Gene expression profiling and functional proteomic analysis reveal perturbed kinase-mediated signaling in genetic stroke susceptibility

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The stroke-prone spontaneously hypertensive rat (SHRSP) is a model of heritable hypertension-associated cerebrovascular injury. This study sought to compare SHRSP to the stroke-resistant SHR strain to identify genes and protein pathways whose expression and/or function was significantly altered between the strains prior to the onset of stroke. Cerebral cortex gene expression profiles from male SHRSPs and matched SHRs were examined by Affymetrix microarray analysis. mRNAs encoding the brain-derived neurotrophic factor receptor (TrkB) and multiple kinases of the MAPK/AKT signaling pathways, including JNK2, AKT2, and PI3K, were differentially expressed between SHRSP and SHR. Because these data suggest altered function in pathways involving MAP and AKT kinase activity, we performed Western blot using phosphorylation state-specific antibodies to characterize activity of MAP kinase and PI3K/AKT pathways. Changes in the levels of the phosphorylated forms of these kinases paralleled the changes in transcript levels observed between the strains. Two-dimensional gel electrophoresis and peptide fragment mass fingerprinting were used to identify altered protein substrates of these kinases. Protein profiling of kinase substrates further supported the notion of perturbed kinase-mediated signaling in SHRSP and identified adenyl cyclase associated protein 2, TOAD-64, propionyl CoA carboxylase, APG-1, and valosin-containing protein as kinase targets whose phosphorylation state is altered between these strains. Altered gene and protein expression patterns in SHRSP are consistent with increased vulnerability of this strain to cerebrovascular injury.

SHR; microarray; kinases

**SUSCEPTIBILITY TO STROKE** in humans has a complex etiology involving both environmental and genetic factors (6, 14). Although a few rare Mendelian syndromes producing stroke have been well characterized, genetic susceptibility to the common form of this disorder is polygenic (22). Little is known, however, about the genes involved or the pathogenetic mechanism these genes direct and which evolves prior to the occurrence of stroke. In particular, very little information has been gained on the antecedent events unfolding in brain tissue that reflect the progression from genetic susceptibility to frank disease.

The stroke-prone spontaneously hypertensive rat (SHRSP) has been developed by selective breeding from the SHR strain. While SHR is resistant to stroke, SHRSP develops cerebrovascular injury at high frequency (21). The genetic mechanism of stroke in SHRSP involves multiple genes. Mapping studies have begun to define three chromosomal loci that may harbor genes responsible for about 28% of the variation in stroke risk between SHRSP and SHR (23). However, little progress has been made in further identifying the localized susceptibility genes or understanding how they initiate or control the disease process.

In the present study we have combined global gene expression and proteomic approaches to examine cerebral tissue prior to the occurrence of stroke in SHRSP. By contrasting SHRSP with age- and sex-matched SHR animals, we have sought to shed light on potential pathogenetic mechanisms and the underlying genes that contribute to this model of polygenic stroke susceptibility. Our results point to alterations in several related protein kinase-mediated signaling pathways that may be relevant to the pathogenesis of stroke in SHRSP and are of potential significance in human stroke.

**MATERIAL AND METHODS**

Animals, sample collection, and RNA extraction. Studies were performed on six male SHRSPs and six male SHRs from breeding colonies maintained by the investigators. These are the same substrains used by others in genetic linkage studies to identify chromosomal regions contributing to stroke (23). Age-matched male rats from both strains were fed a standard rat chow and water ad libitum until age 8 wk, and both strains subsequently received a “stroke-permissive” diet (18.7% protein, 0.63% potassium, 0.37% sodium) and 1% NaCl drinking solution. Animals were killed at age 12 wk. They did not exhibit any evidence of stroke after behavioral assessment (29) and histological examination (3) at the time.
of sampling. Cerebral cortices were dissected and immediately frozen in liquid nitrogen. RNA was extracted using the method of Chomczynski and Sacchi (7) adapted in the RNAzol protocol (TelTest, Friendswood, TX). Each animal was used to generate a single RNA sample and there was no sample pooling.

