Enhanced Mitogen-Activated Protein Kinase Activity and Phosphorylation of the Na^+/H^+ Exchanger Isoform-1 of Human Lymphoblasts in Hypertension

Frank P. Sweeney, Paulene A. Quinn, and Leong L. Ng

Increased activity of the Na^+/H^+ exchanger isoform-1 (NHE-1) is recognized as an intermediate phenotype for hypertension, but the basis for this association is unclear. We have previously demonstrated an increased phosphorylation of NHE-1 in lymphoblasts from hypertensives that was associated with increased cell proliferation. Due to the central importance of mitogen-activated protein kinases (MAPKs) in signaling cascades transducing responses from extracellular growth factors and hormones, we examined the activity of this kinase in a specific peptide phosphorylation assay. Cells from hypertensives showed a significant twofold enhancement of MAPK activity ($P < .001$). This was not associated with any increase in $p42^{mapk}$ and $p44^{mapk}$ protein content. There was no significant increase in the level of tyrosine phosphorylation of MAPK in cells from hypertensives. MAPK activity was correlated with NHE-1 activity ($r_s = .55$, $P < .01$) and phosphorylation ($r_s = .51$, $P < .02$). These findings suggest that the increased cell proliferation rate, NHE-1 activity, and phosphorylation of lymphoblasts from hypertensives may be associated with enhanced MAPK activity, suggesting upregulation of this signaling pathway in hypertension.

THE Na^+/H^+ exchanger isoform-1 (NHE-1) is a membrane-associated glycoprotein that has important roles in the regulation of intracellular pH (pH_i) and volume[1,2] in addition to a permissive role in cell proliferation. Our group and others have previously demonstrated that the activity[3,4] and phosphorylation of NHE-1 are increased in lymphoblasts from patients with hypertension, with the phenotype being preserved despite culture of cells in vitro over several passages. This was not associated with any increased expression of NHE-1 protein.[4] Furthermore, the lymphoblasts exhibited an increased proliferation rate.[3,4] However, the mechanism underlying this hypertensive intermediate phenotype is unknown, but there are several mechanisms that could control NHE-1 activity and phosphorylation upstream of this exchanger.

The mitogen-activated protein kinases (MAPKs) play a central role in the transduction of cell proliferation responses from growth factor and hormone receptors[5-7] to a variety of cellular targets including transcription factors with expression of immediate early genes (such as c-*myc*, c-*fos*, and c-*jun*) and S6 kinases ($p90^{rsk}$ and $p70^{S6kinase}$), with increases in cell proliferation and protein synthesis.[8] Activation of MAPK by the upstream dual-specificity MAPK kinase (MEK) occurs by phosphorylation of tyrosine and threonine residues on subdomain VIII of the catalytic domain, and both phosphorylations are necessary for activity of the enzyme.[6-8] Furthermore, Pages et al[9] have suggested that the regulation of NHE-1 activity may be mediated by the MAPK cascade. In the present study, we have therefore measured the activity of MAPK in cells from hypertensives, which have been demonstrated to possess the intermediate phenotype of increased cell proliferation and NHE-1 activity and phosphorylation. The density of $p42^{mapk}$ and $p44^{mapk}$ was also determined by immunoblotting using specific antibodies. Phospho-specific MAPK antibody was used to probe for the degree of Tyr204 phosphorylation in subdomain VIII of the catalytic domain. Our findings suggest that the increased activity of MAPK in extracts from lymphoblasts of hypertensives coexists with an increased NHE-1 phosphorylation and NHE-1 activity and was not due to increased MAPK protein content. The similar levels of tyrosine phosphorylation of MAPK suggest that enhanced MAPK activation in hypertensive cell extracts could be dependent on the extent of threonine phosphorylation, since both phosphorylations are necessary for MAPK activation.

