JCI The Journal of Clinical Investigation

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J Clin Invest. 1994;93(6):2623-2631. https://doi.org/10.1172/JCI117275.

Research Article

Increased Na+/H+ antiport activity has been implicated in the pathogenesis of hypertension and vascular disease in diabetes mellitus. The independent effect of elevated extracellular glucose concentrations on Na+/H+ antiport activity in cultured rat vascular smooth muscle cells (VSMC) was thus examined. Amiloride-sensitive 22Na+ uptake by VSMC significantly increased twofold after 3 and 24 h of exposure to high glucose medium (20 mM) vs. control medium (5 mM). Direct glucose-induced Na+/H+ antiport activation was confirmed by measuring Na(+)-dependent intracellular pH recovery from intracellular acidosis. High glucose significantly increased protein kinase C (PKC) activity in VSMC and inhibition of PKC activation with H-7, staurosporine, or prior PKC downregulation prevented glucose-induced increases in Na+/H+ antiport activity in VSMC. Northern analysis of VSMC poly A+ RNA revealed that high glucose induced a threefold increase in Na+/H+ antiport (NHE-1) mRNA at 24 h. Inhibiting this increase in NHE-1 mRNA with actinomycin D prevented the sustained glucose-induced increase in Na+/H+ antiport activity. In conclusion, elevated glucose concentrations significantly influence vascular Na+/H+ antiport activity via glucose-induced PKC dependent mechanisms, thereby providing a biochemical basis for increased Na+/H+ antiport activity in the vascular tissues of patients with hypertension and diabetes mellitus.

Glucose-induced Changes in Na^+/H^+ Antiport Activity and Gene Expression in Cultured Vascular Smooth Muscle Cells

Role of Protein Kinase C

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Abstract

Increased Na^+/H^+ antiport activity has been implicated in the pathogenesis of hypertension and vascular disease in diabetes mellitus. The independent effect of elevated extracellular glucose concentrations on Na^+/H^+ antiport activity in cultured rat vascular smooth muscle cells (VSMC) was thus examined. Amiloride-sensitive $^{22}Na^+$ uptake by VSMC significantly increased twofold after 3 and 24 h of exposure to high glucose medium (20 mM) vs. control medium (5 mM). Direct glucose-induced Na^+/H^+ antiport activation was confirmed by measuring Na^+-dependent intracellular pH recovery from intracellular acidosis. High glucose significantly increased protein kinase C (PKC) activity in VSMC and inhibition of PKC activation with H-7, staurosporine, or prior PKC downregulation prevented glucose-induced increases in Na^+/H^+ antiport activity in VSMC. Northern analysis of VSMC poly A^+ RNA revealed that high glucose induced a threefold increase in Na^+/H^+ antiport (NHE-1) mRNA at 24 h. Inhibiting this increase in NHE-1 mRNA with actinomycin D prevented the sustained glucose-induced increase in Na^+/H^+ antiport activity. In conclusion, elevated glucose concentrations significantly influence vascular Na^+/H^+ antiport activity via glucose-induced PKC dependent mechanisms, thereby providing a biochemical basis for increased Na^+/H^+ antiport activity in the vascular tissues of patients with hypertension and diabetes mellitus. (*J. Clin. Invest.* 1994. 93:2623–2631.) Key words: hypertension • diabetes mellitus • atherosclerosis • hyperglycemia • NHE-1 messenger RNA

Introduction

Diabetes mellitus is a potent risk factor for the development of premature and widespread vascular disease. This risk is greatly accentuated by the coexistence of hypertension. In addition, predisposition to hypertension may be an important determinant in a subset of diabetic patients who subsequently develop complications such as diabetic nephropathy and associated vascular disease (1, 2).

Multiple abnormalities in cell membrane transport have been described in hypertension, the most reproducible being an elevation in Na^+/Li^+ countertransport (3–5). The striking similarities between Na^+/Li^+ countertransport and the physiological Na^+/H^+ antiport have led to the assumption that Na^+/Li^+ countertransport is one mode of operation of the ubiquitous Na^+/H^+ antiport (6, 7). In support of this concept, the activity of the Na^+/H^+ antiport has been shown to be increased in erythrocytes, leukocytes, and platelets from patients with essential hypertension and in lymphocytes and vascular smooth muscle cells (VSMC)[1] from the spontaneously hypertensive rat (8–13).

There are notable similarities in the abnormalities of membrane cation transport from patients with essential hypertension and patients with diabetes mellitus who have developed vascular complications. For example, increased erythrocyte Na^+/Li^+ countertransport and increased leukocyte and fibroblast Na^+/H^+ antiport activity have been demonstrated in type I diabetic patients with hypertension and nephropathy (2, 14–16). The fact that this disturbance in membrane cation transport is common to both hypertension and diabetes has led to the suggestion that increased activity of the Na^+/H^+ antiport may play a role in the pathogenesis of hypertension and vascular disease in patients with diabetes mellitus (2, 14, 15).

The mechanisms responsible for diabetes-induced increases in Na^+/H^+ antiport activity are unknown. Calcium/phospholipid-dependent protein kinase C (PKC) activity plays an important role in the regulation of Na^+/H^+ antiport activity in many tissues (17, 18). Of interest, many of the physical and functional abnormalities demonstrated in cell membranes from hypertensive patients, including Na^+/H^+ antiport activation, can be reproduced by the activation of PKC (11). With regard to diabetes mellitus, more recent studies have shown that the PKC inhibitor staurosporine can restore to normal the previously elevated leucocyte Na^+/H^+ antiport activity of diabetic patients, thereby suggesting that diabetes-induced increases in Na^+/H^+ antiport activity may be dependent on PKC activation (19). Diabetes mellitus is characterized by the development of hyperglycemia, and we have recently shown that elevated extracellular glucose concentrations (20 mM) induce a sustained activation of PKC in cultured VSMC (20, 21). Together, these observations prompt the hypothesis that metabolic factors, in particular, hyperglycemia could directly influence the activity of the Na^+/H^+ antiport in the vasculature of diabetic patients via glucose-induced activation of PKC.

Examining this hypothesis in vivo would be hindered by the fact that the development of hyperglycemia is invariably

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Received for publication 26 July 1993 and in revised form 7 January 1994.