Microarray experiments, data analysis, and validation. Microarray analysis was performed as described previously (19). Briefly, 10 μg total RNA from 12 independent samples (6 SHRSPs, 6 SHRs) was used to synthesize cDNA, which was then used as a template to generate biotinylated cRNA. cRNA was fragmented and hybridized to Test2 microarrays to verify quality and quantity of the samples. Each sample was then hybridized to a RG-U34A array (Affymetrix, Santa Clara, CA). After hybridization, each array was washed and scanned, and fluorescence values were obtained using the Affymetrix Microarray Suite version 5.0 software. Data were analyzed using the method develop by Tusher et al. (28) implemented in the SAM (“significance analysis of microarrays”) package. Expression values were normalized within sample, to the median of all measurements in that sample, and within gene, to the median of all measurements for that gene. Analyses were performed on the log scale. The mixture model approach of Tusher et al. (28) takes full advantage of the existence of replicated data to estimate the null distribution of the test statistic directly from the data. For each gene, the “test statistic” d(i), or relative difference in gene expression is given by

\[ d(i) = \frac{\bar{x}_{gp}(i) - \bar{x}_{sg}(i)}{s(i) + s_0} \]

where \(\bar{x}_{gp}(i)\) and \(\bar{x}_{sg}(i)\) are defined as the average levels of expression for gene (i) in SHRSs and SHRs, respectively; \(s(i)\) is the standard deviation of repeated measurements; \(s_0\) is a constant computed to minimize the coefficient of variation of \(d(i)\). The expected value of the test statistic \(d_g(i)\) was calculated after performing a set of 500 permutations of the vector of gene expression measurements over the samples. Genes were considered differentially expressed if \(d(i) - d_g(i)\) was greater than a threshold \(\Delta\). The threshold \(\Delta\) was chosen so that the false discovery rate (FDR) associated with the identified genes is less than or equal to 5% (i.e., among the genes called significant, 5% or less are false positive).

To compare gene expression using a different methodology, we examined expression of a subset of genes using the 5’ nuclease (TaqMan) RT-PCR assay. The same RNA samples as those used in the microarray experiments were used in the validation experiments. Primer and probe sequences are available as Supplemental Table 1, available at the Physiological Genomics web site.1

Western blot analyses. Cortical tissue samples from the same animals as those used in the microarray experiments were Dounce-homogenized in 5 volumes hypotonic lysis buffer containing (in mM) 10 Tris, 10 NaF, 10 sodium pyrophosphate, 10 β-glycerophosphate, 1 Na3VO4, 1 EDTA, adjusted to pH 7.4 and containing 1% Igepal CA-630 nonionic detergent. Following determination of protein concentration by Coomassie dye assay (Pierce), sample aliquots were added to 2 μg/μl and boiled after addition of SDS sample buffer. Samples were resolved on 6–15% SDS-PAGE gels (20 μg/lane), transferred to PVDF membranes, blocked in 5% nonfat dry milk in Tris-buffered saline with 0.05% Tween-20 (TTBS) followed by overnight incubation in primary antibody diluted in 1% milk- TTBS. Blots were rinsed in TTBS and incubated in the appropriate horseradish peroxidase-labeled secondary antibody, either goat anti-rabbit or anti-mouse (Cell Signaling Technology, Beverly, MA), at 1/20,000 in 1% milk-TTBS for 1 h. After rinse, blots were incubated in enhanced chemiluminescent substrate (Pierce SuperSignal West Pico) and exposed to film (Kodak BioMax ML). The following phosphorylation state-specific primary antibodies to specific kinases were used (all from Cell Signaling Technology): extracellular regulated kinase dually phosphorylated at residues Thr202 and Tyr204 (pERK, 1/2,000); 90-kDa ribosomal S6 kinase phosphorylated on Ser380 (pRSK, 1/1,000); Jun terminal kinase phosphorylated at residues Thr183 and Tyr185 (pJNK, 1/1,000); pre-MAPK kinase phosphorylated on Ser473 (pAKT, 1/1,000). We also used antibodies (all from Cell Signaling Technology) to consensus phosphorylation motifs to detect changes in phosphorylation state of substrates of AKT (antibody detects phospho-Ser/Thr preceded by Lys/Arg at positions –5 and –3), 3-phosphoinositide-dependent kinase 1 (pDK1 antibody detects T9F CGT motifs), and MAPK/CDK family of proline-directed kinases (antibody detects phospho-Thr followed by Pro, pTPT motifs) (27). Welch t-tests for equality of means were used to determine whether phosphorylated protein amounts differed among the two groups of rats.

