Genes overexpressed in cerebral arteries following salt-induced hypertensive disease are regulated by angiotensin II, JunB, and CREB

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HYPERTENSION IS A multifactorial disease, linked to both genetic and environmental origins, that ultimately causes direct alterations on the vasculature including smooth muscle cell proliferation and vascular remodeling (2, 34, 48). Rats selectively bred for hypertension studies have been useful models to study hypertension and define signaling pathways important in their regulation. Intact cerebral arteries from Dahl salt-sensitive normotensive and hypertensive high-salt (HS) rats were examined by immunostaining, revealing an increased phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) and expression of the proliferative marker Ki-67 in arteries from hypertensive animals. Arterial RNA analyzed by microarray and validated with RT-quantitative PCR revealed that jun family member junB and matricellular genes plasminogen activator inhibitor-1 (PAI-1) and osteopontin (OPN) were significantly overexpressed in HS arteries. Fisher exact test and annotation-based gene subsets showed that genes upregulated by Jun and Ca2+/cAMP-response element-binding protein (CREB) were overrepresented. A model of cultured rat cerebrovascular smooth muscle cells was used to test the hypothesis that angiotensin II (ANG II), JunB, and CREB are important in the regulation of genes identified in the rat hypertension model. ANG II induced a transient induction of junB and a delayed induction of PAI-1 and OPN mRNA levels, which were reduced by ERK inhibition with U-0126. Silencing junB using small-interfering RNA reduced mRNA levels of OPN but not PAI-1. The silencing of CREB reduced PAI-1 induction by ANG II but enhanced the transcription of OPN. Together, these results suggest that salt-induced hypertensive disease promotes changes in matricellular genes that are stimulated by ANG II, regulated by ERK, and selectively regulated by JunB and CREB.

Microarray; Dahl rat; brain arteries; mitogen-activated protein kinase signaling; transcription factors

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induces the expression of specific ERK- and CREB-regulated genes in cerebral arteries. Microarray analysis was utilized to identify the range of genes affected by hypertensive disease, and these results were complemented by an investigation of ERK activation, cell proliferation, and validation using techniques to quantify mRNA and detect protein expression. ANG II stimulation of cultured cerebrovascular smooth muscle cells (cVSMCs) was further employed to define selective roles for ERK, JunB, and CREB in the signaling mechanisms leading to the upregulation of gene targets identified in the hypertension study.

**METHODS**

Animal protocol, cerebral artery isolation, and cVSMC explants. All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and followed protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Vermont. All arteries were obtained through tissue sharing from University of Vermont investigators M. LeWinter, Joseph Brayden, Victor May, and Natalia Gokina (IACUC 06-100AP); thus no animals were euthanized exclusively for this study.

For intact studies, male Dahl S rats, weighing ~200 g and ~6 wk old, were obtained from a colony supported by Merck Pharmaceuticals and maintained by Taconic (Germantown, NY). The rats were divided into two groups, and at 7 wk (time = 0) their diets were changed to either an 8% NaCl (HS) or a 0.4% NaCl low-salt (LS) diet. Animals were not started earlier to prevent rapid morbidity observed by others (12, 43). For animals used in the blood pressure study, SBPs were measured weekly using a tail-cuff plethysmograph. For study group animals, beginning at 6 wk after starting the diet, echocardiography was performed weekly, and animals were euthanized when left ventricular dysfunction was detected as previously described (24). The LS animals were euthanized at times that corresponded approximately with the HS animals (see Table 1). After euthanasia, brains were removed and primary branch resistance arteries projecting from the Circle of Willis were dissected (middle and posterior cerebral and posterior cerebellar) in cold HEPES-buffered solution (HBS) composed of (in mmol/l) 10 HEPES (pH 7.4), 140 NaCl, 6 KCl, 2 CaCl2, 1 MgCl2, and 10 glucose. For immunocytochemistry, isolated arteries were fixed in 4% formaldehyde and processed as described in *Cerebral artery immunohistochemistry*. For microarray and RT-quantitative (q)PCR, arteries were either snap frozen in liquid N2 and stored at -80°C or submerged in RNAlater (Qiagen, Valencia, CA) and stored at -20°C until processed for RNA extraction.

