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Hypertensive sodium-proton exchanger phenotype persists in immortalized lymphoblasts from essential hypertensive patients. A cell culture model for human hypertension.

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Research Article

An enhancement of sodium-proton exchange activity is a frequently observed ion transport abnormality in essential hypertension. The cellular basis for this has not yet been elucidated. Due to the lack of a specific cell culture system it has been impossible to distinguish between intrinsic cellular abnormalities and influences exerted by the hypertensive neurohumoral milieu. Using Epstein-Barr virus we have immortalized lymphocytes from controls and from patients with essential hypertension that exhibited enhanced sodium-proton exchanger activity. Sodium-proton exchanger activity was determined in cells loaded with the fluorescent cytosolic pH indicator 2'7'-biscarboxyethyl-5,6-carboxyfluorescein acetoxymethylester (BCECF) after pretreatment with 250 nM of the phorbol ester 12-O-tetradecanoylphorbol-13-acetate for 10 min. Cell lines from hypertensive patients displayed higher Vmax values of sodium-proton exchange than those from normotensive controls (129.6 +/- 30.0 vs. 77.1 +/- 13.2 mmol H+/min.; P < 0.001). Hill coefficients for H+ were distinctly lower in hypertension compared to normotension (1.12 +/- 0.12 vs. 1.50 +/- 0.14; P < 0.0001). The enhanced antiporter activity in cell lines from hypertensive patients was not accompanied by a corresponding increase in steady-state NHE-1 mRNA transcript levels, which argues against overexpression of antiporter protein in hypertension. The cells from hypertensive patients with high sodium-proton exchange activity proliferated distinctly faster than those from normotensive controls. These human cell lines represent a novel model to study the mutual interaction between […]

Hypertensive Sodium-Proton Exchanger Phenotype Persists in Immortalized Lymphoblasts from Essential Hypertensive Patients

A Cell Culture Model for Human Hypertension

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Abstract

An enhancement of sodium-proton exchange activity is a frequently observed ion transport abnormality in essential hypertension. The cellular basis for this has not yet been elucidated. Due to the lack of a specific cell culture system it has been impossible to distinguish between intrinsic cellular abnormalities and influences exerted by the hypertensive neurohumoral milieu. Using Epstein-Barr virus we have immortalized lymphocytes from controls and from patients with essential hypertension that exhibited enhanced sodium-proton exchanger activity. Sodium-proton exchanger activity was determined in cells loaded with the fluorescent cytosolic pH indicator 2′7′-biscarboxyethyl-5,6-carboxyfluorescein acetoxymethylester (BCECF) after pretreatment with 250 nM of the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate for 10 min. Cell lines from hypertensive patients displayed higher V_{max} values of sodium-proton exchange than those from normotensive controls (129.6±30.0 vs. 77.1±13.2 mmol H^+/min.; $P < 0.001$). Hill coefficients for H^+ were distinctly lower in hypertension compared to normotension (1.12±0.12 vs. 1.50±0.14; $P < 0.0001$). The enhanced antiporter activity in cell lines from hypertensive patients was not accompanied by a corresponding increase in steady-state NHE-1 mRNA transcript levels, which argues against overexpression of antiporter protein in hypertension. The cells from hypertensive patients with high sodium-proton exchange activity proliferated distinctly faster than those from normotensive controls. These human cell lines represent a novel model to study the mutual interaction between sodium-proton exchange and cell proliferation, and may provide insights into the alterations in ion transport observed in a group of patients with essential hypertension. (*J. Clin. Invest.* 1993. 92:2553–2559.) Key words: essential hypertension • proliferation • pH regulation • gene expression

Introduction

Despite intensive efforts the cellular mechanisms that cause the elevation of blood pressure in essential hypertension (HT)[1] have not yet been defined. Beside environmental influences, there is a known genetic contribution to the observed blood pressure variation. The investigation of intermediate phenotypes, i.e., traits whose underlying causes contribute to the pathogenesis of HT, is an important tool in the analysis of the pathogenesis of HT.

A major abnormality in a group of hypertensive patients consists of an enhanced activity of the Na^+/H^+ exchanger in platelets, lymphocytes, red blood cells, and skeletal muscle (for review see reference 1), which, at least in platelets, is apparently insensitive to pharmacological blood pressure normalization. So far it remained unknown whether the increased Na^+/H^+ exchange activity represents an abnormality on the cellular level or whether it develops secondarily, e.g., in response to circulating vasoactive factors. The Na^+/H^+ exchanger is a member of a gene family that has recently been cloned. The different isoforms are referred to as NHE-1 to NHE-4 (for review see reference 2). While the isoforms NHE-2, NHE-3, and NHE-4 are apparently involved in transepithelial ion transport (for review see reference 3), NHE-1 controls cytosolic pH (pH_i) and cell volume (2). This latter isoform appears to be expressed in all animal cells and may, in addition to the above mentioned functions, also participate in the initiation of cell growth and proliferation (2, 4). The latter property may provide a link between elevated Na^+/H^+ exchange activity and enhanced proliferation of vascular smooth muscle cells that is frequently observed in HT (1).

To test whether the abnormal kinetic behavior of the Na^+/H^+ exchanger is genetically fixed in HT, we immortalized lymphocytes from previously characterized normotensive and hypertensive individuals with "low" and "high" Na^+/H^+ exchange activity in blood platelets, respectively (5). Our results suggest that the "hypertensive" Na^+/H^+ exchanger phenotype is conserved in EBV-immortalized lymphoblasts from patients with HT. In addition, this phenotype is tightly associated with an enhanced proliferation pattern of the corresponding cell lines.

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1. *Abbreviations used in this paper:* BCECF-(AM), biscarboxyethyl-5,6-carboxyfluorescein (acetoxymethylester); EIPA, ethylisopropylamiloride; GAPDH, glyceraldehyde 3 phosphate dehydrogenase; H_i^+, cytosolic protons; $[H^+]_i$, cytosolic proton concentration; HT, essential hypertension; NT, normotension; pH_i, cytosolic pH; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

We propose that we have established the first cell culture model for a certain phenotype frequently observed in HT.