J. Clin. Invest.

0021-9738/94/06/2623/09 $2.00
Volume 93, June 1994, 2623–2631

1. *Abbreviations used in this paper:* NMDG, *N*-methyl-D-glucamine; PKC, protein kinase C; VSMC, vascular smooth muscle cell.

associated with many humoral and metabolic changes, each of which could independently influence the activity of PKC and/or the Na^+/H^+ antiport in vascular tissue. The present study thus utilizes an in vitro cell culture system to examine the direct effects of elevated extracellular glucose concentrations on Na^+/H^+ antiport activity and steady-state Na^+/H^+ antiport mRNA levels in cultured VSMC. In addition, the role of glucose-induced PKC activation as a mediator of glucose-induced changes in vascular Na^+/H^+ antiport activity is determined.

Methods

Culture of rat VSMC. Aortic VSMC were isolated from Sprague-Dawley rats and cultured and characterized as previously described in detail (20, 21). For Na^+ uptake experiments VSMC were grown on 35-mm culture plates in MEM (Gibco, Grand Island, NY) supplemented with 2 mM L-glutamine, 2 g/liter $NaHCO_3$, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS in an incubator at 37°C in 95% humidified air and 5% CO_2. Every 5–10 d, the cells were passaged after trypsin EDTA harvesting. For all experiments, second through sixth passaged VSMC were used.

Experimental design. VSMC were grown to confluence in the culture medium described above. The medium was then removed and replaced with one of four "test media" for 3 h (short-term exposure) or 24 h (sustained exposure). The test media comprised:

(*a*) Control medium: standard tissue culture medium as described above, with a normal D-glucose concentration of 5 mM.

(*b*) High glucose medium: this was similar to control medium except that it was supplemented with D-glucose to increase its concentration to 20 mM.

(*c*) L-glucose osmotic control medium: this was similar to control medium but supplemented with the cell permeable but poorly metabolized hexose, L-glucose 15 mM.

(*d*) Mannitol osmotic control medium: this medium was similar to control medium but was supplemented with the relatively impermeable and nonmetabolized hexose, mannitol 15 mM.

The latter two media served as osmotic controls for the high-glucose medium.

Measurement of $^{22}Na^+$ uptake by VSMC. After exposure to the various test media, VSMC monolayers were washed with a bicarbonate and sodium-free balanced salt solution. This "sodium-free buffer" contained choline chloride 135 mM, KCl 5 mM, $CaCl_2$ 2 mM, $MgSO_4$ 1 mM, Hepes 5 mM (pH 6.9), ouabain 2 mM, and variable amounts of D-glucose, L-glucose, or mannitol to mimic the composition of the test media to which they had been previously exposed. VSMC were preincubated with this buffer for 30 min at 37°C. This preincubation significantly decreases pH_i in VSMC and thus facilitates the measurement of Na^+/H^+ antiport dependent flux of Na^+ into the cells. VSMC pH_i (see below) after this 30-min preincubation did not significantly differ after 3 or 24 h of exposure to either control or high-glucose medium. After 30 min, the preincubation buffer was removed by aspiration and replaced with a similar buffer except that it contained 125 mM NaCl, 10 mM choline chloride, and 1 μCi/ml $^{22}Na^+$, with or without amiloride 1 mM. The influx of $^{22}Na^+$ into VSMC proceeded for 5 min at 37°C and was terminated by 6 × 1-ml rapid washings of the VSMC monolayers with iced-cold 0.1 M $MgCl_2$. The VSMC monolayer was lysed with warm 0.1 N NaOH and 0.1% SDS. A 50-μl aliquot of the lysate was retained for the measurement of cell protein content by the method of Lowry et al. (22) and $^{22}Na^+$ incorporation into the rest of the cell lysate was determined using a gamma counter (Packard Instrument Co., Inc., Meriden, CT) Na^+ uptake by VSMC was corrected for counter efficiency and cell protein content and is expressed as nanomoles of uptake per minute per milligram of cell protein.

Measurement of intracellular pH in VSMC. VSMC were grown to confluence on 13 × 30-mm glass coverslips and then exposed to the various test media for 24 h before the measurement of pH_i, which was measured using the pH-sensitive dye BCECF [(2,7)-biscarboxyethyl-5(6)-carboxyfluorescein] using a modification of the method described by Chaillet and Boron (23) and Bergman et al. (24). VSMC were loaded with the acetoxymethyl derivative of BCECF (5 μM) for 20 min at 37°C in solution A as noted below (pH 7.4). The cell-coated coverslip was then placed in a plastic cuvette in a Deltascan model 4000 fluorescence spectrometer (Photon Technology International Inc., South Brunswick, NJ) and perfused at 12 ml/min with solutions at 37°C as shown in Fig. 1. All solutions contained 1 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM D-glucose, 7 mM Hepes, 1 mM KH_2PO_4 and 4 mM K_2HPO_4. Solution A contained 145 mM NaCl and 5 mM KCl. Solution B contained 145 mM *N*-methyl-D-glucamine (NMDG-Cl) and 5 mM KCl. Solution C contained 120 mM NMDG-Cl, 25 mM NH_4Cl, and 5 mM KCl. pH_i was calculated from the ratio of fluorescence at excitation wavelengths 495 and 440 nm and emission at 535 nm. Calibration of the BCECF excitation ratio for each experiment was determined using the K^+/nigericin technique as previously described (24).

VSMC Na^+/H^+ antiport activity was calculated from the initial rate of Na^+-dependent pH_i recovery after an acid load in the absence of CO_2/HCO_3. Typical tracings with and without amiloride (1 mM) are depicted in Fig. 1. The Na^+-dependent pH_i recovery after an acid load was inhibited 90% by 1 mM amiloride. The initial rate of Na^+-dependent pH_i recovery (dpH_i/dt) was calculated from a line drawn tangential to the initial 30-s deflection after return of Na^+ to the perfusate.

