Glucose-Induced Changes in Activity and Phosphorylation of the Na^+/H^+ Exchanger, NHE-1, in Vascular Myocytes From Wistar-Kyoto and Spontaneously Hypertensive Rats

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Increased Na^+/H^+ exchanger (NHE) activity has been demonstrated in cells from patients with hypertension and diabetic nephropathy. Vascular myocytes from the spontaneously hypertensive rat (SHR) also exhibit increased NHE activity as compared with cells from the normotensive Wistar Kyoto rat (WKY). The interaction of increased glucose concentrations with NHE activity is unclear. The effect of glucose on NHE activity, NHE-1 (isoform 1) protein expression, and phosphorylation of cultured vascular myocytes from these rat strains was thus investigated. NHE activity was determined fluorometrically with 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF). A rabbit NHE-1–specific polyclonal antibody was used (1) to measure NHE-1 abundance in Western blots of cell extracts and (2) for immunoprecipitating ^{32}P-labeled NHE-1. Cells from SHR exhibited increased NHE activity and NHE-1 phosphorylation as compared with cells from WKY, with similar NHE-1 protein content per cell. Incubation in 25 mmol · L^{-1} glucose for 24 hours led to increased NHE activity only in WKY cultures, with no change in NHE-1 protein but a concomitantly reduced NHE-1 phosphorylation. Changes in NHE activity in WKY cells were reversed by inhibition of protein kinase C. Incubation of SHR cells with 25 mmol · L^{-1} glucose did not enhance the increased NHE activity or NHE-1 phosphorylation present in these cells. Thus, high glucose levels have disparate effects on NHE activity and NHE-1 phosphorylation in cells from different rat strains. The glucose-induced increase in NHE-1 turnover number in WKY cells is not mediated by an increase in its direct phosphorylation, but is dependent on protein kinase C.

THE Na^+/H^+ exchanger (NHE) is a membrane glycoprotein mediating the efflux of intracellular H^+ for extracellular Na^+. Since the initial cloning of the ubiquitous isoform 1 of NHE (NHE-1),[1] other isoforms (NHE-2 and -3) located predominantly on gut and renal epithelia have been described.[2,3] NHE-1 may have a role in the regulation of intracellular pH (pH_i) and cell volume and a possible permissive effect on cell growth,[4] and the other isoforms may mediate *trans*-epithelial Na^+ transport.

Due to the importance of ions in vascular function, abnormalities of ion transport have been extensively investigated in hypertension. Among these, elevated NHE activity has been demonstrated in different cell types (such as leukocytes, platelets, red blood cells, vascular myocytes, and skeletal muscle cells) in hypertensive patients and in spontaneously hypertensive rats (SHR).[5] In diabetes, the prevalence of vascular disease is increased and the risk of such complications is higher, especially in those with diabetic nephropathy. Patients with diabetic nephropathy may possess a predisposition to hypertension, in view of the finding of increased red blood cell Li^+/Na^+ exchange[6,7] and elevated leukocyte, lymphoblast, and fibroblast NHE activity.[8-10] The predominant NHE isoform investigated in these studies may be NHE-1 because of the transporter sensitivity to the amiloride derivative, ethyl isopropyl amiloride, and the tissue-specific, mainly epithelial, localization of the other isoforms.

The mechanism underlying the elevated NHE activity in diabetic nephropathy is uncertain. Although parallels between observed changes in NHE activity in diabetic nephropathy and hypertension can be drawn (namely in the similar changes in the NHE kinetics of elevated maximal rate and decreased Hill coefficient of internal H^+ binding[10,11]), there is no definitive evidence that the mechanisms for increased cellular NHE activity, perhaps from increased generation of endogenous diacylglycerol and activation of protein kinase C,[12,13] has been obtained in leukocytes[14,15] and cultured vascular smooth muscle cells (VSMCs).[16] In rat VSMCs, elevation of NHE activity after a 24-hour incubation in 25 mmol/L glucose was prevented by inhibition or downregulation of protein kinase C[16] and was accompanied by increased NHE-1 mRNA expression. Since NHE-1 possesses putative protein kinase C phosphorylation sites on its cytoplasmic domain[2] and phosphorylation is one mechanism that increases NHE activity,[17-19] effects of glucose on NHE activity may be mediated by altered NHE-1 phosphorylation. However, it is uncertain if the already elevated NHE activity of SHR VSMCs could be further increased by incubation with glucose. In the present investigation, we have therefore examined the effect of high glucose levels on NHE activity of cultured VSMCs from both SHR and the normotensive Wistar Kyoto rat (WKY), with concurrent measurement of NHE-1 protein levels and phosphorylation. The studies suggest that high glucose levels lead to an increased NHE activity only in WKY VSMCs, and this was accompanied by no change in NHE-1 protein. This glucose-induced increased turnover number of NHE-1 was not mediated by increased direct phosphorylation of NHE-1, but was dependent on activation of protein kinase C. However, the elevated NHE activity of SHR VSMCs was not dependent on protein kinase C.