Methods

Selection of patients. Nine male normotensive subjects (age 40.3±9.1 yr) and 10 male hypertensive patients (46.1±9.9 yr) were selected from a group of patients and control subjects who had been investigated for Na^+/H^+ exchange in platelets and whose characteristics have been published elsewhere (5). The selection for the current study was based on "low" (normotensive) and "high" (hypertensive) Na^+/H^+ exchange activity in repeated measurements in platelets and lymphocytes. Furthermore, only normotensive subjects were enrolled who denied a positive family history of HT, whereas in all hypertensive patients at least one parent was afflicted. Systolic (150.0±15.1 mmHg vs. 122.1±7.6 mmHg; $P < 0.01$) and diastolic blood pressure (104.0±10.2 mmHg vs. 77.4±5.8 mmHg; $P < 0.01$) were distinctly higher in HT compared to NT. Furthermore, body mass index was also higher in HT than in NT (27.5±4.3 vs. 24.3±1.6 kg/m^2; $P < 0.05$).

Immortalization of lymphocytes and cell culture of lymphoblasts. 50 ml of blood was drawn from a peripheral vein and lymphocytes were isolated on a Ficoll-Diatrizoate density gradient (Ficol Paque; Pharmacia, Freiburg, Germany). B-lymphocytes were immortalized as described (6) by incubation with Epstein-Barr virus–containing supernatant from the B 95-8 cell line (ECACC; Porton Down, UK). They were routinely maintained in culture in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/liter streptomycin (GIBCO, Eggenstein, Germany) and 10% FCS (Vitromex, Vilshofen, Germany). Throughout the course of the experiments the same lot of FCS was used. After immortalization, B-lymphoblasts were grown for 16 wk, stock cultures were routinely frozen, and the cells were used for the experiments. All cell lines were routinely checked for mycoplasma contamination using an enzyme immunoassay (Boehringer, Mannheim, Germany) and tested negative. Passages were performed twice a week and fresh cultures were established from the frozen stocks every 3–4 mo.

Determination of Na^+/H^+ exchange activity. Cells from exponentially growing cultures were seeded at 1×10^6 cells/ml in the standard cell culture medium and grown for 2 d. Cells ($\sim 1 \times 10^8$) were pelleted by centrifugation at 100 *g* for 10 min, resuspended in 5 ml culture medium, and loaded with 5 μM of the fluorescent pH_i indicator 2′7′-biscarboxyethyl-5,6-carboxyfluorescein acetoxymethylester (BCECF-AM; Molecular Probes, Eugene, OR) for 30 min at 37°C. Thereafter, cells were pelleted and resuspended in NaCl buffer composed of (in mM): NaCl 135, KCl 5, $CaCl_2$ 1.0, $MgCl_2$ 1.0, glucose 10, Hepes 20, pH 7.4, at 37°C.

Na^+/H^+ exchange was activated by cell acidification as described (7). Briefly, BCECF-loaded lymphoblasts were suspended in KCl-buffer (composition as NaCl buffer, but NaCl isoosmotically replaced by KCl; for pH lower than 6.8 Hepes was replaced by Mes) of different pH values ranging from 5.8 to 7.2. Nigericin (0.5 μg/ml final; Sigma; Deisenhofen, Germany) was added for 3 min at 37°C to equilibrate extracellular and intracellular pH. Cells were centrifuged at 7,000 *g* for 10 s in a microcentrifuge and incubated for 1 min in a KCl buffer of the same pH containing fatty acid-free BSA (5 mg/ml final; Sigma; Deisenhofen, Germany) to remove the ionophore. Cells were centrifuged again and resuspended in 100 μl Choline chloride buffer (composed as the NaCl buffer, but NaCl isoosmotically replaced by choline chloride), pH 7.4, at 37°C. Fluorescence was measured in a Perkin-Elmer LS50 spectrofluorometer (excitation 500/440 nm, emission 530 nm). One aliquot of cells (50 μl) was added to 2 ml of choline chloride buffer and intracellular buffer capacity for H^+ was determined after addition of 5 mM NH_4Cl and calculated according to Roos and Boron (8). The other aliquot was rapidly added to NaCl buffer, thereby initiating Na^+/H^+ exchange. In the presence of 10 μM ethylisopropylamiloride (EIPA) or in the absence of Na^+ no recovery was observed, thus indicating the sole involvement of Na^+/H^+ exchange in the recovery from acidification in our conditions. Fluorescence ratios were calibrated in terms of pH_i using the high K^+/nigericin method (7). The initial part of each curve was used for calculation of pH_i recovery. These values were converted to H^+ efflux rates by multiplication with the corresponding buffer capacities (7). V_{max} values for Na^+/H^+ exchange activity were obtained experimentally at pH_i 6.0. In cases where V_{max} was not attained at that pH_i, V_{max} values were calculated from Hanes–Woolf plots of the data using only the linear part of the plots as described (9). Hill coefficients and values for $pH_{0.5}$, i.e., the pH_i for half-maximal activation, were calculated using the Hill equation (9).

Northern blot analysis of NHE-1 mRNA expression. Cells were grown for 2 d as described above. Cells were harvested, total RNA was prepared according to Chomczynski and Sacchi (10), and polyA$^+$ RNA was obtained after two passages of total RNA over an oligo-dt-column. PolyA$^+$RNA (15 μg/lane) was fractionated on a 1% agarose, 2% formaldehyde gel and blotted onto a nylon membrane (Genescreen; DuPont, Bad Homburg, Germany). The blot was prehybridized for 4 h at 42°C in 50% formamide, 10% dextranesulfate (wt/vol), 0.5 mg/ml denaturated herring sperm DNA, 0.5% SDS, 5× SSC, 5× Denhardt's solution and hybridized for 20 h in the same solution containing 5×10^6 cpm/ml of a single stranded, ^{32}P-labeled antisense DNA probe. This latter probe was generated by asymmetric PCR from a cloned human lymphocyte Na^+/H^+ exchanger fragment comprising nt −3 to nt 351 of the human NHE-1 (11). Blots were washed to a stringency of 0.1× SSC, 2% SDS at 65°C, and exposed for 24–32 h to Kodak X-Omat AR film. Likewise, double stranded probes for GAPDH were generated by PCR using primers as described (12). Hybridization and washing was identical to the procedure used for the NHE-1 except that filters were exposed for 6 h only.