MATERIALS AND METHODS

Reagents

2,7′Bis-(carboxyethyl)-5,6,-carboxyfluorescein acetoxymethyl ester (BCECF-AM), bovine serum albumin, glutamine, nigericin, monensin, E-64 (*trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane), polyoxyethylene-8-lauryl ether, leupeptin, and aprotinin were purchased from Sigma Chemicals (Poole, Dorset, UK). Media and reagents for cell culture were from Gibco BRL, Life Technologies (Uxbridge, Middlesex, UK). The RPMI growth medium contained 10% fetal calf serum, 2 mmol · L^{-1} L-glutamine, and penicillin/streptomycin (10^5 U of each per liter). Protein A–Sepharose CL4B was from Pharmacia LKB Biotechnology (Uppsala, Sweden). Hybond C–extra nitrocellulose, enhanced chemiluminescence (ECL) Western blotting reagents and [γ ^{32}P]adenosine triphosphate (ATP) were supplied by Amersham International (Little Chalfont, Buckinghamshire, UK). ^{32}P-orthophosphate was obtained from ICN Biomedicals (Thame, Oxfordshire, UK). MAPK-specific polyclonal antibody and the phospho-specific MAPK antibody were from New England Biolabs (Hertfordshire, UK). The phospho-specific MAPK antibody was raised against a synthetic phosphotyrosine peptide coupled to keyhole limpet hemocyanin, corresponding to residues 196 to 209 of human $p44^{mapk}$ (DHTGFLTEY(p)VATRWC), which reacts with $p44^{mapk}$ and $p42^{mapk}$ only when phosphorylated at Tyr204. P81 phosphocellulose paper was obtained from Whatman (Maidstone, UK). The MAPK-specific nonapeptide substrate APRTPGGRR, described by Clark-Lewis et al,[10] was synthesized and the sequence validated by the local peptide synthesis facility at the University of Leicester. HOE694 (3-methylsulfonyl-4-piperidinobenzoyl guanidine), a recently developed specific inhibitor of NHE-1,[11] was obtained from Dr W. Scholz (Hoechst, Frankfurt, Germany).

From the Department of Medicine and Therapeutics, Leicester Royal Infirmary, Leicester, UK.

Submitted July 23, 1996; accepted October 9, 1996.

Supported by the British Heart Foundation and the Wellcome Trust.

Address reprint requests to Leong L. Ng, MD, Department of Medicine and Therapeutics, Leicester Royal Infirmary, Leicester LE2 7LX, UK.

0026-0495/97/4603-0012$03.00/0

Cell Culture

Human lymphoblasts immortalized by Epstein-Barr virus were obtained from Dr A.S. Krolewski (Joslin Diabetes Center, Boston, MA). These cells were cultured in RPMI growth medium and kept at a density between 0.3 and 1 × 10^6/mL. The cells were fed with fresh growth medium 24 hours before harvesting for study. Cell proliferation rates were calculated by counting cells on a Coulter counter (Coulter Electronics, Luton, UK) over a period of 2 days as previously described.[4,12]

Measurement of NHE Activity

Lymphoblasts were incubated with the fluorophore BCECF-AM (5 μmol · L^{-1} in TC199 tissue culture medium) at 37°C for 30 minutes.[4,12] After deesterification of the dye, measurements of pH_i, intrinsic buffering capacity, and H^+ efflux were performed at 37°C as described in detail previously.[4,12] This Na^+-dependent H^+ efflux was fully inhibited by 10 μmol · L^{-1} HOE694,[11] indicating that the H^+ efflux was mediated by NHE-1 and not other NHE isoforms.

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

The lymphoblasts were washed and then resuspended in phosphate-free HEPES-buffered saline (HBSS) composed of (in mmol · L^{-1}) NaCl 130, KCl 5, $CaCl_2$ 1.8, $MgSO_4$ 1, glucose 5, HEPES 20, and glutamine 2, and bovine serum albumin 1 g · L^{-1}, pH 7.4. The cells were then incubated with 50 μCi · mL^{-1} carrier-free ^{32}P-orthophosphate for 3 hours at 37°C. Labeled cells containing equal amounts of NHE-1 protein (as determined by immunoblots[4,12]) were washed briefly with cold HBSS and snap-frozen with liquid nitrogen. Proteins were extracted with 1 mL cold (4°C) extraction buffer composed of 10 g · L^{-1} polyoxyethylene-8-lauryl ether, (in mmol · L^{-1}) Tris 30, NaCl 130, EDTA 5, phenylmethylsulfonyl fluoride 1, *o*-phenanthroline 1, iodoacetamide 1, sodium fluoride 100, sodium orthovanadate 5, ATP 10, sodium pyrophosphate 10, (in mg · L^{-1}) pepstatin A 1, and leupeptin 2, as previously described.[4,12] The suspension was sonicated, and cell debris was removed by centrifugation at 14,000 × *g*. After preabsorbing the supernatant with protein A–Sepharose CL4B beads, the NHE-1–specific polyclonal antibody G253[4,12] was added to the supernatant at a final concentration of 25 μg · mL^{-1} and the samples rotated for 16 hours at 4°C. The phosphorylated NHE-1 bound to G253 was recovered with protein A–Sepharose CL4B beads that had been pretreated with unlabeled lymphoblast extracts to reduce nonspecific binding. Phosphoproteins were resolved on 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels and detected by autoradiography on preflashed x-ray films at −70°C. Densities of the ^{32}P-labeled NHE-1 were measured using a BioRad densitometer.