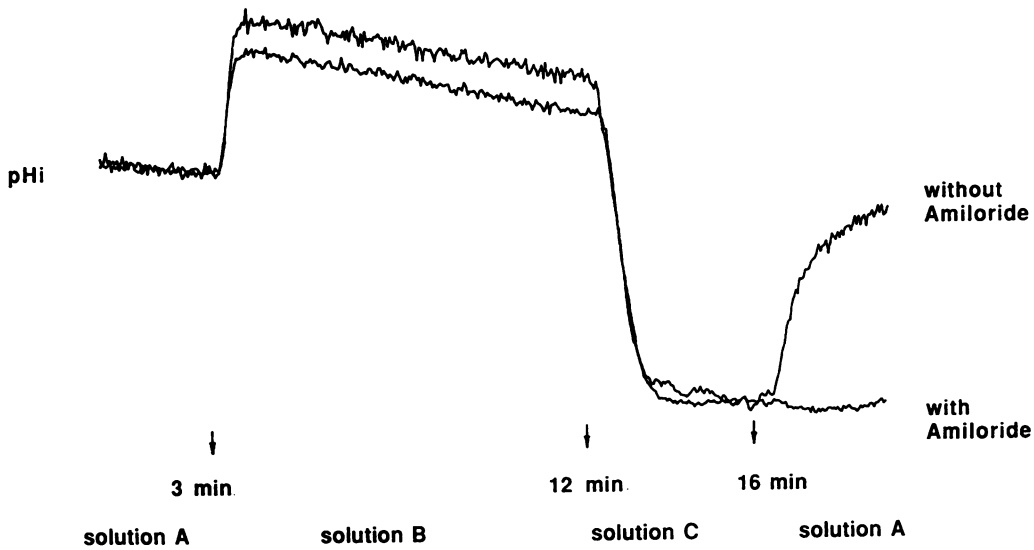


Figure 1. Perfusion schema and effect of 1 mM amiloride on Na^+-dependent recovery of intracellular pH in VSMC monolayers preloaded with BCECF. All solutions contained: 1 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM D-glucose, 7 mM Hepes, 1 mM KH_2PO_4. Solution A contained 145 mM NaCl and 5 mM KCl. Solution B contained 145 mM NMDG-Cl and 5 mM KCl. Solution C contained 120 mM NMDG-Cl. 25 mM NH_4Cl, and 5 mM KCl. Na^+-dependent recovery of intracellular pH was inhibited by 1 mM amiloride.

Intracellular buffering capacity was determined from the pH_i response to removal of NH_3/NH_4^+ using the formula: Buffering capacity $= [NH_4^+]_i/\Delta\ pH_i$, where $[NH^+]_i$ is the intracellular concentration before NH_3/NH_4^+ removal, calculated as $[NH_4^+]_i = [NH_4^+]_0 \times 10^{(7.4-pH_i)}$ and $\Delta\ pH_i$ is the pH_i change on removal of NH_4^+ (25).

Assay of PKC activity in VSMC. The methods used by our laboratory for the in-situ measurement and characterization of PKC activation in VSMC has been previously described in detail (20, 21). Briefly, VSMC were seeded into 96-well microtiter plates and grown to confluence in control medium. Thereafter, the VSMC were exposed to the test media for 3 or 24 h. The test media was aspirated and replaced with 40 μl of a buffered salt solution containing 137 mM NaCl, 5.4 mM KCl, 10 mM $MgCl_2$, 0.3 mM sodium phosphate, 0.4 mM potassium phosphate, 25 mM β-glycerophosphate, 5.5 mM D-glucose, 5 mM EGTA, 1 mM $CaCl_2$, 100 μM [τ-^{32}P]ATP, 50 μg/ml digitonin, and 20 mM Hepes (pH 7.2, 30°C). In addition, 100 μM of a PKC-specific substrate (VRKRTLRRL) was added to the buffer. This short synthetic peptide is based on the sequence surrounding a major PKC-dependent phosphorylation site within the epidermal growth factor receptor (26). This peptide substrate is not phosphorylated by cyclic nucleotide-dependent or Ca^{2+}/calmodulin-dependent protein kinases or S6 kinase and has been extensively characterized to be highly specific for PKC (27, 28). By permeabilizing the VSMC with digitonin, the PKC-specific VRKRTLRRL substrate enters the VSMC along with [τ-^{32}P]ATP to allow a selective and rapid analysis of in situ PKC activity. We have previously demonstrated that the concentration of digitonin used (50 μg/ml) did not modify VSMC morphology or promote cell detachment (20). In addition, the measured PKC activity was retained by the monolayer after permeabilization (PKC activity in the supernatant was 6.6% of total measured PKC activity). This latter finding is consistent with the concept that the PKC activity being assayed was tightly associated with the cell membrane as has been proposed for the active form of the kinase (17). The kinase reaction proceeds for 10 min at 30°C before termination by the addition of 10 μl of 25% (wt/vol) trichloroacetic acid. 45-μl aliquots of the reaction mixture are then blotted onto 2-cm phosphocellulose circles (P81, Whatman Inc., Clifton, NJ) and washed batchwise; three washes with 75 mM phosphoric acid and one wash with 75 mM sodium phosphate (pH 7.5). The basic VRKRTLRRL substrate is retained by the P81 filter and its PKC-dependent phosphorylation is quantified by scintillation counting using a Beta Counter (Packard Instrument Co., Inc.). Background phosphorylation was always < 0.05% of added counts per minute and was not influenced by the different test media. VSMC protein content per well was measured in an NaOH/SDS lysate as described above. Results are expressed as PKC-dependent phosphorylation picomoles per minute per milligram cell protein.

Measurement of steady-state Na^+/H^+ antiport mRNA. VSMC were grown to confluence on 100-mm dishes and exposed to the various test media for 3 or 24 h. Thereafter, total cellular RNA was isolated using the acid guanidium thiocyanate-phenol-chloroform method (29). VSMC from 15 dishes were pooled to yield ~ 3 mg of total cellular RNA. Poly A^+ RNA was isolated using an oligo-Dt spin column and reagents as supplied (5Prime 3Prime Inc., Boulder, CO). For Northern blot analysis, Poly A^+ RNA was separated by electrophoresis in 1% agarose/2.2 M formaldehyde gels, transferred to Nitroplus membranes (Micron Separations Inc., Westboro, MA) by capillary diffusion and immobilized by baking at 80°C in a vacuum oven for 2 h. Prehybridization was performed for 8 h at 42°C in a solution containing 5× SSC, 50% formamide, 5× Denhardt's solution, 0.1% SDS, and 200 μg/ml salmon sperm DNA. Hybridization was performed overnight at 42°C in the above solution containing 1×10^6 cpm/ml ^{32}P-random primer-labeled probe. The Na^+/H^+ antiport probe was a 1.8-kb Bam H1 fragment of the human NHE-1 cDNA (30), a generous gift from Jaques Pouyssegur, Nice, France. The β-actin probe was a 2-kb fragment of human cDNA (Clontech Laboratories Inc., Palo Alto, CA). Band intensities were determined from autoradiograms (Kodak XAR-5) using an Seprascan 2001 (Integrated Separation Systems, Hyde Park, MA).