MATERIALS AND METHODS

Materials

Bovine serum albumin (BSA), nigericin, monensin, TC199, 12-*O*-tetra-decanoyl phorbol-13-acetate (TPA), and isopropyl β-D-

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Submitted February 11, 1995; accepted April 22, 1995.
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0026-0495/96/4501-0017$03.00/0

galactopyranoside (IPTG) were obtained from Sigma Chemical (Poole, UK). TC199 contained 15 mmol · L^{-1} HEPES and 1 g · L^{-1} BSA, with pH adjusted to 7.4 with NaOH. Penicillin and streptomycin were obtained from Gibco BRL, Life Technologies (Uxbridge, Middlesex, UK). Fetal calf serum was supplied by Globepharm (Esher, Surrey, UK). Chick embryo extract, Dulbecco's modified Eagle's medium (DMEM), and ^{32}P-orthophosphate were supplied by ICN Biomedicals (Thame, Oxfordshire, UK). DMEM growth medium contained 15% fetal calf serum, 0.5% (wt/vol) chick embryo extract, 2 mmol · L^{-1} glutamine, and 10^5 IU penicillin · L^{-1}, 100 mg streptomycin · L^{-1}, and 24 mmol · L^{-1} $NaHCO_3$ (pH 7.4 with 5% CO_2 in air). Protein A Sepharose CL4B and glutathione Sepharose 4B were from Pharmacia LKB Biotechnology (Uppsala, Sweden). Hybond C extrasupported nitrocellulose, horseradish peroxidase–conjugated donkey antirabbit antibody, enhanced-chemiluminescence Western blotting reagents, and Aurodye were obtained from Amersham International (Little Chalfont, Buckinghamshire, UK). Molecular weight markers, TEMED, and ammonium persulfate were from Bio-Rad (Fullerton, CA). Protogel acrylamide solution was obtained from National Diagnostics (Atlanta, GA). X-ray film was from Genetic Research Instrumentation (Essex, UK). 2′,7′-Bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF/AM) was from Cambridge Bioscience (Cambridge, UK). All other chemicals were of Analar grade and were from BDH (Poole, UK). The specific protein kinase C inhibitor Ro 318220[20] was obtained from Dr J. Lawton, Roche Products (Welwyn Garden City, Hertfordshire, UK).

Cell Culture

Aortic VSMC cultures were established from WKY and SHR obtained from the Biomedical Services Unit, Leicester University, using methods described previously.[21] VSMC cultures were maintained in DMEM growth medium and were fed every 3 days. All experiments were performed on cultures between passages five and 12, using WKY and SHR cultures matched for passage number. Cells in tissue culture flasks or seeded onto cover slips were grown to confluence (over 4 days) before being serum-deprived for 24 hours. During this period of serum deprivation, cells were incubated with DMEM containing either 5 or 25 mmol · L^{-1} glucose and 1 g · L^{-1} BSA. The cells were then used for fluorometric measurements of pH_i or Western blotting and immunoprecipitation experiments. The effect of the protein kinase C inhibitor, Ro 318220,[20] was investigated by preincubation of cultures with the compound (10 μmol · L^{-1} final concentration) for a half-hour before studies on NHE activity or NHE-1 phosphorylation were performed. Thus, for 24-hour glucose incubations, Ro 318220 was present only for the last half-hour of the experiments. Stimulation with 100 nmol · L^{-1} TPA was performed at 37°C for 20 minutes (in the presence or absence of a 10-minute preincubation with Ro 318220). Cellular morphology was normal and viability maintained (as assessed by trypan blue exclusion >96%) during all incubation conditions.