Two-dimensional gel electrophoresis and mass-spectrometry identification of pTPT substrates. Samples were fractionated on an anion-exchange column (High Q, Bio-Rad). Fractions eluted with an increasing NaCl gradient (0–1,000 mM) were precipitated in 10 vol of acetone (–20°C overnight). The pellets were sonicated in 9 M urea, diluted to a final concentration of 1 μg/μl (9 M urea, 5× SDS buffer), and boiled. We loaded 20 μg per lane (6–15% gradient gels), and Western blotting with the pTPT antibody was performed as described above. Fractions that were enriched for pTPT-immunoreactive bands were precipitated in aceton and resuspended in two-dimensional (2D) electrophoresis buffer (Bio-Rad Ready- Prep 2). Initially, 500 μg was loaded onto a 7-cm wide-range (pH 3–10) IPG strip (Bio-Rad) for isoelectric focusing (10,000 V-h, 4,000 V). After focusing, strips were equilibrated in sample buffer (9 M urea, 150 mM Tris, pH 8.8, 3% SDS) containing first 1% DTT for 10 min followed by 2.5% iodoacacetamide for 10 min. Samples were placed on 6–15% gradient gels, and Western blotting for pTPT was performed. For protein identification, 3 mg protein was loaded onto 17-cm narrow-range (pH 5–8) IPG strips, focused (30,000 V-h, 10,000 V), and resolved by second-dimension SDS-PAGE, and Western blots were probed for pTPT. Replica gels were aligned with the immunoblots, and pTPT-immunoreactive spots were excised and subjected to peptide fragment mass analysis by electrospray mass spectrometry (PerkinElmer/SCIEX API 3000) after in-gel tryptic digestion.

RESULTS

Gene expression array studies. We identified 129 probes, representing 117 unique genes or expressed sequence tags (ESTs), differentially expressed in the cerebral cortex of 12-wk-old SHRSs and SHRs on a stroke-permissive diet (see Supplemental Table 2). The identified genes had known functions in the brain or central nervous system and belonged to major func-

1 The Supplementary Material for this article (Table 1, which is a list of the primer and probe sequences, as well as Supplemental Table 2, which is a comprehensive list of mRNA transcripts showing a differential expression in the cerebral cortex of age- and gender-matched SHRS and compared to SHRs) is available online at http://physiolgenomics.physiology.org/cgi/content/full/00020.2003/DC1.
tional classes such as neurotransmission, intracellular signaling, cell proliferation, metabolism, and RNA transcription (Supplemental Table 2). The transcription factor JunD exhibited the greatest difference in expression among the genes identified (~15-fold lower in SHRSP vs. SHR). The gene encoding the atrial natriuretic peptide (ANP) was expressed at significantly lower levels in SHRSP compared with SHR (average difference was ~3.2-fold across 2 independent probe sets). This is in agreement with earlier reports describing a threefold lower expression of ANP mRNA in the brain of SHRSP compared with SHR (17, 24).

The chromosomal location of the differentially expressed genes was determined using map information from the rat genome assembly (UCSC version 2) and comparative maps of the human, mouse, and rat genomes. Genes mapping to the chromosomal regions previously identified as containing quantitative trait loci (QTLs) influencing stroke in this animal model (23) are shown in Supplemental Table 2. These transcripts were ANP, the potassium voltage-gated channel Kcnab2, and CaMK2 inhibitor α, which map to the QTL on chromosome 5; casein kinase 2, which maps to the QTL on chromosome 3; and Doc2A and sulfotransferase A1, which map to the QTL on chromosome 1.