Materials. All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated. Penicillin, streptomycin, culture media, and FCS were obtained from Gibco Laboratories Life Technology Inc. Acetomethyl ester of BCECF was obtained from Molecular Probes Inc. (Eugene, OR). $^{22}Na^+$ was obtained from Amersham Corp. (Arlington Heights, IL).

Statistical analysis. Results are reported as mean±standard error of the mean. Statistical analysis was performed using an unpaired Student's t test or analysis of variance with a Bonferroni correction as appropriate. A P value of < 0.05 was considered significant.

Results

Effect of elevated extracellular glucose concentrations on Na^+/H^+ antiport activity in VSMC. VSMC were exposed to the various test media for 3 h (short-term exposure) or 24 h (sustained exposure) before the measurement of $^{22}Na^+$ uptake. Basal pH_i was significantly reduced but did not differ after 30 min of exposure to the sodium-free preincubation buffer before measurement of $^{22}Na^+$ uptake by VSMC that had undergone short or sustained exposure to either control or high-glucose media. After a 3-h exposure to the high-glucose medium (20 mM), Na^+ uptake by VSMC was significantly increased by 53% when compared to Na^+ uptake by VSMC exposed to control medium (glucose 5 mM) (Fig. 2). The glucose-induced increase in Na^+ uptake by VSMC was sustained with continued exposure to the high-glucose medium for up to 24 h. Na^+/H^+ antiport activity is a major pathway for Na^+ influx in VSMC and this transport system can be inhibited by the addi-

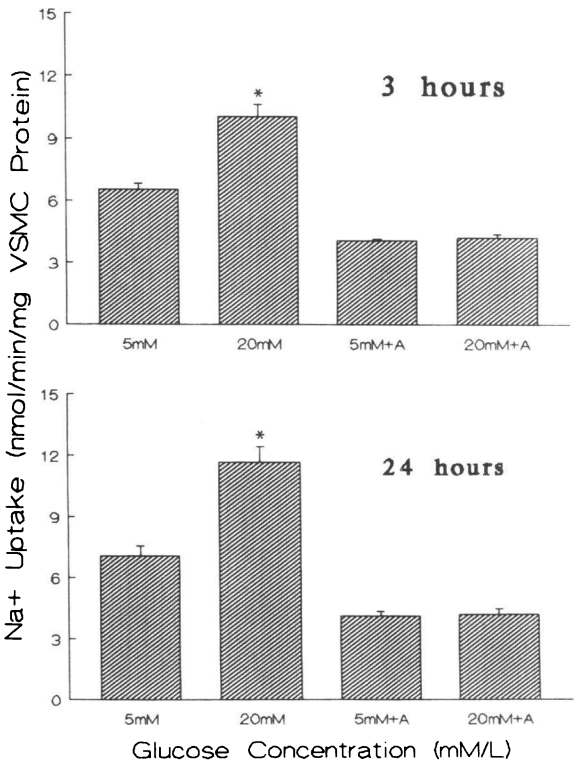


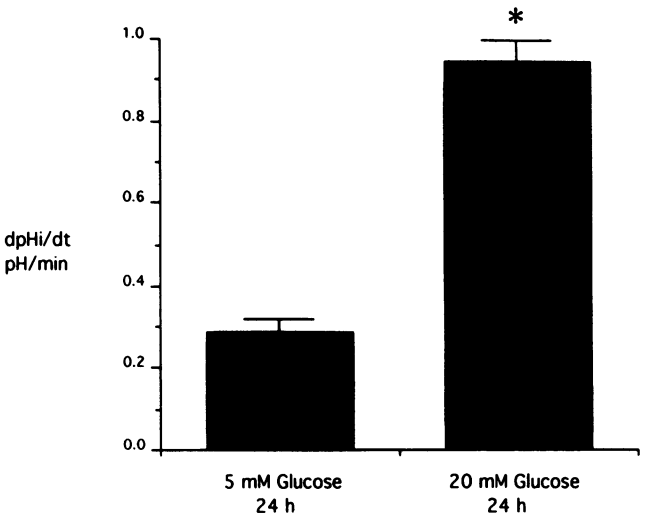
Figure 2. Effect of extracellular glucose concentration on amiloride-sensitive Na^+ uptake by VSMC. VSMC were exposed to control medium (glucose 5 mM) or high-glucose medium (glucose 20 mM) for 3 or 24 h in the absence or presence (+*A*) of amiloride (1 mM) before the measurement of $^{22}Na^+$ uptake. *$P < 0.01$ vs. all other columns; $n = 4$ experiments.

tion of amiloride (31). Amiloride (1 mM) significantly reduced Na^+ uptake by VSMC in the presence of control medium and completely prevented the increase in Na^+ uptake previously observed in the presence of the elevated glucose medium at both 3 and 24 h (Fig. 2). These observations suggest that the glucose-induced increase in Na^+ uptake by VSMC at both 3 and 24 h, was dependent on activation of the Na^+/H^+ antiport.

Osmotic stress is a recognized stimulus for the activation of the Na^+/H^+ antiport in various cell types (32). It was therefore important to determine whether the increased osmolality of the high-glucose medium could account for the glucose-induced activation of the Na^+/H^+ antiport in VSMC. Table I shows that 3 and 24 h of exposure to two osmotic control media (containing concentrations of L-glucose or mannitol designed to mimic the osmolality of the high-glucose medium) had no significant effect on Na^+ uptake by VSMC. This demonstrates that elevated extracellular D-glucose concentrations specifically increased Na^+/H^+ activity in VSMC via mechanisms independent of changes in extracellular osmolality.

Effects of elevated extracellular glucose concentrations on pH_i and intracellular buffering capacity in VSMC. To confirm that elevated glucose concentrations directly and specifically stimulated an increase in Na^+ uptake via the Na^+/H^+ in VSMC, VSMC were exposed to control medium, high-glucose medium, and mannitol osmotic control medium for 24 h before the measurement of Na^+-dependent pH_i recovery from an acid load. Fig. 3 and Table II summarize the results of these experiments. 24 h of preexposure to the high-glucose medium significantly increased dpH_i/dt, approximately threefold, when compared to VSMC incubated with control medium. These differences could not be accounted for by differences in buffering capacity. Moreover, amiloride inhibited the Na^+ dependent recovery in pH_i by 90% in the presence of control medium and eliminated the differences between the control and high-glucose medium, confirming the conclusions of the $^{22}Na^+$ uptake studies, i.e., that elevated glucose concentrations directly activate the Na^+/H^+ antiport in VSMC. Furthermore, Na^+-dependent dpH_i/dt in the presence of the osmotic control medium (mannitol) did not differ from control medium, further confirming that high glucose-induced changes in Na^+/H^+ antiport activity could not be attribute to changes in extracellular osmolality (Table II).