To generate cultured cVSMCs, middle and posterior cerebral and cerebellar arteries were isolated from female Sprague-Dawley rats (~12 wk, 200 g), cut into rings, and placed in 60-mm culture dishes containing smooth muscle growth media 2 (SMGM2; Cambrex, Palo Alto, CA). Rings were cultured at 37°C/5% CO2, and VSMCs that migrated within 7 to 10 days were trypsinized and transferred to 100-mm plates. Cells were used between passages 2 and 4, cVSMCs were grown to ~70–75% confluence and serum starved in DMEM containing 0.5% fetal bovine serum, 2 mmol/l l-glutamine, 1,000 U/ml penicillin, and 1 mg/ml streptomycin for 48 h before treatment.

**Table 1. Dahl S rat study group**

<table>
<thead>
<tr>
<th></th>
<th>Long Wt/ Body Wt, g/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>LS</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>14–24 Wk</td>
</tr>
<tr>
<td></td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>481±8.0</td>
</tr>
<tr>
<td></td>
<td>3.66±0.39</td>
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<tr>
<td>HS</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>14–22 Wk</td>
</tr>
<tr>
<td></td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>415±9.9†</td>
</tr>
<tr>
<td></td>
<td>5.60±2.17*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of experiments. LS, low-salt diet; HS, high-salt diet. *P < 0.05; †P < 0.005 when compared with LS. Dahl S, Dahl salt-sensitive.

ANG II (used at 100 mmol/l) and U-0126 (used at 10 μmol/l) were purchased from Calbiochem (San Diego, CA). SB-203580 (used at 700 mmol/l) was obtained from Biosource (Camarillo, CA), and cell culture reagents were from Gibco (Grand Island, NY). All other reagents were obtained from Sigma (St. Louis, MO).

*Cerebral artery immunofluorescence*. Intact artery immunofluorescence was performed as described previously (37a). Briefly, formaldehyde-fixed intact cerebral arteries, secured to sylgard dishes, were sequentially incubated in blocking solution containing 2% BSA and 0.2% Triton X-100 in PBS (Sigma-Aldrich, St. Louis, MO), followed by rabbit anti-p42/p44-phospho-ERK (anti-pERK1/2; Cell Signaling Technologies, Beverly, MA) diluted 1:250 in blocking solution. Antibodies were then incubated with Cy5 goat anti-rabbit IgG (Jackson ImmunoResearch, Westgrove, PA) diluted 1:500 in blocking solution. YOYO-1 (Molecular Probes, Eugene, OR) diluted 1:10,000 and containing 2 U/ml RNase A was used to counterstain the nuclei. Arteries were mounted on microscope slides, and images were captured using a Bio-Rad 1000 laser-scanning confocal microscope at ×400 magnification. Fluorescence intensity was quantified using mean pixel intensity analysis as described previously (37a). For nuclear intensities, a mask of 25 nuclei was generated from the YOYO images, and the intensity of pERK1/2 was determined within the mask regions.

*Cerebral artery immunohistochemistry*. Formaldehyde-fixed arteries were paraffin embedded, cut in 5-μm sections, and applied to microscope slides. Tissue sections were deparaffinized using standard techniques (54) and exposed to antigen retrieval buffer containing 10 mmol/l sodium citrate (pH 6.0) for 15 min at 95°C. Immunohistochemistry was performed using the EnVision plus Dual Link Peroxidase and Substrate systems according to the manufacturer’s protocol (DakoCytomation, Carpinteria, CA). Rabbit anti-Ki-67 and rabbit anti-osteonectin (OPN) primary antibodies were obtained from Abcam (Cambridge, MA) and used at 1:50 dilution. Sections were counterstained with Mayer’s hematoxylin. Digital images were captured using an Olympus BX50 upright light microscope (Olympus America, Lake Success, NY) with an attached Optronics MagnaFire digital camera and software.