Measurement of NHE Activity and NHE-1 Protein Content in VSMCs

NHE activity in VSMCs derived from SHR and WKY was measured fluorometrically by BCECF, as described in detail previously.[10,21,22] Briefly, cells were seeded onto 9 × 22-mm glass cover slips 4 days before study. When cells were confluent, they were serum-deprived for 24 hours in DMEM containing either 5 or 25 mmol · L^{-1} glucose and 1 g · L^{-1} BSA. Then they were loaded with 2 μmol · L^{-1} BCECF/AM for 1 half-hour in TC199 at 37°C. pH_i was measured in HEPES-buffered saline ([HBSS] composed of NaCl 140 mmol · L^{-1}, KCl 5 mmol · L^{-1}, $CaCl_2$ 1.8 mmol · L^{-1}, $MgSO_4$ 0.8 mmol · L^{-1}, glucose 5 mmol · L^{-1}, HEPES 15 mmol · L^{-1} and BSA 1 g · L^{-1}, pH 7.4), with the cover slip held within a cuvette in a thermostat-equipped sample compartment holder. A dual-grating fluorometer (Deltascan; Photon Technology International, South Brunswick, NJ), with dual-wavelength excitation (500 and 439 nm) and emission at 530 nm was used for fluorescence ratio measurements. Fluorescence ratios were converted to pH_i values by constructing a calibration curve after equalizing pH_i and buffer pH with nigericin and monensin in isotonic KCl buffers as previously described.[10,21,22] pH_i was then clamped to 6.0 (near the maximal rate of NHE[21]), the ionophores were scavenged with BSA, and the rate of change of pH_i was measured in the presence and absence of extracellular Na^+ (replacing Na^+ with *N*-methyl-D-glucamine). Using this double-ionophore technique, we have previously shown that intracellular Na^+ decreases to undetectable levels and intracellular K^+ is equalized with the external isotonic KCl buffer.[23] Thus, any changes in NHE-mediated efflux induced by high glucose levels cannot be attributed to secondary alterations in ionic composition of the cells. Intrinsic buffering capacity was measured with an NH_4Cl pulse. Fluxes mediated by NHE were then calculated as the product of buffering capacity and the Na^+-dependent rate of change of pH_i.[10,21,22]

The NHE-1–specific antibodies, G252 and G253, were raised by immunizing two rabbits with β-galactosidase-NHE-1 C-terminal fusion protein over 6 months.[22] The immunoglobulins were purified from the serum of each rabbit by elution from protein A Sepharose CL4B beads. G252 has been used for Western blotting, and G253 for the immunoprecipitation protocol. Specificities of these antibodies have been previously verified.[10,22,24]

The Western blotting method for detection of NHE-1 in VSMC cultures has been described previously.[10,22,24] Briefly, cells that had been incubated for 24 hours with 5 or 25 mmol · L^{-1} glucose were removed from flasks with EDTA 200 mg · L^{-1} in phosphate-buffered saline. An equal volume of 125 mmol · L^{-1} Tris, pH 6.8, 5% sodium dodecyl sulfate, 20% glycerol, and 0.004% bromophenol blue solution was added to cells resuspended in 50 mmol · L^{-1} Tris, pH 7.4, containing NaCl 150 mmol · L^{-1}, EDTA 5 mmol · L^{-1}, phenylmethylsulfonyl fluoride 1 mmol · L^{-1}, *o*-phenanthroline 1 mmol · L^{-1}, and iodoacetamide 1 mmol · L^{-1}, followed by boiling for 10 minutes. DNA level was measured in cell extracts and extracts from equal cell numbers loaded and resolved on 7.5% SDS-PAGE gels. After electroblotting onto supported nitrocellulose, membranes were blocked with 10% low-fat milk powder in Tris-buffered saline containing 0.1% Tween 20. The membranes were then incubated with 1 μg · mL^{-1} G252 antibody, followed by horseradish peroxidase–linked donkey antirabbit second antibody and detection with enhanced chemiluminescence reagents. Bands obtained on preflashed x-ray film were quantified using a Bio-Rad densitometer software package.

Immunoprecipitation of ^{32}P-Labeled NHE-1 From VSMCs

Serum-deprived VSMC cultures exposed to 5 and 25 mmol · L^{-1} glucose were washed three times in phosphate-free HBSS; 50 μCi · mL^{-1} carrier-free ^{32}P-orthophosphate was then added to each flask, followed by incubation for 3 hours at 37°C. Labeled cells were then quickly snap-frozen with liquid nitrogen. One milliliter of cold (4°C) extraction buffer composed of 10 g · L^{-1} polyoxyethylene-8-lauryl ether, Tris 30 mmol · L^{-1}, NaCl 130 mmol · L^{-1}, EDTA 5 mmol · L^{-1}, phenylmethylsulfonyl fluoride 1 mmol · L^{-1}, *o*-phenanthroline 1 mmol · L^{-1}, iodoacetamide 1 mmol · L^{-1}, Na fluoride 100 mmol · L^{-1}, Na orthovanadate 5 mmol · L^{-1}, ATP 10 mmol · L^{-1}, Na pyrophosphate 10 mmol · L^{-1}, pepstatin A 1 mg · L^{-1}, and leupeptin 2 mg · L^{-1}, was then added to the frozen monolayer, and the cells were scraped off the flasks. These suspensions were then sonicated before centrifugation at 14,000 ×