Resource Q Chromatography of Cell Extracts and MAPK Nonapeptide Phosphorylation Assay

The nonapeptide phosphorylation assay was adapted from that used by Clark-Lewis et al.[10] Briefly, approximately 10^7 lymphoblasts were washed in HBSS three times and resuspended in homogenizing buffer composed of (in mmol · L^{-1}) Tris 20, pH 7.4, EDTA 5, EGTA 12.5, sodium fluoride 50, sodium orthovanadate 2.5, β-glycerophosphate 62.5, phenylmethylsulfonyl fluoride 1, (in μmol · L^{-1}) pepstatin A 1, E-64 20, (in μg · mL^{-1}) aprotinin 2, leupeptin 10, and 0.1% Triton X-100. Cells were snap-frozen in liquid nitrogen. After thawing, extracts were sonicated in a bath for 10 minutes and then centrifuged at 14,000 × *g* for 10 minutes at 4°C.

The resulting supernatant was loaded onto a Pharmacia Resource Q anion-exchange chromatography column that had been previously equilibrated with (in mmol · L^{-1}) Tris 20, β-glycerophosphate 10, EDTA 1, and sodium orthovanadate 0.1, pH 7.4. Elution was made with a linear gradient from 0 to 0.5 mol · L^{-1} NaCl in the same buffer using a flow rate of 1 mL/min. Fractions were collected and assayed for MAPK activity using the nonapeptide phosphorylation assay (described later). Aliquots of the fractions were also immunoblotted for MAPK protein content. MAPK activity in cell extracts could be resolved into two main peaks on Resource Q chromatography, with the first peak corresponding to $p42^{mapk}$ eluting at a concentration of 140 to 150 mmol · L^{-1} NaCl and the second corresponding to $p44^{mapk}$ eluting at a concentration of 190 to 200 mmol · L^{-1} NaCl.[13] Having confirmed a previous study showing that the nonapeptide phosphorylation assay was specific for MAPK activity,[10] we then proceeded to analyze the total MAPK activity of cell extracts from lymphoblasts of normotensive and hypertensive subjects.

Supernatants were used immediately for the assay after determination of protein using a BioRad detergent-compatible assay kit (BioRad, Hertfordshire, UK). An aliquot of the extract containing 5 μg protein was added to assay buffer containing (final concentrations) 20 mmol · L^{-1} $MgCl_2$, 1 mmol · L^{-1} substrate peptide APRTPGGRR, and 50 μmol · L^{-1} ATP with 3 μCi [γ ^{32}P]ATP in a total volume of 25 μL. Incubations were performed for 20 minutes at 30°C. Reactions were terminated by addition of an equal volume of 20% trichloroacetic acid. An aliquot of the supernatant was then spotted onto pieces of P81 phosphocellulose paper and washed extensively in 75 mmol · L^{-1} phosphoric acid, and ^{32}P incorporation was determined by Cerenkov counting. Control incubations were performed with all extracts in the absence of the peptide substrate. All measurements were made in triplicate and expressed as nanomoles ATP incorporated per gram protein per minute. Cell extracts were assayed on three separate occasions, and the mean values are presented.

Determination of MAPK Protein

Extracts containing 80 μg protein were resolved on 10% gels and electroblotted onto Hybond C–extra nitrocellulose. Membranes were blocked in 10% dried milk in TBS Tween (Tris 20 mmol · L^{-1}, NaCl 137 mmol · L^{-1}, and Tween 0.1%, pH 7.4) and then incubated 16 hours at 4°C with anti-MAPK polyclonal antibody (1:1,000 dilution) in 5% dried milk in TBS Tween. After extensive washes, the second antibody (donkey anti-rabbit immunoglobulin G coupled to horseradish peroxidase) was added at a 1:1,500 dilution. Membranes were washed and developed using the ECL reagents as previously described.[4,12] Cell extracts were studied on three separate occasions. Proteins were quantified by scanning densitometry, and the mean values are reported.