Multiple genes encoding members of the serine/threonine kinases superfamily were found differentially expressed between SHRSP and SHR. Among those with a higher expression in SHRSP were the catalytic β-subunit of cAMP-dependent protein kinase (1.5-fold), the AKT2 kinase (1.9-fold, averaged over two probe sets; \( P = 0.01 \)), the Janus protein tyrosine kinase kinase 2 (2.3-fold), the JNK2 isoform (1.3-fold), and the protein kinase C α-subunit (1.9-fold). The cGMP-dependent protein kinase 2, the casein kinase 2α (CK2), and the glycogen synthase kinase 3β (GSK3) had a significantly lower expression in the SHRSP compared with SHR (~1.5-fold, ~1.6-fold, and ~1.8-fold, respectively). Known regulators of these serine/threonine kinases were also found differentially expressed between the two strains. For example, expression of DUSP6, the dual specificity phosphatase that selectively inactivates ERK2, was significantly decreased in SHRSP (1.4-fold, average of two probes). Similarly, the phosphoinositide 3-kinase (PI3K) p85, which stimulates the activity of the AKT kinases, also had a significantly lower expression in SHRSP (~3-fold, average of two probes). Expression of Map4k1, a specific regulator of the JNK pathway, was significantly lower in SHRSP compared with SHR (~9-fold).

The neurotrophin receptor protein tyrosine kinase, TrkB, was also found significantly differentially expressed. The TrkB gene encodes two major isoforms by alternative splicing: the full-length mRNA (FL-TrkB) generates a 145-kDa transmembrane tyrosine kinase and neurotrophin receptor. An alternatively spliced mRNA generates a truncated form of the receptor lacking the intracellular tyrosine kinase domain (95 kDa). Two probe sets, specific to each isoform, were present on the array, but the differences in expression between SHRSP and SHR for these probes were in the opposite direction. The FL mRNA had a 1.4-fold lower expression in SHRSP compared with SHR, while the truncated form was expressed at a higher level of ~2-fold (not significant).

**Independent verification of array findings.** To confirm the microarray results, we measured the relative expression of a subset of the identified genes by the TaqMan assay. We observed good concordance between the array data and the TaqMan data for the direction of changes in expression between the strains (Fig. 1). However, estimates of the magnitude of the expression

![Fig. 1. TaqMan assay validation for a subset of differentially expressed genes. Standard curves were generated using serial dilution of known quantities of RNA. TaqMan assays for ribosomal RNA 18S were run in parallel on each sample for subsequent normalization of the data. Assays were run in triplicate on cDNA synthesized by reverse transcription from the same RNA samples as those used in the microarray experiments. Changes in expression levels were calculated using SHR animals as the reference group. *Significantly different mean gene expression between stroke-prone spontaneously hypertensive rat (SHRSP) and SHR in the TaqMan assay (t-test; \( P < 0.05 \)). Anp, atrial natriuretic peptide; Ck2, casein kinase 2; Cplx2, complexin 2; Scd2, stearoyl CoA desaturase 2; Gpam, glycerol-3-phosphate acyltransferase; Itpr1, inositol 1,4,5-triphosphate receptor.](http://physiolgenomics.physiology.org/)
differences between SHRSP and SHR were consistently lower by the TaqMan assay. This assay was unable to detect small but consistent differences in gene expression that were consistently detected by multiple independent probe sets on the array (e.g., stearoyl CoA desaturase, Scd2, 3 independent probe sets, detected fold change for each set: 1.4, 1.5, and 1.5).

**Western blot analysis to follow-up altered TrkB transcript abundance.** In light of the evidence for perturbed gene expression of components of protein kinase-mediated signaling pathways in the SHRSP and the important role of such pathways in susceptibility and response to cerebral ischemia, we sought to determine whether the observed differences might be translated at the level of protein expression and/or function. A Western blot analysis of the TrkB receptor was carried out using a monoclonal antibody that allows determination of both the full-length and the 95-kDa isoforms of the protein (BD Biosciences). As in the gene expression analyses, we observed a significant decrease in protein abundance of the FL-TrkB receptor in SHRSP compared with SHR (fold change = −2.3, \( P = 0.005 \)), whereas the level of the TrkB 95-kDa isofrom was significantly increased (fold change = 2.6, \( P = 0.003 \)) (Fig. 2).