g. The supernatant was then preabsorbed with protein A-Sepharose CL4B beads, and the samples were recentrifuged. The NHE-1–specific polyclonal antibody, G253 (100 $\mu g \cdot mL^{-1}$), was then added to the supernatant, and the samples were rotated end on end for 2 hours at 4°C. Protein A-Sepharose CL4B beads that had been treated with unlabeled rat VSMC extracts (to reduce nonspecific binding) were then added to recover the immunoprecipitates. After extensive washes in extraction buffer containing 1 $g \cdot L^{-1}$ ovalbumin, the beads were boiled in Laemmli sample buffer for 3 minutes. Extracts were then resolved on 7.5% SDS-PAGE gels. The gels were stained and dried, and phosphoproteins were detected by autoradiography on preflashed x-ray films at −70°C. Intensities of phosphoprotein bands were measured using a Bio-Rad densitometer (Bio-Rad, Hemel Hempstead, Hertfordshire, UK). Concurrent measurement of NHE-1 abundance by Western blotting of extracts from similar flasks enabled calculation of a phosphorylation index per NHE-1 protein. The NHE-1 phosphorylation index was normalized to a value of 1 for WKY cultures in media containing 5 mmol/L glucose.

In WKY VSMC cultures exposed to 5 and 25 $mmol \cdot L^{-1}$ glucose, specific activity of ^{32}P-ATP was also measured. Cells were labeled with 250 $nCi \cdot mL^{-1}$ carrier-free ^{32}P-orthophosphate and snap-frozen with liquid nitrogen. One milliliter of cold 10-$mmol \cdot L^{-1}$ Tris, pH 7.4, containing 5 $mmol \cdot L^{-1}$ EDTA was then added to the frozen monolayer, and the cells were scraped off the plastic flask and stored in liquid nitrogen until analysis. Defrosted samples were vortexed and centrifuged at 13,000 × *g* for 10 minutes. ATP specific activity was determined by high-performance liquid chromatography using a 10SAX Partisil anion column (Whatman International, Maidstone, Kent, UK). Elution of ATP was achieved with a linear gradient of 0% to 67% 1.4-$mol \cdot L^{-1}$ ammonium dihydrogen orthophosphate. Total ATP levels were determined by absorption at 254 nm with reference to known ATP standards and ^{32}P incorporation into ATP measured by Cerenkov-counting the fractions obtained on chromatography.

Statistics

Results are expressed as the mean ± SEM, and comparisons were by ANOVA and Student's *t* test, performed on an Oxstat statistics package (Microsoft, Reading, UK). Two-tailed *P* values less than .05 were considered significant.

RESULTS

The pH_i of SHR vascular myocytes cultured in 5 $mmol \cdot L^{-1}$ glucose was higher than that of WKY myocytes ($P < .05$; Fig 1). We also confirmed our previous observations of increased NHE activity of SHR vascular myocytes at pH_i 6.0[21] (Fig 1). Culture of SHR cells in media containing 25 $mmol \cdot L^{-1}$ glucose led to no significant change in the elevated NHE activity. However, WKY myocytes showed a significantly increased NHE activity when cultured in 25 $mmol \cdot L^{-1}$ glucose ($P < .002$; Fig 1). Although pH_i values were lower in cells from both rat strains cultured in 25 $mmol \cdot L^{-1}$ glucose, these were not statistically significant.