**RNA isolation from intact cerebral arteries**. Each starting sample (n) consisted of arteries pooled from three brains (~2 mg wet tissue wt) with an average yield of 340 ng total RNA. Total RNA was extracted from intact cerebral arteries that had been stored at ~80°C or at ~20°C in RNAlater using a modified RNeasy protocol for isolation from animal tissues with a DNase I digestion step (Qiagen). To improve tissue disruption and increase RNA yield, the protocol for tissue homogenization was modified to include ~25 mg of 200-μm silicon carbide abrasive beads (MoBio, Carlsbad, CA) in the homogenizing buffer and use of a Pellet Pestle system (Kontes, Vineland, NJ). RNA was eluted with 25 μl RNAse-free H2O. RNA concentrations were determined using a Nanodrop spectrophotometer, and RNA quality was assessed by an Agilent 2100 bioanalyzer (Agilent, Palo Alto, CA).

**Cerebral artery RNA amplification and microarray**. Each sample (n) consisted of RNA pooled from three different rats on their respective diets (9 total rats/group). RNA samples (n = 3) per group were analyzed to achieve an estimated statistical power of 0.8. RNA amplification and microarray analysis were performed by the University of Vermont DNA facility. RNA samples were double amplified to generate cRNA from the reverse-transcribed cDNA using the method outlined in the Affymetrix Technical Manual (36). After a second cycle, second-strand cDNA synthesis and cleanup, biotinylated cRNA was prepared by in vitro transcription, fragmented, and stained using a streptavidin-phycocerythrin conjugate before application to Affymetrix GeneChip Rat Expression Set 230A (RAE230A) (Affymetrix, Santa Clara, CA). Biotinylated antistreptavidin antibodies were used to amplify the signal. A total of 10 μg of amplified cRNA was applied to each gene chip.
Cerebral artery real-time RT-qPCR. For validation experiments, cDNA was reverse transcribed from different sample pools of RNA than those used in the array analysis. Reverse transcription of 160 ng total RNA from intact cerebral arteries was carried out using an Omniscrypt reverse transcriptase. cDNA was then amplified by TaqMan qPCR, as previously described (37, 38). Expression levels of target genes were determined using hypoxanthine-guanine phosphoribosyl transferase (hp rt) as the internal standard. All samples were run in duplicate from at least three independent experiments, and the comparative cycle threshold (Ct) method for relative quantity (RQ) value was used to calculate relative mRNA expression among samples.

Cerebral artery real-time RT-qPCR. Total RNA was extracted from cultured vSMCs using the RNeasy PLUS protocol for total RNA isolation from animal cells (Qiagen). cDNA was reverse transcribed from a total of 500 ng total RNA, and qPCR was performed as described for intact arteries in Cerebral artery real-time RT-qPCR. All samples were run in duplicate with at least three independent experiments, and the comparative Ct method for RQ was used to calculate relative mRNA levels among samples.

