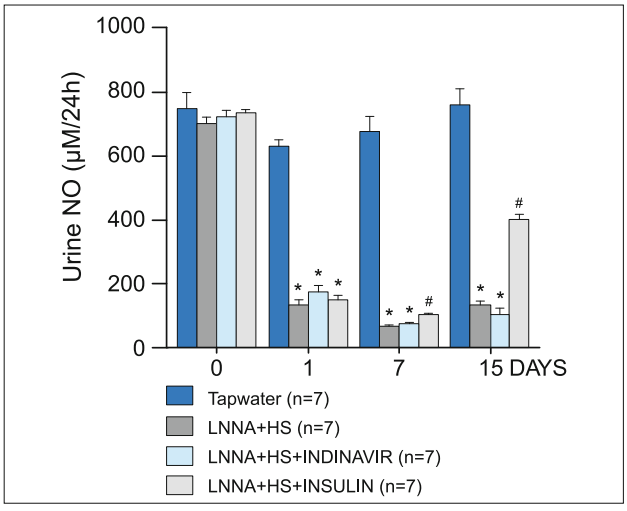

Fig. 7. Urinary NO excretion in LNNA/HS (4 % NaCl diet)-treated rats receiving vehicle (0.05 M citric acid), indinavir (80 mg/kg/day) or insulin (1 U/day) for 15 days (* p < 0.01 vs control; # p < 0.01 vs LNNA/HS/indinavir). Control animals were placed on tap water.

decreased significantly (2796.59 ± 250.5 mM/24 h) compared to the indinavir group (Fig. 6).

There was a significant increase in $U_{NOX}V$ in the LNNA/HS/insulin group on day 7 (55.19 ± 11.6 µM, $p < 0.05$) and day 15 (406.43 ± 34.27 µM, $p < 0.01$) compared to LNNA/HS/indinavir group at day 7 (55.19 ± 11.6 µM) and day 15 (124.09 ± 46.24 µM), respectively (Fig. 7).

In the indinavir-treated animals, $U_{Na}V$ was increased by benzamil (2.0 fold, $p < 0.01$) and hydrochlorothiazide (2.5 fold, $p < 0.05$) compared to the control. $U_{Na}V$ also increased by 2.3 fold ($p < 0.01$) in the control group in indinavir-treated rats compared to the control group in vehicle-treated rats (Fig. 8).

In Figure 9, the control rats, urine volume was increased by furosemide (2.5 fold; $p < 0.01$), benzamil (2.1 fold, $p < 0.01$), hydrochlorothiazide (1.8 fold, $p < 0.05$) and dimethylamiloride (2.1 fold, $p < 0.01$). In the indinavir-treated animals, there was no change in urine volume induced by the diuretics when compared to the control but there was a 1.8 fold increase in the control group of indinavir-treated rats compared to control group of the vehicle-treated rats.

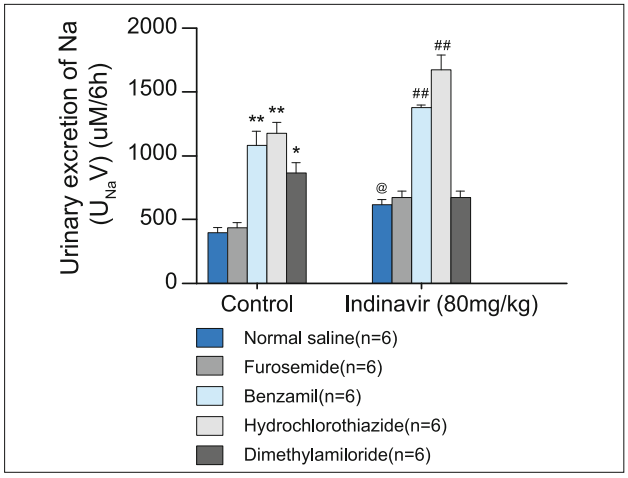

Fig. 8. Effect of furosemide, benzamil, hydrochlorothiazide and dimethylamiloride on urinary Na excretion after treatment with indinavir (80 mg/kg/day) for 15 days or its vehicle (0.05 M citric acid) for 15 days (*p < 0.05, **p < 0.01 vs vehicle); $^{\#\#}$p < 0.01 vs indinavir; $^{@}$p < 0.05 vs vehicle).

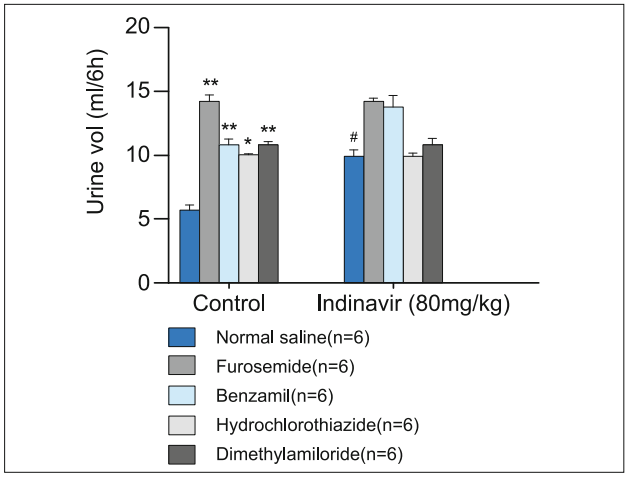

Fig. 9. Effect of furosemide, benzamil, hydrochlorothiazide and dimethylamiloride on urine volume after treatment with indinavir (80 mg/kg/day) for 15 days or its vehicle (0.05 M citric acid) for 15 days (*p < 0.05, **p < 0.01 vs vehicle); $^{\#\#}$p < 0.01 vs indinavir; $^{@}$p < 0.05 vs vehicle).