Role of PKC activation in mediating glucose-induced increases in Na^+/H^+ antiport activity in VSMC. PKC has been shown to play an important role in the regulation of Na^+/H^+ antiport activity in many cell types. We have previously demonstrated that elevated glucose concentrations induce a sustained activation of PKC in VSMC (20). Table III shows that a 3-h exposure to high-glucose medium markedly increases PKC activity in VSMC, and that this activity is sustained for at least 24 h providing the extracellular glucose concentrations remain elevated. This effect is specific for D-glucose and is not reproduced by either of the osmotic control media. The addition of two dissimilar PKC inhibitors, H-7 (10^{-5} M) or staurosporine (10^{-6} M) (33, 34), had little effect on basal PKC activity at these concentrations but markedly inhibited glucose-induced



	5 mM Glucose 24 h	20 mM Glucose 24 h
Baseline pHi	7.31±0.04	7.31±0.03
Trough pHi	6.34±0.02	6.32±0.02
Buffering Capacity mmol/L x pH	18.4±1.7	17.7±1.8
N	15	16

Figure 3. Effect of the test media on Na^+/H^+ antiport activity in confluent VSMC. VSMC were pretreated for 24 h with the test media shown and then loaded with BCECF. Na^+/H^+ antiport activity was measured as the 30-s recovery from NH_4Cl-induced intracellular acidosis. Buffering capacity was calculated as indicated in Methods. pH_i = intracellular pH, dpH_i/dt = change in intracellular pH with time. *$P < 0.001$; n, number of experiments.

Table I. Effect of Various Test Culture Media on Sodium Uptake by VSMC

Culture media added to cultured VSMC	Sodium uptake by VSMC	
	3-h exposure	24-h exposure
	nmol/min per mg cell protein	
Control	6.53±0.27	6.25±0.17
Mannitol control	6.53±0.28	6.08±0.30
L-Glucose control	6.45±0.34	7.15±0.13
High glucose	10.1±0.50*	10.45±0.39*

Measurement of $^{22}Na^+$ uptake by VSMC after 3 or 24 h of exposure to the various test culture media. * $P < 0.01$ vs. results with all other test media; $n = 4$ experiments.

Table II. Effect of Mannitol Osmotic Control Medium on Na^+/H^+ Antiport Activity in VSMC

Measurements	Glucose 5 mM	Mannitol control
dpH_i/dt pH/min	0.22±0.03	0.23±0.03
Baseline pH_i	7.24±0.05	7.22±0.07
Trough pH_i	6.31±0.03	6.27±0.02
Buffering capacity	17.1±2.7	18.4±2.5

Confluent VSMC were pretreated with the test media shown and loaded with BCECF. Na^+/H^+ antiport activity was measured as the 30-s recovery from NH_4Cl-induced intracellular acidosis. Buffering capaity = mM × pH. $n = 13$. There were no significant differences in any parameter measured between the 5 mM glucose and mannitol osmotic control media.

Table III. VSMC PKC Activity after Exposure to Various Test Media

Culture media added to VSMC	Protein kinase C activity	
	3-h exposure	24-h exposure
	pmol phosphorylation/min per mg cell protein	
Control	40.0±4.4	42.1±5.3
Mannitol control	38.6±4.9	41.7±6.1
L-Glucose control	41.8±5.1	43.6±4.8
High glucose	79.4±7.7*	83.6±8.3*
Control + H-7	35.8±5.7	33.7±3.9
Control + ST	38.4±6.3	36.5±6.1
High glucose + H-7	49.7±8.2‡	52.1±7.1‡
High glucose + ST	51.6±6.8‡	54.3±7.5‡

PKC activity in VSMC after 3 or 24 h of exposure to the various test media. In some experiments, inhibitors of PKC activity; H-7 (50 μM) or staurosporine (ST) (1 μM) were coincubated with the control or high-glucose media. * $P < 0.01$ vs. control, mannitol, or L-glucose media. ‡ $P < 0.05$ vs. high glucose. There was no significant difference between high glucose + PKC inhibitors vs. all test media except the high-glucose medium alone; $n = 3$.

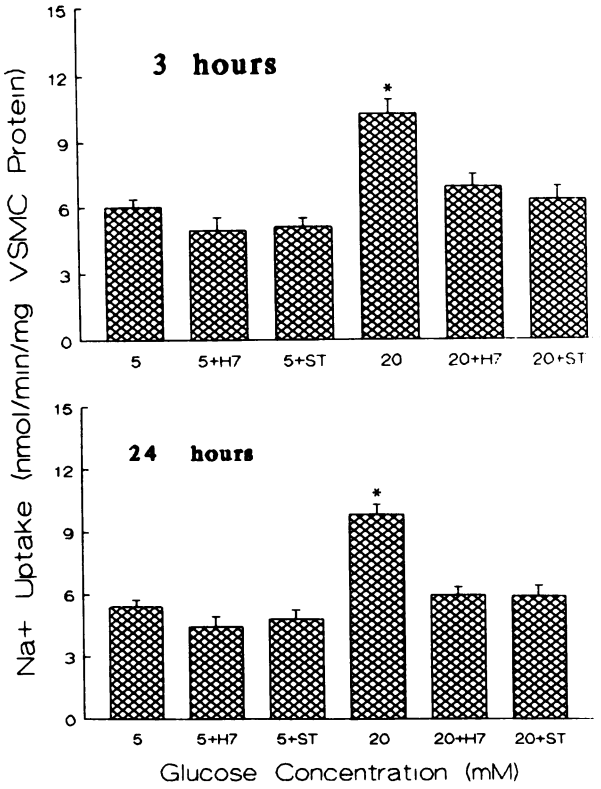


Figure 4. Effect of PKC inhibitors on glucose-induced changes in Na^+ uptake by VSMC. VSMC were exposed to control or high-glucose medium for 3 or 24 h, in the absence or presence of H-7 (50 μM) or staurosporine (ST) (1 μM). * $P < 0.05$ vs. all other columns; $n = 3$ experiments.