Determination of cell proliferation. EBV-immortalized lymphoblasts from normotensive and hypertensive donors were seeded on day 0 at a cell concentration of 1×10^6/ml. Cell concentrations were determined daily using a CASY Cell Analyzer System (Schärfe Systems Co., Reutlingen, Germany). DNA synthesis was estimated from the incorporation of [^{3}H]thymidine (sp act 6.7 Ci/mmol). Cells were prepulsed with 1 μCi [^{3}H]methyl-thymidine (DuPont, Bad Homburg, Germany) 16 h before harvesting.

Results

Kinetic properties of the Na^+/H^+ exchanger in HT and NT. Effect of phorbol ester treatment. We first searched for an increased V_{max} of Na^+/H^+ exchange in cell lines of hypertensive origin and observed that pH_i recovery from acidification to pH_i 6.0 was distinctly faster in lymphoblasts from HT (102±15 mmol H^+/min.; $n=6$) compared to NT (70±20 mmol H^+/min.; $n=6$; $P < 0.02$). However, we also noticed a progressive decrease in pH_i and a shift of the entire pH_i activation curve to more acidic pH values, while V_{max} remained rather constant. This phenomenon initially complicated the determination of complete pH_i activation curves for each cell line. When cells were treated for 10 min. with 250 nM of the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) directly before the measurements, the time-dependent decrease in pH_i and the acidic shift in the pH_i activation curve could be efficiently prevented. This behavior is in accordance with the known delicate regulation of the antiporter's phosphorylation state (13) and may suggest that the Na^+/H^+ exchanger in lymphoblasts was slowly dephosphorylated during storage of cells in our conditions.

In untreated cells derived from a normotensive subject the activity of the antiporter depended on the cytosolic proton concentration, $[H^+]_i$, in a typical sigmoidal fashion (Fig. 1 *A*). TPA treatment of cells markedly altered the pH_i activation curve (Fig. 1 *A* and *B*). We observed an alkaline shift in steady-

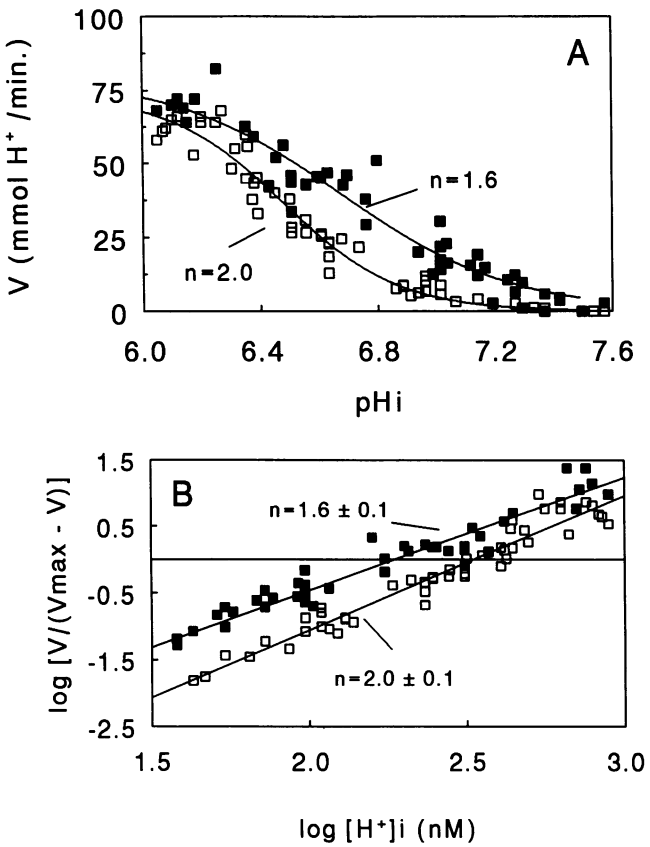

Figure 1. Effect of phorbol ester treatment on the kinetic parameters of the Na^+/H^+ exchanger in EBV-immortalized lymphoblasts from a normotensive subject. (*A*) effect of TPA (250 nM) on the pH_i dependence of the Na^+/H^+ exchanger in lymphoblasts from a normotensive donor. □, control; ■, TPA-treated. (*B*) Hill plots of the data shown in *A*. □, control; ■, TPA-treated.

state pH_i by 0.2 U and $pH_{0.5}$ increased from 6.5 to 6.7 (Fig. 1 *A* and *B*). The Hill coefficient for H^+ decreased from 2.0±0.1 to 1.6±0.1 (Fig. 1 *A* and *B*). A similar effect of phorbol ester on the Hill coefficient of the antiporter has recently been reported for A 431 cells (14). This finding may suggest that dephosphorylation dampens the antiporter since pH_i might rise above 7.4 under the influence of the Na^+ gradient. V_{max}, on the other hand, remained unchanged in TPA-treated cells. The subsequent measurements of Na^+/H exchange activity in all individual cell lines were conducted on three to four different cell preparations that were always pretreated with 250 nM TPA.