**Protein kinase activation in SHRSP cerebral cortex.** We next performed a Western blot analysis with phosphoprotein-specific antibodies to activated kinases to determine whether the observed differences in gene expression of several serine/threonine kinases were accompanied by differences in phosphorylation level of these proteins. Dually phosphorylated ERK protein abundance was significantly increased (1.8-fold) in SHRSP compared with SHR (\( P = 0.002 \)) (Fig. 3A). An antibody to the total ERK protein did not, however, detect any significant difference in the amount of either total ERK1 or ERK2 between the two strains (\( P = 0.80 \) and 0.58, respectively; not shown). Increased level in SHRSP cortex of pRSK (Ser380) (3.7-fold; \( P = 0.005 \)), a known substrate of ERK, is also consistent with the increase in SHRSP ERK activity, as measured by its level of phosphorylation (Fig. 3B). We also investigated whether JNK phosphorylation level was significantly different between the two strains. As shown in Fig. 3C, there was a significant increase (\( \sim 1.7 \)-fold) in pJNK in SHRSP (\( P = 0.03 \)). Total JNK protein was also significantly increased in SHRSP (JNK1, 1.7-fold, \( P = 0.01 \); JNK2, 1.3-fold, \( P = 0.04 \)) (not shown). We were unable to detect p38, another member of the mitogen-activated protein kinase (MAPK) superfamily. This is likely due to its predominantly nuclear location. Gene expression data also suggested differences between SHRSP and SHR in the AKT signaling pathways. We observed a small, but significant increase in pAKT in SHRSP compared with SHR (1.3-fold, \( P = 0.01 \)) (Fig. 3D), whereas no difference between the strains was detected in the levels of total AKT protein (\( P = 0.80 \)) (not shown).

**Altered kinase substrate phosphorylation in SHRSP.** To further corroborate our hypothesis that MAPK- and AKT kinase-mediated signaling is perturbed in SHRSP cortex prior to the onset of stroke, we next screened cortical tissues from SHRSP and SHR for differences in abundance of phosphorylated substrates of these kinases using phosphorylation state-specific antibodies. ERK, JNK, and GSK3 belong to the family of proline-directed serine/threonine kinases, which phosphorylate proteins at threonine or serine residues followed by a proline residue. By Western blot analysis using an antibody that specifically recognizes phospho-threonine-proline (pTP) motifs, we detected the presence of several phosphorylated substrates whose level of expression is increased in cortical tissue from SHRSP compared with SHR (Fig. 4A). These data extend and confirm those which show that activity of proline-directed kinases is increased in SHRSP, as increased substrate phosphorylation is likely a functional consequence of increased ERK/MAPK, JNK, and/or other proline-directed kinase activity.

We next determined whether the altered expression of genes belonging to the AKT pathway observed in the microarray analyses was functionally relevant, assessed by examining the abundance of substrates phosphorylated by AKT and PDK1. We observed significant differences in abundance of AKT-phosphorylated substrates between SHRSP and SHR (Fig. 4B). Similarly, we showed significant differences in abundance of

![Fig. 2. A: Western blot analysis of TrkB in SHRSP and SHR. The monoclonal antibody recognizes two isoforms of the receptor: full-length (145 kDa) and short (95 kDa) isofrom. Equal amounts (10 µg) of proteins were loaded in each lane. B: comparison of mean difference in protein abundance between 6 SHRSPs and 6 SHRs \((P < 0.05, t\text{-test})\). IOD, integrated optical density.](http://physiolgenomics.physiology.org/content/journal/physiolgenomics/15/4/figure/fig2)
phosphorylated PDK1 substrates between the two strains (Fig. 4C). These data further demonstrate evidence of perturbations in multiple kinase-mediated signaling pathways in SHRSP prior to the onset of stroke.