We used the NHE-1–specific polyclonal serum G252 to probe Western blots of cell extracts from both cell strains.[10,22,24] This antibody reacted with a band of approximately 95-kd molecular weight in both SHR and WKY vascular myocyte cultures (Fig 2),[22] consistent with *N*-glycosylation of NHE-1.[17] This reactivity was abolished by coincubation of the primary antiserum G252 with NHE-1

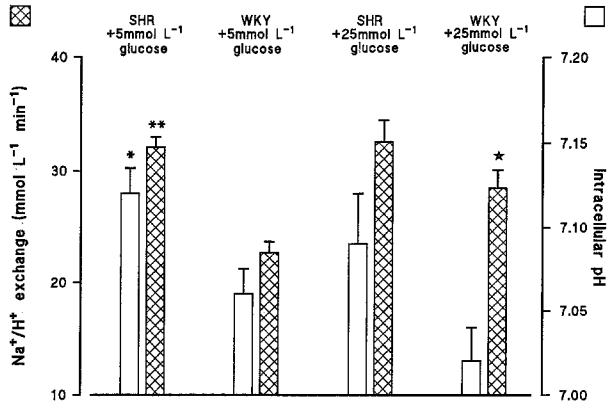

Fig 1. Effect of incubation of SHR and WKY VSMCs in DMEM containing 5 or 25 $mmol \cdot L^{-1}$ glucose for 24 hours on pH_i and NHE-mediated H^+ efflux at pH_i 6.0. Results are the mean ± SEM. *$P < .05$, **$P < .001$: *v* WKY myocytes in 5 $mmol \cdot L^{-1}$ glucose. ⋆$P < .002$ *v* WKY myocytes in 5 $mmol \cdot L^{-1}$ glucose; n = 15 experiments in each group.

C-terminal fusion protein (data not shown, but illustrated in Siczkowski et al[22]). There was no difference in cellular abundance of NHE-1 in SHR or WKY myocytes cultured in 5 $mmol \cdot L^{-1}$ glucose (Fig 2 and Table 1). Moreover, cells cultured in 25 $mmol \cdot L^{-1}$ glucose for 3 or 24 hours showed no significant change in NHE-1 protein content (Fig 2 and Table 1). Thus, it is likely that the increased NHE-mediated H^+ efflux in WKY cells induced by 25 $mmol \cdot L^{-1}$ glucose is mediated by an increased turnover number of NHE-1, rather than by an increased amount of NHE-1 protein.

Among posttranslational processes that may alter NHE activity, phosphorylation of the cytoplasmic domain of the transporter has been demonstrated to increase NHE activity.[17-19] Furthermore, the cytoplasmic domain of rat NHE-1 contains a consensus sequence for protein kinase C[2]. We therefore examined the effects of a specific protein kinase C inhibitor, Ro 318220,[20] on the glucose-induced stimulation of NHE activity in WKY vascular myocytes. Figure 3 shows that incubation of WKY vascular myocytes with Ro 318220 in 5 $mmol \cdot L^{-1}$ glucose media did not alter NHE activity significantly. However, the elevated NHE activity demonstrated in cells cultured in 25 $mmol \cdot L^{-1}$ glucose was reduced to levels comparable to cultures in 5 $mmol \cdot L^{-1}$ glucose media following incubation with Ro 318220 (Fig 3;

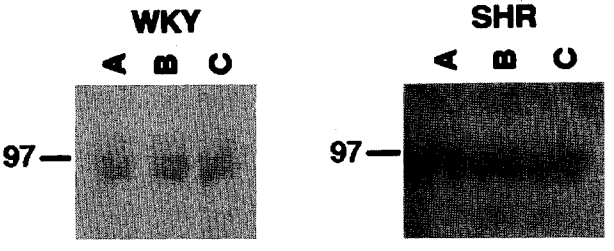

Fig 2. Western blot showing NHE-1 in extracts of VSMCs from WKY and SHR cultures maintained in 5 $mmol \cdot L^{-1}$ glucose media (A) or incubated for 3 hours (B) or 24 hours (C) in media containing 25 $mmol \cdot L^{-1}$ glucose. Equal numbers of cells (1.5×10^6) were loaded per lane. Molecular weight marker for 97 kd is shown.

Table 1. Relative Abundance of NHE-1 Protein (mean ± SEM)

Cell Type	5 mmol · L^{-1} Glucose	25 mmol · L^{-1} Glucose	
		3 h	24 h
WKY	1.00 ± 0.12	1.05 ± 0.05	0.95 ± 0.02
SHR	0.98 ± 0.03	1.00 ± 0.08	1.01 ± 0.07

NOTE. No significant differences were demonstrated between WKY and SHR cultures, and glucose had no significant effect on NHE-1 protein content in both cell types (n = 4 separate experiments in each group).

$P < .01$ *v* WKY cells incubated with 25 mmol · L^{-1} glucose alone, n = 6). This suggests that the elevated NHE activity in WKY cells exposed to high glucose concentrations is mediated by protein kinase C.