Typical pH_i activation curves of a "normotensive" and a "hypertensive" cell line are displayed in Fig. 2 *A*. The activity (v) of the antiport in the normotensive cell displayed the typical sigmoidal activation by $[H^+]_i$ (n = 1.7) and V_{max} was reached at pH_i 6.0. The antiport in lymphoblasts from the hypertensive subject did not reach V_{max} at pH_i 6.0 and the allosteric activation of transport activity by $[H^+]_i$ was almost absent (Fig. 2 *A*). Hill plots of these data (Fig. 2 *B*) revealed that the lymphoblast Na^+/H^+ exchanger of normotensive and hypertensive origin differed distinctly in Hill coefficients (1.7 vs. 1.2) and in the values of $pH_{0.5}$. The kinetic parameters of the antiport from all individual cell lines established are displayed in Fig. 3. Baseline pH_i in TPA-stimulated cells was not significantly different in normotension and hypertension (7.55±0.09 vs. 7.53±0.08; P = 0.7; Fig. 3 *A*). Values for $pH_{0.5}$ (Fig. 3 *B*) and Hill coefficients, n (Fig. 3 *C*), were markedly different in normotension and hypertension and averaged 6.72±0.15 vs. 6.34±0.23 ($P < 0.002$), and 1.50±0.14 vs. 1.12±0.12 ($P < 0.0001$), respectively. Calculated V_{max} values were significantly lower in cells derived from normotensive subjects, than in those from hypertensive patients (Fig. 3 *D*) and averaged 77.1±13.2 and 129.6±30.0 mmol H^+/min ($P < 0.001$), respectively. In agreement with an earlier report by Canessa et al. on red blood cells (9), V_{max} was attained at pH_i 6.0 in cell lines of normotensive origin, whereas the latter value was calculated to be ~ pH 5.0 in hypertensive ones. H^+ buffer capacities, on the other hand, were identical in both groups (data not shown). EIPA strongly inhibited the Na^+/H^+ exchanger in cells of hypertensive and normotensive origin and K_i values were calculated at 0.05 μM. There was no evidence for involvement of other pH_i regulating systems besides Na^+/H^+ exchange in nominally bicarbonate-free buffer.

Northern blot analysis of NHE-1 specific mRNA transcripts. To determine potential differences in the steady-state mRNA levels of NHE-1 as a possible reason for the distinctly different V_{max} values in both groups, mRNA from "hypertensive" and "normotensive" cell lines was subjected to Northern blot analysis. Hybridization was performed using a single

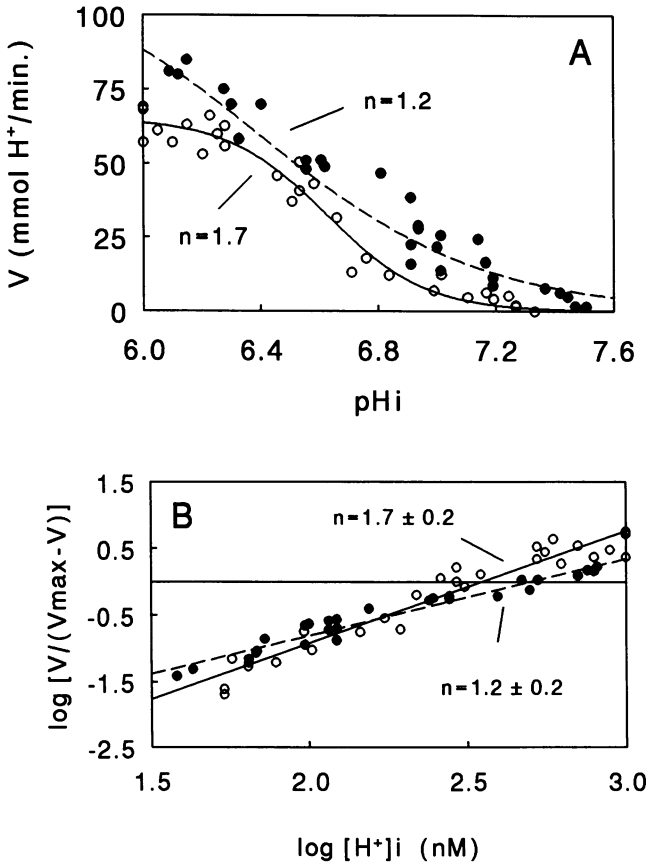

Figure 2. Kinetic parameters of the Na/H exchanger in lymphoblasts from a normotensive and a hypertensive individual. (*A*) pH_i dependence of the Na^+/H^+ exchanger in lymphoblasts from a normotensive (○) and a hypertensive donor (●) (*B*) Hill plots of the data of *A*. ○, normotension; ●, hypertension. All experiments were conducted on cells treated with 250 nM TPA