activation of PKC during the 3- or 24-h incubation with the high-glucose medium. The use of these two PKC inhibitors thus provided a useful experimental tool with which to assess the role of glucose-induced PKC activation in mediating glucose-induced increases in Na^+/H^+ antiport activity in VSMC. To do this, VSMC were exposed to control or high-glucose medium for 3 and 24 h in the absence or presence of either of the two PKC inhibitors, before the measurement of $^{22}Na^+$ uptake by VSMC. Fig. 4 shows that glucose-induced increases in Na^+ uptake by VSMC were almost completely prevented by inhibition of glucose-induced PKC activation with H-7 or staurosporine. Because H-7 and staurosporine are not specific in their inhibition of PKC, further experiments were performed to clarify the relevance of PKC activation in the mediation of glucose-induced changes in VSMC Na^+/H^+ antiport activity. VSMC were preexposed to PMA (10^{-7} M) for 24 h. We have previously demonstrated that this maneuver downregulates PKC activity in VSMC (20). The VSMC were then exposed to either control or high-glucose medium for 3 or 24 h in the continued presence of PMA. As shown in Table IV, basal Na^+/H^+ antiport activity was not significantly affected by PKC downregulation, however, both the short-term and sustained high-glucose–induced increase in Na^+ uptake was significantly attenuated by PKC downregulation. Together, these results suggest that both short-term and sustained glucose-induced increases in Na^+/H^+ antiport activity in VSMC occur via mechanisms dependent on glucose-induced activation of PKC.

Effects of cycloheximide and actinomycin-D on glucose-induced increases in Na^+/H^+ antiport activity. To examine whether gene transcription and protein synthesis were required for the initiation and maintenance of glucose-induced changes in Na^+/H^+ antiport activity, VSMC were incubated with actinomycin-D (10 nM), an inhibitor of gene transcription or cycloheximide (10 μM), an inhibitor of protein synthesis. Confluent VSMC monolayers were exposed to these two inhibitors throughout a 3- or 24-h incubation with control or high-glucose (20 mM) media. Cycloheximide (10 μM) inhibited protein synthesis by ~ 80% (measured by [^{3}H]leucine incorporation into cell protein). Fig. 5 shows that at 3 h the presence of actinomycin-D or cycloheximide had no effect on high-glucose–induced increases in Na^+/H^+ antiport activity in VSMC. These results suggest that short-term (3 h) glucose-induced activation of the Na^+/H^+ antiport in VSMC occurs independent of gene transcription or protein synthesis. In contrast, in-

Table IV. Effect of PKC Downregulation on Sodium Uptake by VSMC

Culture media added to VSMC	Sodium uptake by VSMC	
	3-h exposure	24-h exposure
	nmol/min per mg protein	
Control (glucose 5 mM)	6.1±0.43	5.96±0.3
Control + PMA	6.95±0.63	6.68±0.57
High glucose (20 mM)	10.69±0.61*	10.92±0.54*
High glucose + PMA	7.19±0.6	7.04±0.66

Measurement of $^{22}Na^+$ uptake by VSMC after 3 or 24 h of exposure to either control or high-glucose culture medium. To down-regulate PKC activity, in some experiments (+PMA) and VSMC were pretreated with PMA (10^{-7} M) for 24 h and during the 3- or 24-h exposure to the control or high-glucose culture medium. * $P < 0.05$ vs. control ± PMA and high glucose + PMA; $n = 4$.

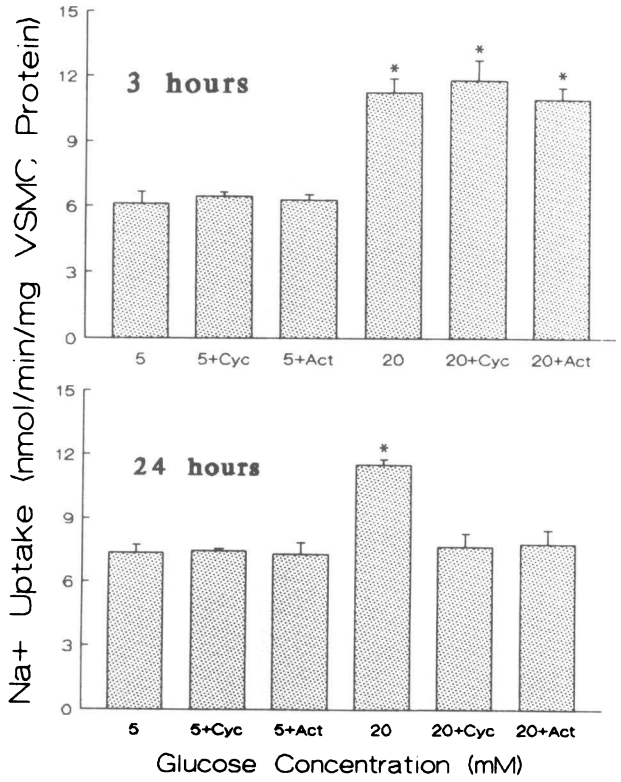


Figure 5. Effect of inhibitors of transcription and translation on glucose-induced changes in Na^+ uptake by VSMC. VSMC were exposed to control or high-glucose medium for 3 or 24 h, in the absence or presence of actinomycin-D (10 nM) or cycloheximide (10 μM) before the measurement of Na^+ uptake by VSMC. $*P < 0.05$; $n = 3$ experiments.

creased Na^+/H^+ antiport activity in response to sustained exposure to high glucose (24 h) was prevented by coincubation with actinomycin-D and cycloheximide. Neither agent inhibited glucose-induced PKC activation at 24 h and there was no measurable VSMC toxicity as indicated by no change in cell detachment rates, lactate dehydrogenase release, or trypan blue exclusion (data not shown). These results suggest that sustained glucose-induced activation of Na^+/H^+ in VSMC requires gene transcription and translation.

Effect of elevated extracellular glucose on Na^+/H^+ antiport mRNA in VSMC. The above experiments suggested that transcription and translation were involved in the increase in Na^+/H^+ antiport activity observed within 24 but not 3 h of incubation with the high-glucose medium. The effects of high-glucose concentrations on steady-state Na^+/H^+ antiport (NHE-1) mRNA levels in VSMC was thus examined. Northern blot analysis revealed a single band of 4.8–5.0 kb, as previously described for NHE-1 mRNA in cultured VSMC (35). Northern blot analysis revealed an approximately threefold increase in steady-state NHE-1 mRNA concentration normalized for β-actin in VSMC after 24 h of incubation with the high-glucose (20 mM) medium when compared with control medium (glucose 5 mM) (Fig. 6). There was no difference in steady-state levels of β-actin mRNA between the control and high-glucose group; thus the increase in the NHE-1/β-actin ratio was due to a true increase in the abundance of NHE-1 mRNA. There was no difference in steady-state NHE-1 mRNA in VSMC exposed to the control or high-glucose media at 3 h (data not shown).