Determination of Tyrosine-Phosphorylated MAPK Protein

Cell extracts electroblotted onto nitrocellulose membranes were blocked in 10% dried milk in TBS Tween and then incubated with the phospho-specific MAPK antibody (1:1,000 dilution). This antibody detected only Tyr204-phosphorylated MAPK (both $p42^{mapk}$ and $p44^{mapk}$ isoforms), being unreactive with even 1 μg non–tyrosine-phosphorylated MAPK. Detection was made with ECL as described earlier. Densitometric data from cell extracts on two separate occasions are presented as the mean values.

Statistics

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

RESULTS

NHE-1–Mediated H^+ Efflux and NHE-1 Phosphorylation in Lymphoblasts From Hypertensives

Table 1 lists characteristics of the normotensive and hypertensive subjects studied. None of the subjects had renal impairment

Table 1. Clinical Characteristics of the Subjects

Characteristic	Normotensives	Hypertensives	*P*
No. of subjects (male)	10 (4)	10 (5)	
Age (yr)	45 ± 5	50 ± 4	NS
Systolic blood pressure (mm Hg)	109 ± 4	148 ± 4	<.001
Diastolic blood pressure (mm Hg)	76 ± 3	98 ± 1	<.001
Family history of hypertension (n)	0	8	
Antihypertensive therapy (n)	0	3	
NHE-1 H^+ efflux rate ($mmol \cdot L^{-1} \cdot min^{-1}$)	13.5 ± 0.7	18.7 ± 0.5	<.001
NHE-1 phosphorylation (arbitrary units)	1.00 ± 0.08	1.95 ± 0.18	<.001
Lymphoblast proliferation rate ($\times 10^{-3} \cdot h^{-1}$)	17.6 ± 1.0	25.5 ± 2.0	<.005
MAPK activity ($nmol \cdot g^{-1} \cdot min^{-1}$)	6.8 ± 1.2	13.2 ± 1.1	<.001
$p44^{mapk}$ (arbitrary density units)	1.00 ± 0.10	1.25 ± 0.15	NS
$p42^{mapk}$ (arbitrary density units)	1.77 ± 0.26	2.24 ± 0.22	NS
Phospho-$p44^{mapk}$ (arbitrary density units)	1.00 ± 0.09	0.98 ± 0.15	NS
Phospho-$p42^{mapk}$ (arbitrary density units)	1.25 ± 0.22	1.36 ± 0.15	NS

NOTE. HOE-694–sensitive NHE-1–dependent H^+ efflux rates at pH_i 6.0 and NHE-1 phosphorylation in cell extracts from normotensives and hypertensives are presented. NHE-1 phosphorylation was normalized to a value of 1 in the normotensive group. MAPK activity, MAPK isoform protein contents, and phospho-MAPK content were determined, and all densitometric data were normalized to a value of 1 for the normotensive $p44^{mapk}$ values. The mean ± SEM are reported.

Abbreviation: NS, not significant.

or diabetes. All but two of the hypertensive subjects had a family history of hypertension, whereas none of the normotensive subjects had such a family history. Only three hypertensives were on antihypertensive therapy at the time of study.

HOE-694–sensitive NHE-1–mediated H^+ efflux rates were significantly higher when pH_i was clamped at 6.0, reflecting the increased V_{max} of NHE-1 that had been previously reported.[3,4] When lymphoblasts containing equal amounts of NHE-1 protein as detected by immunoblotting[4,12] were labeled with ^{32}P-orthophosphate and the NHE-1 immunoprecipitates prepared from cell extracts using the specific antibody G253,[4] we confirmed an elevated NHE-1 phosphorylation of approximately twofold in the cells of the hypertensives ($P < .001$). The cell proliferation rate was also elevated in lymphoblasts derived from hypertensives ($P < .005$). These changes in cell proliferation rate and NHE-1 activity and phosphorylation may represent an intermediate phenotype of hypertension.

The mechanism of the increased NHE-1 phosphorylation in cells from hypertensives was unclear. However, it has been proposed that NHE-1 activation may be downstream of MAPK,[9] the central kinase in a cascade transducing signals from growth factor and hormone receptors to other intracellular targets mediating protein synthesis and cell proliferation.[7,8] We therefore examined the hypothesis that MAPK activation may be increased in cell extracts from hypertensives.