We performed further control experiments examining the possibility that protein kinase C stimulation with TPA could increase NHE activity. Figure 4 shows that NHE activity of both WKY and SHR cells measured at pH_i 6.0 was significantly stimulated following a 20-minute incubation with 100 nmol · L^{-1} TPA (in media containing 5 mmol · L^{-1} glucose). The protein kinase C inhibitor, Ro 318220, abolished this stimulation of NHE activity by TPA in both cell lines. Furthermore, Ro 318220 alone had no significant effect on NHE activity of quiescent SHR or WKY cells, indicating that this NHE-mediated H^+ efflux was independent of protein kinase C activity.

The possibility of direct changes in NHE-1 phosphorylation induced by high glucose levels was then examined using cultures exposed to 25 mmol · L^{-1} glucose media for 3 or 24 hours and labeled with ^{32}P-orthophosphate. Figure 5 illustrates that SHR vascular myocytes cultured in 5 mmol · L^{-1} glucose had higher levels of phosphorylation than WKY cells in a similar medium ($P < .001$, n = 5). However, incubation of these SHR cells in 25 mmol · L^{-1} glucose for 3 or 24 hours did not significantly alter the level of NHE-1 phosphorylation. In contrast, incubation of WKY cells in 25 mmol · L^{-1} glucose led to decreased NHE-1 phosphorylation at 24 hours ($P < .007$; Figs 5 and 6, n = 5). Addition of Ro 318820 to WKY cultures treated with 25 mmol · L^{-1}

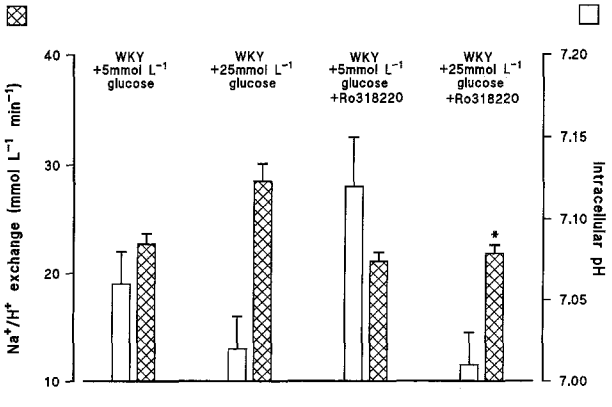

Fig 3. Effect of incubating WKY VSMCs in media containing 5 or 25 mmol · L^{-1} glucose for 24 hours in the absence or presence of the protein kinase C inhibitor, Ro 318220, at a concentration of 10 μmol · L^{-1} for 0.5 hours at 37°C. Results are the means ± SEM. *$P <$ .01 *v* WKY myocytes cultured in 25 mmol/L glucose; n = 12 experiments in each group.

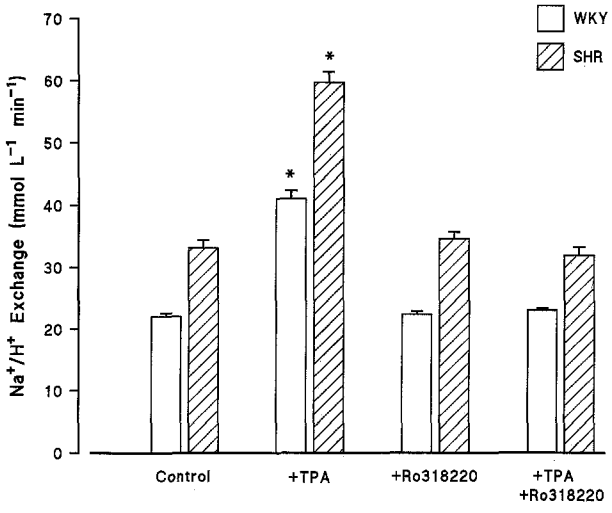

Fig 4. Effect of 100 nmol · L^{-1} TPA on NHE activity (measured at pH_i 6.0) of WKY and SHR VSMCs. TPA led to significant stimulation of NHE activity in both WKY and SHR cells (*$P <$ .001 *v* control cells). The protein kinase C inhibitor, Ro 318220 (10 μmol · L^{-1} × 0.5 h), completely abolished stimulation of NHE activity by TPA in both cell types, but the inhibitor alone had no significant effect on basal NHE activity of either cell type. Results are the means ± SEM; n = 4 separate experiments in each group.