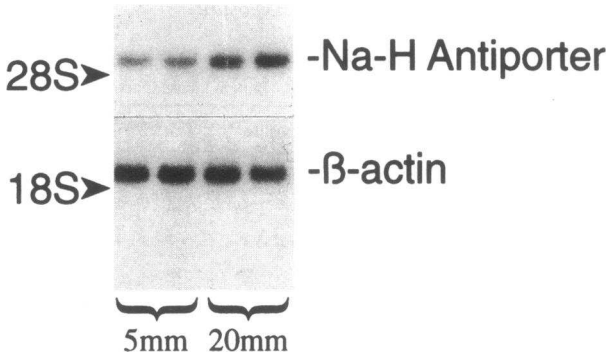


Figure 6. Effect of extracellular glucose concentration on steady-state Na^+/H^+ antiport mRNA after 24 h of incubation. VSMC were treated and Northern blot performed as indicated in Methods. This autoradiogram shows the Na^+/H^+ antiport mRNA in the upper portion of the figure at 4.8–5.0 kb. The lower portion shows the β-actin mRNA from the same membrane. The 28S and 18S markers are shown for reference.

Exposing VSMC to the mannitol osmotic control media for 3 or 24 h did not change NHE-1 mRNA levels vs. glucose 5 mM (not shown) confirming that the high-glucose-induced increase in VSMC NHE-1 mRNA was unrelated to changes in extracellular osmolality.

Discussion

The major finding of the present study is that elevated extracellular glucose concentrations exert a significant effect on the regulation of Na^+/H^+ antiport activity in vascular tissue. Elevated glucose concentrations compatible with those attained in poorly controlled diabetic patients stimulated an increase in the activity of the VSMC Na^+/H^+ antiport within hours and this effect was sustained for up to 24 h providing the glucose concentrations remained elevated. This effect was specific for D-glucose and was unrelated to changes in extracellular osmolality. In addition, both the acute and longer-term activation of the VSMC Na^+/H^+ antiport by high glucose were dependent on glucose-induced PKC activation.

The Na^+/H^+ antiport is a major pathway for the regulation of Na^+ uptake by VSMC (31). Previous studies have emphasized that the activity of Na^+/H^+ antiport in many cell types, including VSMC, is influenced by the activation of PKC (11, 18, 36–42). The present study demonstrates that elevated extracellular glucose concentrations induce a sustained activation of PKC in VSMC. We have previously characterized this effect of glucose in VSMC in detail (20). Both the short-term and sustained high-glucose–induced activation of the VSMC Na^+/H^+ antiport appear to be strongly dependent on glucose-induced PKC activation. This conclusion is based on the fact that two dissimilar inhibitors of glucose-induced PKC activity (H-7 and staurosporine) prevented short-term and sustained glucose-induced Na^+/H^+ antiport activation. The Na^+/H^+ antiport has been shown to be regulated by a number of kinases other than PKC, notably; cAMP-dependent protein kinase and calcium calmodulin-dependent multiprotein kinase (43). Because neither of the PKC inhibitors used in the present study are entirely specific for PKC, the possibility remained that other kinases that are also nonspecifically inhibited by these agents may have contributed to the glucose-induced response.

However, the important role of PKC activation in mediating glucose-induced changes in Na^+/H^+ antiport activity is further emphasized by our observation that prior downregulation of PKC by prolonged exposure to PMA prevents glucose-induced Na^+/H^+ antiport activation.

The finding that short-term (3 h) exposure to high glucose produces a PKC-dependent increase in Na^+/H^+ antiport activity in VSMC which was not blocked by coincubation with actinomycin-D or cycloheximide suggested that this acute effect of high glucose occurred independently of the need for transcription and translation. This conclusion is further supported by the observation that short-term exposure of VSMC to high glucose did not increase the abundance of NHE-1 mRNA. Previous studies of rat proximal tubule cells have demonstrated that acute activation of the Na^+/H^+ antiport after direct PKC activation with phorbol esters, also occurs without the need for transcription or translation (42).

There are numerous potential mechanisms to explain the acute, PKC-dependent, glucose-induced increase in Na^+/H^+ antiport activity in VSMC. One possibility is a phosphorylation-dependent increase in the activity of existing antiporters or the activation of "dormant" membrane-associated antiporters. In support of this concept, Sardet et al. (41) demonstrated that the Na^+/H^+ antiport is rapidly phosphorylated in response to various mitogens and concluded that this phosphorylation of the Na^+/H^+ antiport is temporally correlated with its activation. That the magnitude of phosphorylation could regulate the rate of Na^+/H^+ exchange is also suggested by the finding that vanadate, an inhibitor of phosphatases, activates Na^+/H^+ countertransport in A431 cells (44). Additional experiments support the hypothesis that PKC is one of the kinases responsible for this phosphorylation in VSMC (38–40). It is conceivable, however, that PKC-dependent activation of the Na^+/H^+ antiport could occur via phosphorylation of an ancillary, regulatory protein rather than direct phosphorylation of the antiport itself. An alternative mechanism of Na^+/H^+ antiport activation is that glucose-induced PKC activation could promote the exocytic insertion into the plasma membrane of antiporters previously stored in the cytoplasm. Such a "shuttling" process has been demonstrated for angiotensin II–induced increases in Na^+/H^+ antiport activity in renal proximal tubular cells (45) and many other transporters in various cell types (46–49). Finally, direct PKC activation by PMA has been shown to increase Na^+/H^+ exchange by causing an alkaline shift in the pH_i dependence of the antiport in rat thymic lymphocytes (50) and NHE-1–transfected cell lines (51). This could occur via a PKC-dependent alkaline shift in the pH_i responsiveness of an allosteric modifier site on the cytoplasmic surface of the cell membrane (50, 52–54). There are therefore, numerous potential mechanisms whereby glucose-induced PKC activation could promote an acute increase Na^+/H^+ antiport activity in VSMC.