MAPK Activity and MAPK Protein and Phospho-MAPK Content in Cell Extracts

Total MAPK activity in cell extracts was assayed by a modification of a previously described nonapeptide phosphorylation assay.[10] Incorporation of ^{32}P into the nonapeptide was linear up to 40 minutes in the incubations, and further studies were thus performed using a 20-minute incubation.

Using this specific peptide phosphorylation assay, MAPK activity in cell extracts was markedly enhanced by approximately twofold ($P < .001$) in cell lysates from hypertensives, indicating that this central signaling kinase exhibits increased activity in these cells (Fig 1 and Table 1). We then examined whether this elevated MAPK activity could be due to increased MAPK protein content by immunoblotting cell extracts and detecting the MAPK isoforms using a specific polyclonal MAPK antibody. Figure 2 shows a typical blot, where 80 μg protein from different cell extracts of hypertensives and normotensives was resolved in each lane. The antibody detected both $p42^{mapk}$ and $p44^{mapk}$, but the increased immunoreactivity toward $p42^{mapk}$ may be a property of this antibody rather than an actual increased $p42^{mapk}$ content relative to $p44^{mapk}$ content. Quantitation of these bands by densitometry did not show any significant differences in the content of these MAPK isoforms between extracts from normotensives and hypertensives (Table 1), although there was a trend toward higher values in cell extracts from hypertensives. These data suggest that cell extracts from both groups of subjects contain similar amounts of these MAPK isoforms, indicating that the increased MAPK activity in cells from hypertensives was not due to increased MAPK protein content, but to increased activation of the MAPK cascade.

The phospho-specific MAPK antibody detected Tyr204-phosphorylated MAPK of both the $p42^{mapk}$ and $p44^{mapk}$ isoforms. Figure 3 illustrates a typical immunoblot using the phospho-specific MAPK antibody to detect tyrosine-phosphorylated MAPK. The level of tyrosine-phosphorylated MAPK of both isoforms was similar in extracts from both normotensive and hypertensive subjects. The mean levels of phospho-MAPK

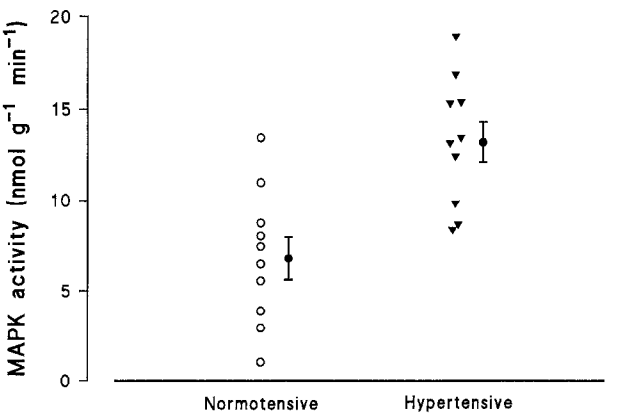

Fig 1. MAPK activity of cell extracts derived from normotensive or hypertensive subjects. Mean data over 3 separate determinations for each subject are presented.

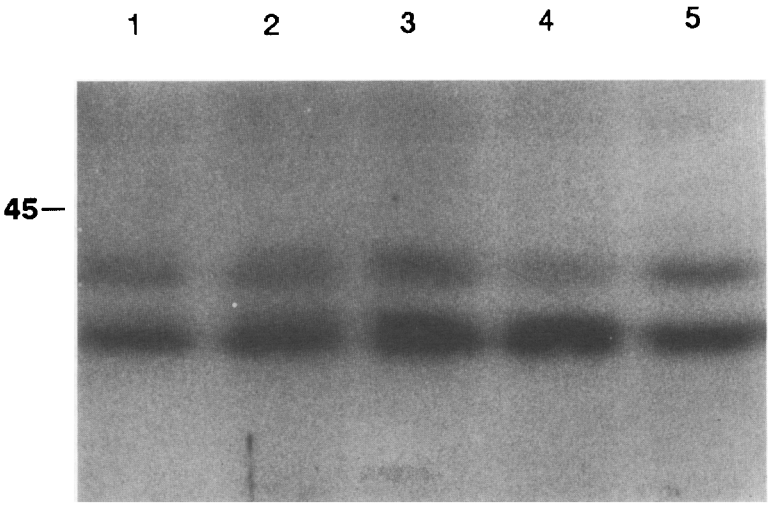

Fig 2. Typical immunoblot of cell extracts from normotensives (lanes 1, 3, and 5) or hypertensives (lanes 2 and 4), using a specific MAPK antibody to detect p42^{mapk} and p44^{mapk} isoforms. The molecular weight marker for 45 kd is shown.

in both groups over two separate experiments are reported in Table 1, with no significant differences between the groups.