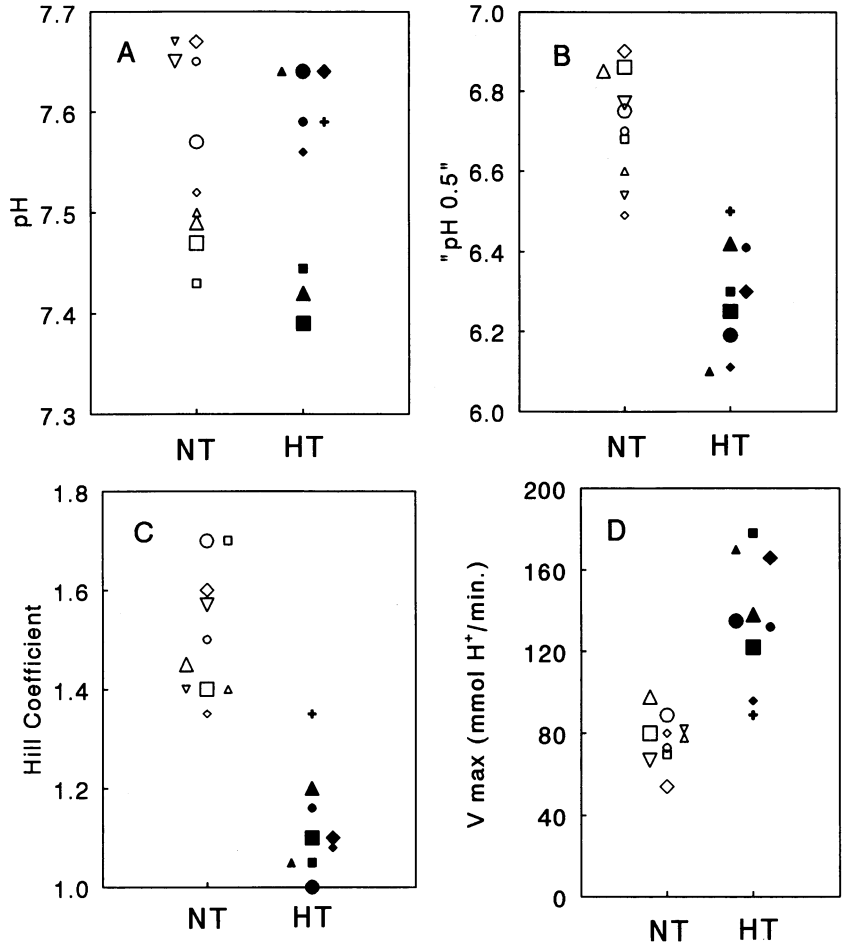

Figure 3. Kinetic parameters of the Na^+/H^+ exchanger in individual EBV-immortalized cell lines from normotensive and hypertensive individuals. (*A*) basal values of pH_i. Means are 7.55±0.09 in normotension (NT) and 7.53±0.08 in hypertension (HT); $P = 0.7$; (*B*) calculated values for $pH_{0.5}$; means are 6.72±0.15 (NT) and 6.34±0.23 (HT; $P < 0.002$); (*C*) calculated Hill coefficients; means are 1.50±0.14 (NT) and 1.12±0.12 (HT; $P < 0.0001$); (*D*) calculated V_{max} values; means are 77.1±13.2 (NT) and 129.6±30.0 (HT; $P < 0.001$). Parameters were determined as described in Methods. All determinations of Na^+/H^+ exchange activity were made in cells pretreated with 250 nM TPA for 10 min. Open symbols, normotensive (NT), closed symbols, hypertensive (HT) subjects. Each symbol represents values from one single cell line and is the mean of two separate determinations.

stranded antisense probe encoding for a fragment of the human lymphocyte NHE-1. A typical result, representative of 10 other determinations is displayed in Fig. 4. A single transcript of 5.2 kB (Fig. 4, *upper band*) was detected in all cell lines examined. To determine the relative abundance of NHE-1 mRNA, the blots were rehybridized to a probe specific for glyceraldehyde 3 phosphate dehydrogenase (GAPDH; Fig. 4, *lower band*), which is assumed to be constitutively expressed. Densitometric analysis of these signals yielded no significant differences between cell lines of hypertensive and normotensive origin. Hence, the increased V_{max} in "hypertensive" cell lines is not accompanied by an increased steady-state level of NHE-1 mRNA.

Cell growth and DNA synthesis. Since the growth factor-activatable Na^+/H^+ exchanger may participate in the initiation of cell growth and proliferation (4), we investigated whether or not "hypertensive" cell lines with altered antiporter properties would also display different proliferation characteristics. Cells derived from hypertensive patients proliferated distinctly faster than those from control subjects (Fig. 5 *A*). Mean cell concentrations were 1.57±0.48, 2.62±0.88, and 3.08±0.47 in HT compared to 1.04±0.15, 1.62±0.33, and 2.1±0.54 × 10^6 in NT (all differences $P < 0.01$) on days 1, 2, and 3 after seeding cells at an initial density of 1.0 × 10^6 cells/ml. Note that the cell concentration in cell lines immortalized from hypertensive patients tended to decrease on day 3, presumably due to an exhaustion of the cell culture medium, which was not replaced during the experiment. On the other hand, cell lines from normotensive subjects reached the same cell density on days 4–5. Hence, the cell lines of normotensive and hypertensive origin only differ in the rate of proliferation. In order to exclude that these differences in proliferation kinetics were of accidental nature only, we repeatedly determined cell growth of normotensive and hypertensive cell lines. For this purpose cell lines were repeatedly seeded at an initial density of 1 × 10^6 cells/ml and counted 2 d later. As displayed in Fig. 5 *B*, the enhanced proliferation of cell lines of hypertensive origin was highly reproducible: whereas the cell count of lymphoblasts from normotensive controls increased by a mean factor of 1.5, a mean factor of 2.4 was calculated for lymphoblasts from hypertensive subjects.

In addition, we determined DNA synthesis in cell lines of both groups. Lymphoblasts derived from patients with HT incorporated distinctly more [^{3}H]thymidine (32.7±6.3 × 10^3 cpm; $n = 5$) than cells from normotensive controls (17.8±7.7 × 10^3 cpm; $n = 5$; $P < 0.02$).

Discussion

Recent studies have revealed an increased activity of Na^+/H^+ exchange in different blood cells ex vivo and an accelerated pH_i-recovery in skeletal muscle in vivo in a group of essential hypertensive patients (1). To separate the potential effect of genetic factors on Na^+/H^+ exchange from those mediated by