Continued exposure to high extracellular glucose concentrations for up to 24 h induced a sustained activation of the Na^+/H^+ antiport in VSMC, also via a PKC-dependent process. The kinetic basis for this glucose-induced increase in Na^+/H^+ antiport activity was not specifically examined in the present study. It is important to note, however, that the assays of Na^+ uptake and Na^+-dependent pH_i recovery were performed using extracellular Na^+ concentrations (Na_o^+) of 125 and 145 mM, respectively. These Na_o^+ concentrations greatly exceed the Na^+/H^+ antiport K_m for Na_o^+ ($\sim$ 51 mM) (52); thus it is extremely unlikely that the measured increase in Na^+/H^+ antiport activity using these experimental conditions represents anything other than an increase in the V_{max} of the antiporter. Concordant with this conclusion, in almost all instances, sustained increases in Na^+/H^+ antiport activity in response to a variety of biological stimuli have been associated with an increase in antiporter V_{max}, which in some instances has been associated with increased synthesis of transporters (55–62).

Our observation that sustained high-glucose–induced stimulation of the VSMC Na^+/H^+ antiport was inhibited by coincubation with actinomycin-D or cycloheximide also suggested that transcription and translation were required, perhaps indicating the need for the synthesis of new Na^+/H^+ antiporters to sustain the high-glucose–induced effect. This hypothesis is supported by our finding that sustained exposure to high-glucose medium also caused a threefold increase in the abundance of NHE-1 mRNA in VSMC. Whether this increase in steady-state mRNA was due to glucose-induced changes in transcription rate or message stability has yet to be determined. Our observation that sustained activation of the Na^+/H^+ antiport by glucose requires the induction of transcription and translation is compatible with the studies of Berk et al. (37), who concluded that the long-term regulation of the Na^+/H^+ antiport in VSMC involves alterations in gene expression. Moreover, the response of the Na^+/H^+ antiport to a variety of chronic stimuli in different tissues appears to require gene transcription and translation to sustain an increase in Na^+/H^+ antiport activity (18, 42, 59, 60, 63). It is noteworthy, however, that there may not always be a good correlation between steady-state NHE-1 mRNA abundance and Na^+/H^+ antiport activity (35). Moreover, the results of the cycloheximide and actinomycin-D experiments are also consistent with the possibility that sustained activation of the Na^+/H^+ antiport requires the synthesis of a regulatory protein rather than direct synthesis of antiporters.

The intracellular signaling mechanism responsible for the high-glucose–induced increase in NHE-1 mRNA abundance has not been defined in the present study. Nevertheless, the fact that the sustained increase in VSMC Na^+/H^+ antiport activity is dependent on both glucose-induced PKC activation and gene transcription suggests that glucose-induced PKC activation may signal the increase in Na^+/H^+ mRNA. Concordant with this hypothesis, Horie et al. (42) recently demonstrated that long-term phorbol ester–mediated PKC activation leads to a chronic increase in both Na^+/H^+ antiport activity and mRNA expression in cultured proximal tubular cells. In this regard, PKC has been shown to regulate the expression of numerous different genes. These genes usually express "PKC responsive elements," i.e., various consensus sequences in the 5′ flanking region of the gene that mediate PKC-responsive regulation of gene expression (64–66). In various studies, PKC-induced regulation of these genes has been shown to be due to the binding of AP-1 or fos/jun dimers, to an AP-1 binding site in the regulatory region of the gene (64). With this in mind, it is notable that the 5′ flanking regulatory region of the gene coding for the Na^+/H^+ antiport examined in the present study contains three consensus sequences for AP-1 binding sites (67, 68).

Together the aforementioned observations suggest that glucose-induced PKC activation leads to the activation of the Na^+/H^+ antiport in VSMC via an increase in antiport V_{max}. The acute activation occurs independently of new protein syn-

thesis and gene transcription, whereas more sustained Na^+/H^+ antiport activation by elevated glucose concentrations does require protein synthesis and gene transcription and is associated with a threefold increase in NHE-1 mRNA abundance.

Glucose-induced increases in Na^+/H^+ antiport activity in vascular tissue could have considerable significance with regard to the role of hyperglycemia in the pathogenesis of vascular disease and hypertension in patients with diabetes mellitus. The Na^+/H^+ antiport has been shown to play a key role in the regulation of intracellular pH, cell volume, growth, differentiation, and contractility (7, 35, 69–72). Disordered VSMC growth is a pathologic feature of atherosclerosis and hypertension (73, 74) and increased Na^+/H^+ antiport activity has been implicated in the development of these vascular growth abnormalities (13, 35, 75, 76). It has also been suggested that Na^+/H^+ antiport hyperactivity could explain the relationship between abnormal Na^+ homeostasis and Ca^{2+} metabolism in essential hypertension and thereby contribute directly to enhanced vascular contractility (77). The hypothesis that Na^+/H^+ antiport hyperactivity plays an important role in the pathogenesis of hypertension and vascular disease is supported by reports that both essential hypertension and diabetes mellitus are associated with increased Na^+/H^+ antiport activity in many tissues (2–5, 8–16). It has been suggested that increased Na^+/H^+ antiport activity in these circumstances is genetically determined and that interaction between genetic predisposition, environmental, metabolic and other factors eventually determines the evolution of hypertension and vascular disease in the individual (2, 4–6, 16). For example, it is established that risk for vascular disease in diabetic patients is accentuated by the coexistence of hypertension and poor glycemic control (2). An interaction between genetic influences on Na^+/H^+ antiport activity and our finding of a direct effect of glucose on antiport activity could provide an explanation for this interaction at the cellular level, based on additive stimulation of the antiport. Furthermore, essential hypertension is also associated with abnormal glucose homeostasis (78–80). The findings of the present study provide a novel mechanism whereby metabolic factors, in addition to genetic factors, could directly influence Na^+/H^+ antiport activity in essential hypertension.

In conclusion, hyperglycemia is recognized to be an independent risk factor for the development of vascular disease. Hyperactivity of the Na^+/H^+ antiport has been implicated in the pathogenesis of hypertension and vascular injury. The present study uniquely demonstrates that elevated glucose concentrations induce a sustained activation of the Na^+/H^+ antiport and an increase in NHE-1 mRNA levels in vascular tissue via glucose-induced PKC-dependent mechanisms. These observations suggest a novel cellular mechanism that could explain the apparent synergism that exists between hyperglycemia and genetic factors in the clinical expression of vascular disease and hypertension in patients with abnormal glucose homeostasis.

Acknowledgments

Bryan Williams is supported by grants IRF 296 and PG 92/61 from the British Heart Foundation. Randy Howard is a recipient of a National Institutes of Health Physician Scientist Award DK-02116.

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