MAPK activity was significantly correlated with systolic ($r_s = .55$, $P < .01$) and diastolic ($r_s = .61$, $P < .005$) blood pressure. Furthermore, MAPK activity was correlated with NHE-1 phosphorylation ($r_s = .51$, $P < .02$), NHE-1 activity ($r_s = .55, P < .01$), and cell proliferation rate ($r_s = .46, P < .05$). In addition, the cell proliferation rate was significantly related to the level of NHE-1 phosphorylation ($r_s = .62$, $P < .004$). Tyrosine-phosphorylated MAPK levels were not correlated with NHE-1 phosphorylation, NHE-1 activity, cell proliferation rate, or MAPK activity.

DISCUSSION

Our group and others have previously demonstrated that hypertensives possess an intermediate phenotype consisting of enhanced cellular NHE-1 activity, and that this phenotype persists despite culture of cells in vitro.[3,4,14] This exchanger phenotype has also been described in a wide variety of cells, including vascular smooth muscle cells, leukocytes, platelets, and red blood cells.[14] This enhanced NHE-1 activity is associated with no increase in NHE-1 protein expression,[4] but NHE-1 phosphorylation may be increased and may contribute to the expression of the hypertensive intermediate phenotype. These findings of increased NHE-1 activity and phosphorylation have also been described in the genetic model of hypertension, the insulin-resistant spontaneously hypertensive rat (SHR).[15,16]

Although extracellular growth factors and hormones may activate NHE-1 activity and phosphorylation,[17] the precise pathways used in signal transduction are unclear. Pages et al[9] have suggested that MAPK may be a focal point where intracellular signals converge, and subsequently could lead to NHE-1 activation. We have therefore examined the activity of this central signaling kinase in extracts of immortalized lymphoblasts derived from normotensives and hypertensives. The specific MAPK substrate peptide phosphorylation assay exhibits markedly enhanced activity in extracts of cells from hypertensive subjects, and our current experiments demonstrate the coexistence of this enhanced activity with the intermediate phenotype of enhanced NHE-1 activity and phosphorylation. Since the cells from hypertensives proliferated at a faster rate than those from normotensives, it was not surprising that MAPK activity was higher in the cells from hypertensives, and there was a correlation between cell proliferation rate and MAPK activity. Withdrawal of serum to make the cells quiescent led to changes in viability that could affect interpretation of the data. Thus, our findings may reflect possible changes at any level in the cascade from growth factor receptors to the signaling kinase rather than specifically at the level of MAPK itself.

Despite the twofold enhancement of MAPK activity in cell extracts from hypertensives, there was no concomitant overexpression of the MAPK isoforms p44^{mapk} and p42^{mapk}, indicating that enhanced MAPK activity may result from its phosphorylation. Since MAPK is activated by phosphorylations on both Tyr and Thr residues by the dual-specificity kinase MEK, we examined the level of Tyr phosphorylation of MAPK from the cell extracts using a phospho-specific MAPK antibody that detects phosphorylation of Tyr204 in subdomain VIII of MAPK. Our results suggest that the level of Tyr phosphorylation was similar in extracts from hypertensives and normotensives. Since MAPK activity was higher in extracts from hypertensives, one possibility is that the level of Thr phosphorylation may be higher in these cells. It is known that both Tyr and Thr phosphorylations are necessary for full activation of MAPK[18,19]

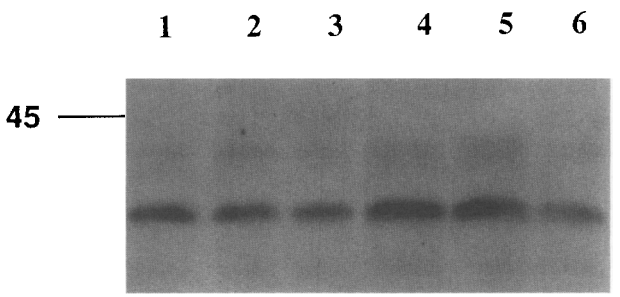

Fig 3. Immunoblot of cell extracts from normotensives (lanes 1, 3, and 5) or hypertensives (lanes 2, 4, and 6), using the phospho-specific MAPK antibody to detect tyrosine 204–phosphorylated p42^{mapk} and p44^{mapk} isoforms. The molecular weight marker for 45 kd is shown.