Identification of altered kinase substrates in SHRSP. Identification of specific targets of activated proline-directed and AKT kinases may provide further information about the pathophysiological consequences of perturbations in kinase-mediated signaling in SHRSP. We were able to identify by mass spectrometry several kinase-regulated substrates. Anion-exchange chromatography was first used to enrich for the pTP substrates, and gel electrophoresis was used to determine which purified proteins were of differential abundance. Four proteins which show significant difference in abundance between SHRSP and SHR in Western blotting with pTP antibody (Fig. 5A, proteins indicated with arrows) appear in the 300 mM NaCl anion-exchange eluate (Fig. 5B). The 300 mM NaCl fraction was then resolved by 2D electrophoresis (Fig. 5C). The corresponding spots on the gel were punched, trypsinized, and analyzed by peptide mass fingerprinting using mass spectrometry. Three of these proteins could be identified: adenylyl cyclase associated protein 2, TOAD-64, and propionyl CoA carboxylase. The band from the 600 mM fraction (Fig. 5B) was also resolved by 2D electrophoresis and was shown to be two separate proteins of similar mass, APG-1 and valosin-containing protein.

DISCUSSION
To improve our understanding of the molecular mechanisms involved in the SHRSP predisposition to
spontaneous brain damage, we have carried out microarray gene expression profiling and functional proteomic analysis of cortical tissue from age-matched SHRSP and SHR prior to stroke occurrence. The TrkB receptor is the high-affinity receptor for two neurotrophins: brain-derived neurotrophic factor (BDNF) and neurotrophin 4/5. Both are important promoters of neuronal survival, growth, and differentiation and regulate synaptic transmission. Evidence is accumulating for a crucial role of TrkB in protection from neuronal damage after ischemia. In adult rats subjected to permanent middle cerebral artery (MCA) occlusion, both TrkB mRNA and expression of the FL-TrkB protein were significantly increased in cortical neurons distant from the infarct (2, 10). This increase was associated with a decreased neuronal cell death in the penumbra area and appeared to be mediated through elevation in BDNF levels (10). Similar findings were obtained in a gerbil model of global ischemia (9). In the present studies we found that TrkB was significantly differentially expressed between the SHRSP and SHR strains.

The TrkB receptor is expressed as multiple isoforms produced by alternative mRNA splicing. Both the FL and the truncated forms of the receptor were differentially expressed between SHRSP and SHR, but in the opposite direction. The FL receptor was significantly downregulated, and the truncated form was significantly upregulated in SHRSP. The FL form of the receptor possesses a tyrosine kinase domain that is lacking in the truncated form. Although the intracellular signaling mechanisms used by FL-TrkB to promote neurotrophic actions are well characterized, the physiological functions of the truncated receptors remain poorly understood. Recent data have suggested that they inhibit FL-TrkB-mediated cell survival by acting as a dominant-negative receptor either by preventing FL-TrkB activation or by trapping the ligand and preventing its interaction with FL-TrkB (8, 12). The role of the truncated form of the receptor in TrkB-mediated neuroprotection from ischemic injury has been examined in a recent study by Saarelainen et al. (25), which reports that mice overexpressing truncated TrkB receptor in neurons suffer much greater cortical damage after focal cerebral ischemia than their wild-type littermates. These data are consistent with the hypothesis that aberrations in TrkB expression may promote SHRSP vulnerability to cerebrovascular lesions.

The TrkB receptor promotes neurotrophic events through actions mediated by at least three types of multifunctional, highly conserved serine/threonine protein kinases: CaMK2, CK2, and MAPKs (4). All three pathways were found significantly differentially expressed between SHRSP and SHR. More recently, the PI3K/AKT pathway was also implicated in Trk-receptor-mediated neurotrophins action (16). We found a significant increase in AKT2 gene expression and a significant decrease in PI3K gene expression in SHRSP. Moreover, a known target of the AKT signaling pathway, the GSK3, was also significantly differentially expressed between the two strains. Perturbations in expression of these kinases may represent a functional consequence of altered TrkB expression; alternatively, they may contribute to SHRSP stroke vulnerability by mechanisms independent from TrkB.

MAPKs regulate gene expression and protein synthesis. There is growing evidence that MAPKs play pivotal roles in the regulation of neuronal survival and apoptosis. Alessandrini et al. (1) reported that inhibition of the MAPK/ERK pathway affords significant neuroprotection from brain injury resulting from MCA occlusion. More recently, inhibition of ERK2 phosphorylation has been directly correlated with inhibition of neuronal death in an animal model of ischemia/reperfusion injury (20). We report evidence for an activation of the ERK and JNK pathways in the cortex of SHRSP prior to the onset of stroke. JNK gene expression and activated ERK and JNK protein abun-
dance were increased in SHRSP compared with age-matched SHR. The increased ERK1/2 protein activity, as measured by its level of phosphorylation, observed in the cortex of SHRSP is consistent with the enhanced vulnerability of this model to cerebrovascular injury.