glucose for 24 hours did not alter NHE-1 phosphorylation significantly (remaining at 0.61 ± 0.12 arbitrary units). Specific activity of ^{32}P-ATP was measured in WKY and SHR cultures incubated in 5 or 25 mmol · L^{-1} glucose for 24 hours to determine whether changes in labeling efficiency could account for an apparent decrease in NHE-1 phosphorylation. This parameter was similar in WKY and SHR cells exposed to 5 mmol · L^{-1} glucose (Fig 5). Incubation of SHR cells with 25 mmol · L^{-1} glucose for 24 hours did not alter ^{32}P-ATP specific activity, whereas specific activity was significantly increased in WKY cells ($P < .04$, n = 4 for each group). Thus, the decrease in NHE-1 phosphorylation

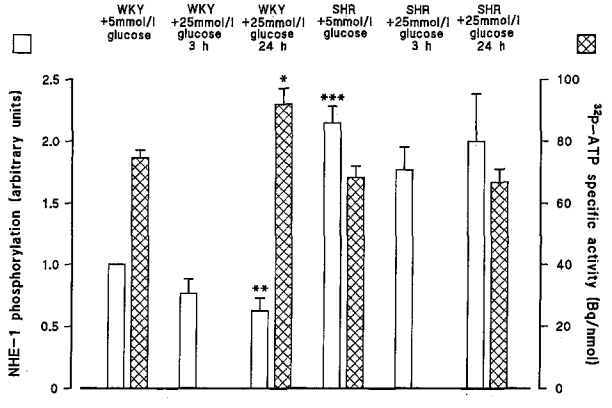

Fig 5. Bar graph showing level of NHE-1 phosphorylation in SHR and WKY cultures incubated in 5 mmol · L^{-1} glucose media or treated with 25 mmol · L^{-1} glucose for 3 and 24 hours (n = 5 experiments in each group). Specific activity of ^{32}P-ATP from the cell extracts (with cells incubated in 5 or 25 mmol · L^{-1} glucose for 24 hours) was also determined by HPLC (n = 4 experiments in each group). Results are the mean ± SEM. *$P <$ .05, **$P <$ .01, ***$P <$ .001: *v* WKY myocytes cultured in 5 mmol · L^{-1} glucose.

in WKY cells following incubation in 25 mmol · L^{-1} glucose for 24 hours was more pronounced than the raw data suggested in Figs 5 and 6.

DISCUSSION

Previous studies have demonstrated increased NHE activity in a variety of cells in hypertension[5] and diabetic nephropathy,[8-10] suggesting a predisposition to hypertension in patients with diabetic nephropathy. However, in the diabetic nephropathy studies, it was unclear whether the elevated NHE activity could have resulted from increased glucose levels or whether transporter activity was predominantly dependent on genetic determinants. Thus, in the present investigation, we used VSMCs from a genetic model of hypertension (SHR) and its normotensive counterpart (WKY) to assess the effect of high glucose levels on this membrane transport marker of hypertension.

The present study confirms previous observations that NHE activity is elevated in SHR VSMC cultures as compared with WKY cultures.[21,25] One previous study has demonstrated that incubation of rat VSMCs in 25 mmol · L^{-1} glucose media for 24 hours led to an increase in NHE activity concomitantly with an elevated protein kinase C activity, and this was accompanied by a relative increase in NHE-1 mRNA.[16] In the present study, we could only reproduce the finding of increased NHE activity in WKY cells incubated with 25 mmol · L^{-1} glucose. It is possible that the elevated NHE activity found in SHR cells prevented an additional effect mediated by the high-glucose medium. However, since the phorbol ester, TPA, stimulated NHE activity in both WKY and SHR cells that was reversed by coincubation with Ro 318220 (a specific protein kinase C inhibitor), the lack of glucose-induced stimulation of NHE activity in SHR cells was not due to a ceiling of activity above which the cells could not be elevated. The activity of NHE in quiescent SHR and WKY cells was not affected by Ro 318220, suggesting that the elevated basal NHE activity of SHR cells was not dependent on isoforms of protein kinase C susceptible to this inhibitor. However, the increased NHE activity in WKY cells following incubation in high-glucose media was reversed by treatment of the cells with Ro 318220. This confirms a role for protein kinase C in the stimulation of NHE activity in WKY cells with high-glucose media, which has been previously demonstrated in leukocytes[15] and rat VSMCs.[16] Moreover, previous studies have demonstrated increased protein kinase C activity in cells exposed to high glucose concentrations, from endogenous generation of diacylglycerol.[12,13]

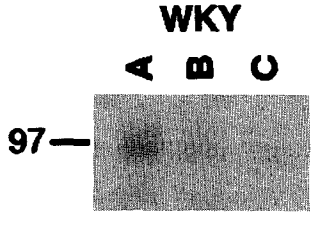

Fig 6. ^{32}P-labeled NHE-1 was immunoprecipitated from WKY cultures maintained in 5 mmol/L glucose (A) or incubated for 3 hours (B) or 24 hours (C) in media containing 25 mmol/L glucose. Molecular weight marker for 97 kd is shown.