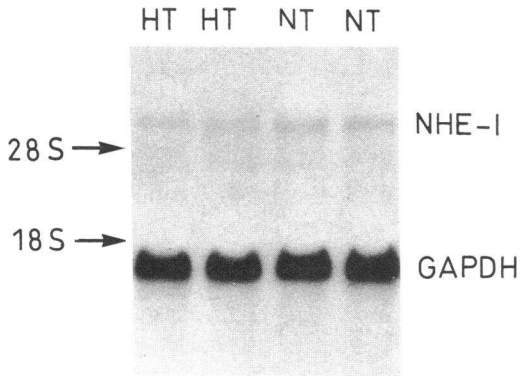

Figure 4. Northern blot analysis of Na^+/H^+ exchanger-specific and GAPDH-specific mRNA in EBV lymphoblasts from normotensives and hypertensives. Shown are representative experiments on cell lines derived from two normotensive (NT) and two hypertensive (HT) donors. The upper band was obtained after hybridization of the blot with a NHE-1-specific probe, the lower band developed after hybridization with a GAPDH-specific probe. Densitometric evaluation of these signals yielded relative values for the NHE-1/GAPDH ratio of 0.038 and 0.056 in hypertension (HT) and 0.042 and 0.046 in normotension (NT), respectively. Note that these figures are relative values only, which cannot be used for comparison of absolute amounts of NHE-1-specific and GAPDH-specific transcripts. Similar experiments were conducted on polyA$^+$RNA preparations from other established cultures. Cell lines derived from hypertensive patients never exhibited increased levels of NHE-1-specific mRNA (after correction for GAPDH abundance) in comparison to cell lines established from normotensive controls.

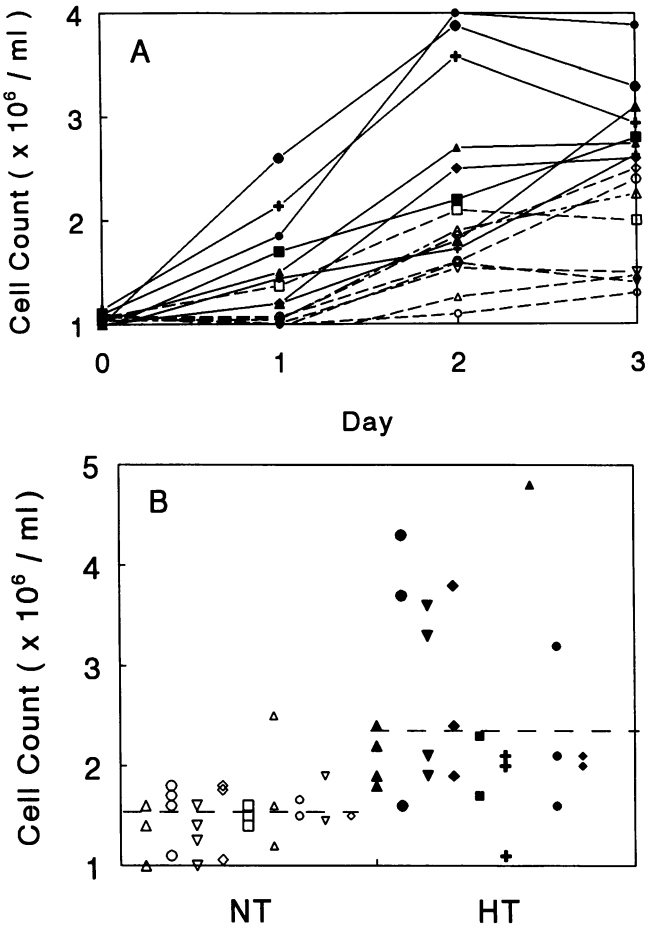

Figure 5. Growth pattern of EBV lymphoblasts from normotensive and hypertensive individuals. (*A*) Time course of the proliferation of EBV-immortalized lymphoblasts from normotensive (*open symbols; dashed lines*) and hypertensive subjects (*closed symbols; straight lines*). Each symbol represents an individual cell line. Cells were seeded at an initial density of 1×10^6 cells/ml and counted daily for 3 d. (*B*) Reproducibility of the enhanced proliferation of immortalized lymphoblasts from hypertensive donors. Cells from normotensive (NT; *open symbols*) and hypertensive (HT; *closed symbols*) donors were seeded on day 0 at an initial density of 1×10^6 cells/ml and counted on day 2. Each symbol represents an individual cell line; the number of symbols indicates the number of determinations performed. The horizontal dashed lines indicate mean cell concentrations on day 2 of all "hypertensive" and all "normotensive" cell lines.

the neurohumoral milieu in vivo, we established an EBV-transformed lymphoblast cell culture model to study Na^+/H^+ exchange in vitro after several cell cycles. This approach allows to exclude effects potentially arising from the hypertensive environment and to investigate for genetically determined abnormalities. The presumed multifactorial etiology of essential hypertension forced us to investigate highly selected hypertensive patients and normotensive controls. Only male hypertensive patients were enrolled in the study, who reported a strong family history of essential hypertension, and who exhibited elevated Na^+/H^+ activity in lymphocytes and blood platelets in repeated measurements. Conversely, male normotensive control subjects were selected with "normal" Na^+/H^+ exchange activity in repeated examinations and whose parents were not affected by hypertension. To avoid the generation of oligo- or monoclonal cell lines, a high number of lymphocytes (1.5×10^8 cells) was transformed of each individual and fresh cultures were established from frozen stocks every 3–4 mo.

One major finding of the present work is, that the kinetic abnormalities of Na^+/H^+ exchange are conserved in immortalized lymphoblasts of hypertensive origin and that this phenotype is associated with an enhanced cell proliferation. Our results on the kinetic abnormalities of Na^+/H^+ exchange in cell lines of hypertensive origin are almost identical to those of Canessa and colleagues, who found an increased V_{max} and a lowered Hill coefficient in erythrocytes of essential hypertensive patients (9). This suggests that the altered kinetic behavior of the Na^+/H^+ antiport of certain hypertensive individuals determined ex vivo is not a mere epiphenomenon arising secondary to the elevated blood pressure in vivo.

The exact molecular mechanism(s) underlying this finding remain to be defined. The increase in V_{max} in the cell lines of hypertensive origin cannot be attributed to an elevated steady-state mRNA level for NHE-1, suggesting that an overexpression of Na^+/H^+ exchanger protein is unlikely. However, this issue has to be reinvestigated, using specific antibodies against the NHE-1 to finally exclude an overexpression of NHE-1 in cell lines of hypertensive origin. On the other hand, a simple overexpression would not explain the observed differences in Hill coefficients and $pH_{0.5}$ values.