Previous studies have reported increased ERK and JNK activities in the left ventricle and aorta of SHRSP compared with the normotensive closely related strain, WKY, and have proposed a role of these kinases in the development of hypertensive cardiovascular disease (13, 18). The effect of increased ERK and JNK activities on the development of left ventricular hypertrophy in SHRSP was shown to be independent from any effect on blood pressure and appeared to be mediated by distinct mechanisms. Although further studies are needed to elucidate the mechanisms and significance of the increase in cortical ERK and JNK in SHRSP, this is, to our knowledge, the first report suggesting a perturbed MAPK-mediated signaling in the brain of SHRSP prior to stroke occurrence.

JunD, a member of the Jun family of transcription factors, is a known target of the MAPK cascade and was differentially expressed between SHRSP and SHR both consistently and at high levels. The significance of this result is unclear. A previous study examining JunD expression in the rat hippocampus after transient ischemia has shown that JunD induction after ischemia represents a protective reaction to ischemic

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**Fig. 5. Identification of pTP substrates of different abundance between SHRSP and SHR.**

A: four pTP substrates (indicated by arrows) showed markedly increased levels in cerebral cortex from SHRSP. B: anion-exchange chromatography to enrich for pTP substrates shows that all four substrates elute from the 300 mM NaCl elution fraction. C: the 300 mM fraction was resolved by two-dimensional electrophoresis. The pTP Western blot (red overlay) is superimposed on a Coomassie-stained replicate gel from which spots were punched and analyzed by mass spectrometry.
stress (15). Thus the reduced levels of JunD expression in SHRSP corroborate our finding of patterns of gene expression in SHRSP consistent with an increased vulnerability of this strain to cerebrovascular lesions.

Both SHRSP and SHR develop hypertension at early age but by 10 wk of age, SHRSP's blood pressure levels are greater than SHR's. Our animals were sampled at 12 wk of age. We, therefore, cannot exclude the possibility that some observed differences in gene expression may be due to differences in blood pressure between the two strains. It is well established that high levels of blood pressure contribute to expression of the stroke phenotype in the SHRSP and that lowering of blood pressure greatly reduces the occurrence of stroke in these animals (5). It is, therefore, likely that among the genetic factors that confer stroke susceptibility to the SHRSP, some will contribute to stroke via effects on blood pressure. Identification and characterization of such genes will be important to the understanding of the mechanisms underlying hypertension-associated stroke susceptibility in this model.

The goal of studies employing genome-scale gene expression profiling to genetic disease susceptibility is to shed light on a broad range of systems that may be altered in the disease model and to make relevant correlations that may indicate fruitful targets for more focused investigation. As such, this study has provided the first evidence that gene and protein expression levels of the TrkB receptor and protein activity of multiple kinases mediating its signaling are altered in the cortex of SHRSP prior to the onset of stroke. Additional gene expression profiling experiments by others may verify the data and inferred hypothesis reported here (26). The present study does not permit to firmly establish a causal relationship between changes in gene expression, functional alterations in the corresponding protein pathways, and stroke susceptibility. However, the hypothesis that the observed perturbations are consistent with an enhanced vulnerability of the SHRSP to neuronal injury and thus may play an important role in the pathophysiology of cerebrovascular lesions in this animal model is intriguing and provides the basis for further targeted investigations (11). In particular, we have begun to identify and characterize sequence variation in genes whose expression was significantly different between the two strains (11). Resequencing of the TrkB mRNA sequence has already identified two variants: a synonymous A to G substitution at position 1450, and a C to T substitution at position 1224 which results in an amino acid change from an alanine to a valine in the second cysteine-cluster domain of the receptor involved in ligand binding (not shown). Future analyses will determine whether this mutation cosegregates with stroke and stroke-related phenotypes in crossbred animals to assess its role in disease etiology.

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DISCLOSURES

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