The elevated NHE activity in WKY VSMCs with glucose incubation was not associated with an increased NHE-1 protein content. Although a previous study had demonstrated that rat VSMCs have an increased expression of NHE-1 mRNA, the level of NHE-1 protein was not measured.[16] Our present findings in WKY cells are thus not consistent with an effect of glucose on increasing mRNA transcription leading to increased NHE-1 protein levels, as suggested previously,[16] but could be interpreted as an increased turnover number of NHE-1, mediated by a direct posttranslational effect on the transport protein.

Since phosphorylation has been implicated as an important modulator of NHE activity,[17-19] we immunoprecipitated ^{32}P-labeled NHE-1 from VSMCs of both rat strains, incubated in either 5 or 25 mmol · L^{-1} glucose media. In SHR cells, no significant change in NHE-1 phosphorylation could be demonstrated in cells exposed to high glucose concentrations, resembling the findings on NHE activity. In WKY cells, high glucose levels paradoxically led to decreased NHE-1 phosphorylation. Concomitant determination of specific activity of intracellular pools of ^{32}P-ATP showed an increased specific activity in cells incubated in high glucose levels, so that the true decrease in NHE-1 phosphorylation in WKY cells was greater than the uncorrected data reported. If protein kinase C was implicated in the direct phosphorylation of NHE-1, we would have expected an increased level of phosphorylation in glucose-treated WKY cells. Further evidence against a direct role for protein kinase C in modulating NHE-1 phosphorylation is provided by the lack of an effect of Ro 318220 on the decreased NHE-1 phosphorylation in WKY cells incubated in high-glucose media. This contrasts with the findings regarding glucose-induced stimulation of NHE activity, which was protein kinase C–dependent. Thus, glucose-induced enhancement of NHE activity in WKY cells can be dissociated from changes in NHE-1 phosphorylation, which was protein kinase C–independent.

The mechanism of glucose-induced activation of NHE-1 in WKY VSMCs is therefore undefined, but is protein kinase C–dependent. Direct NHE-1 phosphorylation was excluded as a possible mechanism. Although early studies on agonists demonstrated that increased NHE activity is associated with increased NHE-1 phosphorylation,[17,18] Rao et al[26] have since demonstrated in granulocytic HL-60 cells that stimulation of NHE activity by phorbol esters is not associated with an increased but with a decreased NHE-1 phosphorylation.[26] Thus, NHE activity can be dissociated from alterations in its phosphorylation depending on the cell type studied. Furthermore, other recent studies have pointed to other potential regulatory factors, including calmodulin, which could directly interact with and activate NHE-1, independent of NHE-1 phosphorylation.[27,28] These mechanisms remain to be explored as possible routes of glucose-induced activation of NHE-1 in WKY VSMCs.

In conclusion, we have demonstrated that the glucose-induced response of VSMCs from different rat strains may

be heterogeneous with respect to stimulation of NHE activity. When incubated in high-glucose media, only WKY cells demonstrated an enhanced NHE activity that was protein kinase C–dependent. The increased NHE activity was not associated with increased NHE-1 protein content, and was therefore attributed to an increased turnover number of the transporter. This increased NHE activity was not associated with an increase in NHE-1 phosphorylation, but a significant protein kinase C–independent decrease in phosphorylation was revealed. Thus, a dissociation between activity and phosphorylation of NHE-1 was evident in WKY cells in conditions mimicking hyperglycemia. In contrast, SHR VSMCs did not exhibit any change in NHE activity, NHE-1 protein content, or NHE-1 phosphorylation with high-glucose media, although values of NHE activity and its phosphorylation were elevated as compared with WKY cells in normal-glucose media. The increased NHE activity in quiescent SHR cells was not dependent on protein kinase C. Our findings suggest that the mechanism(s) leading to elevated NHE activity and phosphorylation in cells from hypertensive subjects may have a basis different from the elevation of NHE activity resulting from exposure to a hyperglycemic milieu. The precise factors leading to increased NHE activity when cells of normotensive animals are incubated in high-glucose media remain to be determined.

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