Thus, it was interesting to investigate for mutations in the NHE-1 gene. Consistent with the findings by Lifton et al., who excluded the NHE-1 as a candidate gene in essential hypertension (15), we were unable to demonstrate a sequence difference in the cDNAs encoding for the NHE-1 in lymphoblasts of

hypertensive and normotensive origin (Flake, A., and W. Siffert, unpublished results). Another possible explanation for the observed kinetic alterations in lymphoblasts of hypertensive origin is a different expression of several isoforms of Na^+/H^+ exchangers. However, pH_i recovery was highly sensitive to inhibition by EIPA. The isoforms NHE-2 (16), NHE-3, and NHE-4 have been reported to be amiloride-resistant (for review see reference 3), which makes a significant contribution of these antiporters to lymphoblast pH_i regulation at least unlikely. Furthermore, Rao et al. have recently demonstrated that NHE-3 and NHE-4 mRNA are barely detectable in HL-60 cells (17), which confirms the notion that these isoforms are predominantly expressed in epithelia. Whether or not these isoforms are expressed in lymphoblasts and, eventually, selectively in those of hypertensive origin remains to be studied. Further studies with specific antibodies will also be necessary to delineate possible differences concerning antiporter-oligomerization, its posttranslational modification, its glycosylation, and its interaction with cytoskeletal elements. The NHE-1 is a phosphoglycoprotein that exhibits a subtle regulation by different protein kinases, such as protein kinase C, tyrosinkinase(s), or Ca^{2+}/calmodulin-dependent kinases, and by phosphatases, such as phosphatase P1 and 2A (for review see reference 2). The antiporter is phosphorylated at different sites and it was proposed that the activation via different signaling pathways is integrated by a NHE-1 specific kinase or a mitogen-activated protein kinase (MAP-kinase) before transmittal to the antiporter protein (2, 18). Although we tried to fully activate the Na^+/H exchanger by treating cells with a high concentration of phorbol ester, this experimental maneuver does not ultimately exclude that differences in phosphorylation account for the observed kinetic differences in NHE-1 activity. Activation of the NHE-1 by phosphorylation constitutes a relatively distal step resulting from multiple cellular signaling pathways. Therefore, it is also very attractive to suggest, that the altered kinetic behavior of the Na^+/H^+ exchanger in "hypertensive" cells results from abnormalities in the more proximal signaling network. On the other hand, the antiport can be activated without a change in its phosphorylation state. Hyperosmolar stress increases Na^+/H^+ exchange activity without an increase in antiporter phosphorylation (19). An antibody directed against the cytoplasmic domain of the protein has been reported to block endothelin- and thrombin-induced activation of the Na^+/H^+ exchanger while leaving its activation by phorbol esters and osmotic shrinkage intact (20). Thus, our present knowledge only permits the statement that the altered Na^+/H^+ exchange activity in essential hypertension represents a conserved marker or phenotype of one or perhaps several other cellular abnormalities.

The NHE-1 is expressed in all mammalian cell types investigated so far, including vascular smooth muscle cells (21). This may also explain why "hypertensive" antiporter characteristics have been observed in different blood cells ex vivo as well as in skeletal muscle in vivo (1).

The second important observation of our report is the distinctly increased proliferation of cell lines of hypertensive origin, and similar findings have so far only been reported for animal models of genetic hypertension (22, 23). The association of altered Na^+/H^+ exchange in "hypertensive" cell lines with enhanced proliferation is of special interest since current hypothesis suggest that the enhanced peripheral resistance in essential hypertension partially develops on the basis of an increased proliferation of vascular smooth muscle cells in resistance vessels (24, 25). It was also suggested that the cardiac hypertrophy in essential hypertension precedes the increase in blood pressure. Normotensive children of hypertensive parents have an enlarged mass of the left heart ventricle (26). Finally, renal hypertrophy was reported to coincide with essential hypertension (27). Thus, an abnormal growth pattern of cells in the cardiovascular system appears a potential pathogenetic factor in the development of hypertension. Whether or not activation of the Na^+/H^+ exchanger plays an important role in cell proliferation remains controversial (4). It appears likely, however, that the enhanced V_{max} and the reduced Hill coefficient in "hypertensive" cells enables them to rapidly and efficiently counteract any cytosolic acidification due to metabolically produced protons and may, thus, constitute a growth-promoting advantage.

In summary we believe that the cell culture model described here for a certain phenotype of essential hypertension provides a valuable tool to further investigate the mutual relationship between Na^+/H^+ exchange and proliferation and to get more insight into the cellular peculiarities that may ultimately cause hypertension.

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References

1. Rosskopf, D., R. Düsing, and W. Siffert. 1993. Membrane sodium-proton exchange and primary hypertension. *Hypertension (Dallas)*. 21:607–617.

2. Wakabayashi, S., C. Sardet, P. Fafournoux, L. Counillon, S. Meloche, G. Pagés, and J. Pouysségur. 1992. Structure function of the growth factor activatable Na^+/H^+ exchanger (NHE1). *Rev. Physiol. Biochem. Pharmacol.* 119:157–186.

3. Counillon, L., and J. Pouysségur. 1993. Molecular biology and hormonal regulation of vertebrate Na^+/H^+ exchanger isoforms. *In* Molecular biology and function of carrier proteins. L. Reuss, J. M. Russell, and M. L. Jennings, editors. The Rockefeller University Press, New York. 169–185.

4. Grinstein, S., D. Rotin, and M. Mason. 1989. Na^+/H^+ exchange and growth factor-induced cytosolic pH changes. Role in cellular proliferation. *Biochim. Biophys. Acta.* 988:73–97.

5. Rosskopf, D., G. Siffert, U. Osswald, K. Witte, R. Düsing, J. W. N. Akkerman, and W. Siffert. 1992. Platelet Na^+/H^+ exchange activity in normotensive and hypertensive subjects: effect of enalapril therapy upon antiport activity. *J. Hypertens.* 10:839–847.