and that during stimulation Tyr phosphorylation occurs before Thr,[20,21] so that only molecules phosphorylated on both residues are fully active. Furthermore, dephosphorylation of MAPK with the phosphotyrosine-specific protein phosphatase or the phosphoserine- and phosphothreonine-specific phosphatase 2A[18] leads to loss of activity of the kinase, suggesting that another possibility for the differences we observed could be altered dephorphorylation of MAPK between normotensive and hypertensive cells. Others have recently observed differences in MAPK inactivation following stimulation by agonists in SHR vascular smooth muscle cells with inactivation being more rapid in SHR cells.[22] Moreover, differences in activation of MAPK were also observed, with the agonist-induced stimulation of MAPK in SHR cells being more dependent on Ca^{2+}.[22] These possible differences in MAPK activation and deactivation remain to be explored in the human hypertensive lymphoblast model.

The association between increased NHE-1 activity or phosphorylation and increased MAPK activity is also present in vascular smooth muscle cells derived from the SHR model of genetic hypertension.[13,15] In SHR vascular smooth muscle cells, MAPK activation by agonists such as angiotensin II and phorbol esters is enhanced compared with that in Wistar Kyoto normotensive rat cells,[13] and basal NHE-1 phosphorylation is also enhanced approximately twofold in SHR cells.[15] Thus, the increased MAPK activity could be a central component of the hypertensive intermediate phenotype that manifests as the increased NHE-1 activation and phosphorylation that has been reported in diverse cell types.[3,4,14-16] The underlying mechanism leading to increased MAPK activation is currently not known and remains to be investigated further. Since these phenotypic changes are preserved in cultured cells, they cannot result from an increased blood pressure itself, but may be dependent on the genetic program of the cells from hypertensives. Moreover, if the increased MAPK activation occurs in vivo, it may account for the increased protein synthesis (from $p70^{S6kinase}$ and $p90^{rsk}$ activation)[5-7] and cell proliferation (from activation of transcription factors with expression of immediate early genes such as c-*myc*, c-*fos*, and c-*jun*)[8] that have been described in hypertrophied hypertensive tissues such as the heart and blood vessels.

A recent study has also demonstrated that agonists such as vasopressin and phorbol esters could stimulate MAPK activity and NHE-1 activity in a coordinated fashion in platelets.[23] However, only phorbol esters lead to activation of NHE-1 phosphorylation, and it was suggested that although both agonists could stimulate NHE-1 activity via the MAPK cascade without altering NHE-1 phosphorylation, phorbol esters could increase NHE-1 activity further by its direct phosphorylation.[23] If this interpretation is correct, then our findings may result from a signaling defect upstream of MAPK and protein kinase C leading to increases in MAPK activity, NHE-1 activity, and NHE-1 phosphorylation. However, some caution must be exercised in interpreting these results from platelets, since there may be differences in the response of NHE-1 phosphorylation to phorbol esters in different tissues; for example, we have demonstrated that phorbol esters actually lead to NHE-1 dephosphorylation in vascular smooth muscle cells while NHE-1 activity was increased.[24]

In conclusion, this is the first demonstration that cell extracts from cultured lymphoblasts derived from hypertensive subjects exhibit enhanced MAPK activity with no significantly increased MAPK protein content and no significant increase in the level of tyrosine phosphorylation of MAPK. This enhanced MAPK activity is associated with the increased NHE-1 activity and phosphorylation that have been described in such cells. These findings concur with those reported in vascular smooth muscle cells from the SHR, and may represent a further component of the hypertensive intermediate phenotype. However, the mechanisms leading to these changes are currently unknown. Possible mechanisms are differences in the signaling cascade upstream of MAPK or in the phosphatases that inactivate MAPK, and these remain to be elucidated.

ACKNOWLEDGMENT

We thank Dr Andrzej Krolewski Genetics Core, Diabetes and Endocrinology Research Center, Joslin Diabetes Center, Boston, MA, for the lymphoblast cell lines.