Discussion

In our experiments, LNNA/HS increased basal MABP and decreased CBF and MBF. There was a greater reduction in MBF by subchronic inhibition of GLUT4 with indinavir compared to LNNA/HS-treated group implying that GLUT4 is involved in increasing MBF probably through increase in NO production (22) and previous reports showed that renal MBF which is strongly influenced by NO production is thought to be an important component of blood pressure regulation and sodium balance (23). The further reduction in MBF by subchronic inhibition of GLUT4 with indinavir compared to LNNA/HS-treated group also suggests that there was an incomplete inhibition of NO synthase with the dose of LNNA used in these studies. Subchronic treatment with insulin, a GLUT4 activator, improved basal CBF and MBF in rats with hypertensive nephropathy. This effect may result from vasodilating action of insulin (24– 25). Insulin may stimulate endothelial nitric oxide production (22) or may act directly on vascular smooth muscle via stimulation of the Na^{+}-H^{+} exchanger and Na^{+}/K^{+}-ATPase, leading to hyperpolarisation of the cell membrane and consequent closure of voltage-gated Ca^{2+} channels and subsequent vasodilation (26). Several studies have shown decreased GLUT4 expression in vascular smooth muscles of several animal models of hypertension (9–10). Renal and cardiac hypertrophy induced by LNNA/HS treatment was not worsened by GLUT4 inhibition in as much as the kidney weight and the heart weight indices of indinavir-treated rats were not different from that of the LNNA/HS or insulin-treated rats.

Proteinuria, a measure of renal injury in this hypertensive animal model was markedly increased after subchronic GLUT4 inhibition in indinavir-treated rats compared to the LNNA/HS; however proteinuria was markedly decreased in insulin-treated rats implying that GLUT4 may be renoprotective in hypertensive nephropathy. Comparing the $U_{Na}V$ and urine volume (UV) between indinavir and LNNA/HS-treated groups, GLUT inhibition increased urine volume and sodium excretion and in insulin,treated animals there was a significant decrease in $U_{Na}V$ and UV in this hypertensive model. These data are congruent with previous reports (28–30) showing insulin-enhanced sodium reabsorption and reduced urine output. Urinary NO excretion ($U_{NOX}V$) decreased significantly after induction of hypertension in this animal model; this is a prominent feature of the experimental animal model (27). $U_{NOX}V$ was slightly lower in the indinavir-treated group compared to the LNNA/HS group suggesting there may be a further inhibition of NO production by GLUT4 inhibition. The significant increase in urinary NO production in rats treated with insulin on Day 15 may also suggest that GLUT4 activation may be involved in NO production that may be involved in its renoprotective ability in hypertensive rats.

Based on our results on the urinary sodium excretion and urine volume, we sought to determine specific ion channels involved in the observed GLUT4-antinatriuretic effects. This was determined by using differential response to diuretics as an indirect measure of *in vivo* ion transport (19, 31) by treating rats with benzamil, an ENaC inhibitor, hydrochlorothiazide, an NCC inhibitor, furosemide,, an NKCC2 inhibitor or dimethylamiloride, an NHE inhibitor. Sodium is the predominant extracellular cation and is of critical importance to the maintenance of extracellular fluid volume. The ability of kidneys to absorb large amounts of sodium with exquisite control relies on sequential actions of various segments of the nephron, each with highly specialized transport capabilities. The major apical Na transporters that contribute significantly to this regulation are the type 3 Na/H exchanger (NHE3) in the proximal tubule, bumetanide-sensitive type 2 Na-K-2Cl cotransporter (NKCC2) in the thick ascending limb of Henle, thiazide-sensitive Na-Cl cotransporter (NCC) in the distal convoluted tubule, and amiloride-sensitive epithelial Na channel (ENaC) in the connecting tubules and col-

lecting ducts (32). In the present study, the increase in $U_{Na}V$ in indinavir-treated rats compared to vehicle-treated rats suggests that GLUT4 increases sodium reabsorption in the renal tubules. We interpret the increase in $U_{Na}V$ in benzamil and hydrochlorothiazide groups treated with indinavir to imply that inhibition of GLUT4 further enhanced the blockade of ENAC and NCC in the renal tubules by benzamil and hydrochlorothiazide, respectively. Hence, GLUT4 may be increasing sodium reabsorption in the nephron by enhancing the ion transport activity of ENaC in the connecting tubules and collecting ducts and NCC in the distal convoluted tubule. This is in agreement with previous reports (33, 34). In the indinavir-treated animal, there was no change in urine volume induced by the diuretics when compared to the control but urine output was significantly greater in control group of indinavir-treated rats compared to that of the vehicle-treated rats. Normally, an increase in urinary excretion of sodium is followed by an increase in urine volume, the variance between $U_{Na}V$ and UV resulting from the inhibition of NCC, ENaC, NKCC2 or NHE channels in the indinavir-treated rats may be due to the involvement of GLUT4 in mechanisms controlling the concentration of urine in the medulla.

In conclusion, GLUT4 exerts a renoprotective role in hypertensive nephropathy and this effect seems to be related to increase in nitric oxide production resulting from the stimulation of GLUT4. The antinatriuretic effects of GLUT4 appear to be due to the enhancement of ion transport activity of ENaC and NCC at the distal and connecting/collecting tubules.

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