6. Walls, E. V., and D. H. Crawford. 1987. Generation of human B lymphoblastoid cell lines using Epstein-Barr virus. *In* Lymphocytes—A Practical Approach. G. G. B. Klaus, editor. IRL Press, Oxford. 149–162.

7. Strazzullo, P., and M. Canessa. 1990. Kinetics of the human lymphocyte Na^+/H^+ exchanger. *Clin. Sci. (Lond.)*. 79:531–536.

8. Roos, A., and W. F. Boron. 1981. Intracellular pH. *Physiol. Rev.* 61:296–434.

9. Canessa, M., K. Morgan, R. Goldszer, T. J. Moore, and A. Spalvins. 1991. Kinetic abnormalities of the red blood cell sodium-proton exchange in hypertensive patients. *Hypertension (Dallas)*. 17:340–348.

10. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156–159.

11. Quednau, B., D. Rosskopf, H. Weiler-Güttler, and W. Siffert. 1991. Detection of Na^+/H^+ exchanger mRNA in human neutrophils and lymphocytes using the polymerase chain reaction. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 289:221–223.

12. Yun Tso, J., X.-H. Sun, T. Kao, K. S. Reece, and R. Wu. 1985. Isolation

and characterization of rat and human glyceraldehyde 3-phosphate dehydrogenase cDNAs: genomic complexity and molecular evolution of the gene. *Nucleic Acids Res.* 13:2485–2502.

13. Bianchini, L., M. Woodside, C. Sardet, J. Pouyssëgur, A. Takai, and S. Grinstein. 1991. Okadaic acid, a phosphatase inhibitor, induces activation and phosphorylation of the Na^+/H^+ antiport. *J. Biol. Chem.* 266:15406–15413.

14. Takaichi, K., D. F. Balkovetz, E. Van Meir, and D. G. Warnock. 1993. Cytosolic pH sensitivity of an expressed human NHE-1 Na^+-H^+ exchanger. *Am. J. Physiol.* 264:C944–C950.

15. Lifton, R. P., S. C. Hunt, R. R. Williams, J. Pouysségur, and J.-M. Lalouel. 1991. Exclusion of the Na^+/H^+ antiporter as a candidate gene in human essential hypertension. *Hypertension (Dallas).* 17:8–14.

16. Tse, C.-M., S. A. Levine, C. H. C. Yun, M. H. Montrose, P. J. Little, J. Pouysségur, and M. Donowitz. 1993. Cloning and expression of a rabbit cDNA encoding a serum-activated ethylisopropylamiloride-resistant epithelial Na^+/H^+ exchanger isoform (NHE-2). *J. Biol. Chem.* 268:11917–11924.

17. Rao, G. N., C. Sardet, J. Pouysségur, and B. C. Berk. 1993. Phosphorylation of Na^+-H^+ antiporter is not stimulated by phorbol ester and acidification in granulocytic HL-60 cells. *Am. J. Physiol.* 264:C1278–C1284.

18. Sardet, C., P. Fafournoux, and J. Pouysségur. 1991. α-Thrombin, epidermal growth factor, and okadaic acid activate the Na^+/H^+ exchanger, NHE-1, by phosphorylating a set of common sites. *J. Biol. Chem.* 266:19166–19171.

19. Grinstein, S., M. Woodside, C. Sardet, J. Pouysségur, and D. Rotin. 1992. Activation of the Na^+/H^+ antiporter during cell volume regulation. Evidence for a phosphorylation-independent mechanism. *J. Biol. Chem.* 267:23823–23828.

20. Winkel, G. K., C. Sardet, J. Pouysségur, and H. E. Ives. 1993. Role of cytoplasmic domain of the Na^+/H^+ exchanger in hormonal activation. *J. Biol. Chem.* 268:3396–3400.

21. Rao, G. H. R., C. Sardet, J. Pouysségur, and B. C. Berk. 1990. Differential regulation of Na^+/H^+ antiporter gene expression in vascular smooth muscle cells by hypertrophic and hyperplastic stimuli. *J. Biol. Chem.* 265:19393–19396.

22. Berk, B. C., G. A. Vallega, A. J. Muslin, H. M. Gordon, M. Canessa, and R. W. Alexander. 1989. Spontaneously hypertensive rat vascular smooth muscle cells in culture exhibit increased growth and Na^+/H^+ exchange. *J. Clin. Invest.* 83:822–829.

23. Socorro, L., G. Vallega, A. Nunn, T. J. Moore, and M. Canessa. 1990. Vascular smooth muscle cells from the milan hypertensive rat exhibit decreased functional angiotensin II receptors. *Hypertension (Dallas).* 15:591–599.

24. Aalkjaer, C., H. Eiskjaer, M. J. Mulvany, B. Jespersen, T. Kjaer, S. S. Sorensen, and E. B. Pedersen. 1989. Abnormal structure and function of isolated subcutaneous resistance vessels from essential hypertensive patients despite antihypertensive treatment. *J. Hypertens.* 7:305–310.

25. Mulvany, M. J. 1991. Structure and function of resistance vessels in hypertension. *In* Cellular Aspects of Hypertension. G. Bruschi and A. Borghetti, editors. Springer Verlag, Berlin, Heidelberg, New York. 3–11.

26. Hansen, H. S., J. R. Nielsen, N. Hyldebrandt, and K. Froberg. 1992. Blood pressure and cardiac structure in children with a parental history of hypertension: The Odense Schoolchild Study. *J. Hypertens.* 10:677–682.

27. Nosadini, R., A. Semplicini, P. Fioretto, L. Lusiani, R. Trevisan, V. Donadon, G. Zanette, G. L. Nicolosi, V. Dall'Aglio, D. Zanuttini, and G. Viberti. 1991. Sodium–lithium countertransport and cardiorenal abnormalities in essential hypertension. *Hypertension (Dallas).* 18:191–198.