REFERENCES

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

2. Mahnensmith RL, Aronson PS: The plasma membrane sodium-hydrogen exchanger and its role in physiological and pathophysiological processes. Circ Res 56:773-788, 1985

3. Rosskopf D, Fromter E, Siffert W: Hypertensive sodium-proton exchanger phenotype persists in immortalized lymphoblasts from essential hypertensive patients. A cell culture model for human hypertension. J Clin Invest 92:2553-2559, 1993

4. Ng LL, Sweeney FP, Siczkowski M, et al: Na^+/H^+ antiporter phenotype, abundance and phosphorylation of immortalized lymphoblasts from humans with hypertension. Hypertension 25:971-977, 1995

5. Boulton TG, Yancopoulos GD, Gregory JS, et al: An insulin-stimulated protein kinase similar to yeast kinases involved in cell cycle control. Science 249:64-67, 1990

6. Boulton TG, Nye SH, Robbins DJ, et al: ERKs: A family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. Cell 65:663-675, 1991

7. Davis RJ: The mitogen-activated protein kinase signal transduction pathway. J Biol Chem 268:14553-14556, 1993

8. Cano E, Mahadevan LC: Parallel signal processing among mammalian MAPKs. Trends Biochem Sci 20:117-122, 1995

9. Pages G, Lenormand P, L'Allemain G, et al: Mitogen-activated protein kinases $p42^{mapk}$ and $p44^{mapk}$ are required for fibroblast proliferation. Proc Natl Acad Sci USA 90:8319-8323, 1993

10. Clark-Lewis I, Sanghera JS, Pelech SL: Definition of a consensus sequence for peptide substrate recognition by p44mpk, the meiosis-activated myelin basic protein kinase. J Biol Chem 266:15180-15184, 1991

11. Scholz W, Albus U, Lang HJ, et al: HOE 694, a new Na^+/H^+ exchange inhibitor, and its direct effect in cardiac ischaemia. Br J Pharmacol 109:562-568, 1993

12. Ng LL, Davies JE, Siczkowski M, et al: Abnormal Na^+/H^+ antiporter phenotype and turnover of immortalized lymphoblasts from type 1 diabetic patients with nephropathy. J Clin Invest 93:2750-2757, 1994

13. Wilkie N, Ng LL, Boarder MR: Angiotensin II stimulation of MAP kinases in cultured vascular smooth muscle cells from hyperten-

sive rats: Protein kinase C–dependent and –independent mechanisms. Br J Pharmacol 117:41P, 1996 (abstr)

14. Rosskopf D, Dusing R, Siffert W: Membrane sodium-proton exchange and primary hypertension. Hypertension 21:607-617, 1993

15. Siczkowski M, Davies JE, Ng LL: Na^+/H^+ exchanger isoform 1 phosphorylation in normal Wistar-Kyoto and spontaneously hypertensive rats. Circ Res 76:825-831, 1995

16. Siczkowski M, Ng LL: Glucose-induced changes in activity and phosphorylation of the Na^+/H^+ exchanger NHE-1 of vascular myocytes from Wistar-Kyoto and spontaneously hypertensive rats. Metabolism 45:114-119, 1996

17. Sardet C, Counillon L, Franchi A, et al: Growth factors induce phosphorylation of the Na^+/H^+ antiporter, a glycoprotein of 110 kD. Science 247:723-726, 1990

18. Anderson NG, Maller JL, Tonks NK, et al: Requirement for integration of signals from two distinct phosphorylation pathways for activation of MAP kinase. Nature 343:651-653, 1990

19. Posada J, Cooper JA: Requirements for phosphorylation of MAP kinase during meiosis in Xenopus oocytes. Science 255:212-215, 1992

20. Haystead TA, Dent P, Wu J, et al: Ordered phosphorylation of $p42^{mapk}$ by MAP kinase kinase. FEBS Lett 306:17-22, 1992

21. Robbins DJ, Cobb MH: Extracellular signal–regulated kinases autophosphorylate on a subset of peptides phosphorylated in intact cells in response to insulin and nerve growth factor: Analysis by peptide mapping. Mol Biol Cell 3:299-308, 1992

22. Lucchesi PA, Bell JM, Willis LS, et al: Ca^{2+}-dependent mitogen-activated protein kinase activation in spontaneously hypertensive rat vascular smooth muscle defines a hypertensive signal transduction phenotype. Circ Res 78:962-970, 1996

23. Aharonovitz O, Granot Y: Stimulation of mitogen-activated protein kinase and Na^+/H^+ exchanger in human platelets. Differential effect of phorbol ester and vasopressin. J Biol Chem 271:16494-16499, 1996

24. Siczkowski M, Ng LL: Phorbol ester activation of the rat vascular myocyte Na^+/H^+ exchanger isoform 1. Hypertension 27:859-866, 1996