cVSMC Western blot analysis. After treatments, cell plates were transferred to ice, media was aspirated, and the cells were washed first with cold PBS followed by cold hypotonic lysis buffer (HLB) containing (in mmol/l) 25 Tris (pH 8.0), 2 MgCl2, and 5 KCl. Cells were collected by scraping in 60 μl of cold HLB supplemented with a protease/phosphatase inhibitor cocktail containing 1 mmol/l phenylmethyl-sulfonamide, 20 μg/ml aprotinin, 4 μg/ml leupeptin, 2 mmol/l Na+ orthovanadate, and 2 mmol/l Na+ pyrophosphate. Cell lysates were homogenized, and DNA was sheared by passing the extract through a 26-gauge needle 20 times. Protein concentration was determined using a Bradford protein assay (Bio-Rad, Hercules, CA), and 10 μg of protein were separated by 10% SDS-PAGE and transferred to nitrocellulose. Western blot analysis was performed using antibodies specific to CREB (mouse anti-CREB; Cell Signaling Technologies; 1:1,000), OPN [rabbit polyclonal anti-OPN (AB8448); Abcam; 1:1,000], JunB (rabbit polyclonal anti-JunB; Santa Cruz Biotechnology, Santa Cruz, CA; 1:500), and β-actin (rabbit polyclonal anti-β-actin; Cell Signaling Technologies; 1:1,000). All antibodies were diluted in 3% BSA in Tris-buffered saline/0.1% Tween 20 (TBST) and blocked in 3% nonfat dry milk dissolved in TBST. Binding of primary antibody to nitrocellulose blots was detected with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Santa Cruz Biotechnology; 1:5,000) or HRP-conjugated anti-mouse IgG (Santa Cruz Biotechnology; 1:5,000), followed by chemiluminescence using Lumiglo (Kirkgaard and Perry, Gaithersburg, MD).

Statistical analysis. For immunofluorescence, immunohistochemistry, and RT-qPCR data, statistical significance between two groups was determined using a two-tailed unpaired Student’s t-test (significance at P < 0.05). Where multiple comparisons were required, significance was assessed using ANOVA for multiple comparisons (Holm-Sidak method). Results are presented as means ± SE.

For statistical analysis of microarray data, signal intensities were assigned to probes in each sample using Affymetrix software (GeneChip operating software). With the use of BioConductor software (www. bioconductor.org), probe-level expression data was normalized using the qsp line method of Workman et al. (55), and the robust multichip average (RMA) expression statistics were calculated for each probe set and sample using the method of Speed and coworkers (4, 21). The complete gene expression omnibus (GEO) data set is available under accession No. GSE5488.

Each probe set was associated with the magnitude of differential expression between LS- and HS-treatment groups (M, the average difference of RMA expression statistics), as well as a sample standard deviation (s), a P value (p) based on the Student’s t-test, and degrees of freedom (d). Genes identified as differentially expressed (see RESULTS) were judged statistically significant by two methods. Regions describing the joint distribution of M and −log10p under the null hypothesis were constructed after calculating M and −log10p for each of the 10 distinct permutations of the sample labels and contouring a two-dimensional histogram.

Regions describing the joint distribution of M and standard deviation were obtained by explicitly estimating the probability density of the standard deviation, p(σ), using an inverted gamma distribution. The probability of obtaining a differential expression statistic greater than or equal to the observed value, conditional upon the estimated standard deviation σ, is

\[ p(M \geq m|σ) = \frac{1}{p(σ)} \int_{m}^{\infty} p(σ)p(σ)p(M \geq m|σ)dσ \]

where

\[ p(σ) = \int_{m}^{\infty} p(σ)dσ \]

RESULTS

Dahl S animal study group. The Dahl S model of hypertensive disease is characterized by elevated blood pressure and the development of heart failure in response to a HS diet (22, 24). SBP measurements in Dahl S animals confirmed an early onset and sustained increase in blood pressure in animals fed a 8% NaCl diet (HS) (supplementary Fig. 1; all supplementary material can be found with the online version of this article), and previous experiments found no significant difference in blood pressures between 6 and 12 wk on the HS diet (24). Animals in the study group were evaluated by echocardiography, and HS animals with their LS controls were euthanized when left ventricular dysfunction was detected, after an average of 11 wk on the HS diet. As shown in Table 1, when compared with LS controls, animals receiving a HS diet exhibited a significant decrease in body weight and an increase in lung-body weight ratio, indicating the development of heart failure as a result of malignant hypertension.

Cerebral arteries from salt-induced hypertensive rats have significantly higher ERK activity than LS controls. To determine whether hypertensive disease is associated with an up-regulation of cerebral artery ERK activity, cerebral arteries from HS and LS animals were dissected and analyzed by immunofluorescence to detect pERK1/2. As represented in Fig. 1, arteries from HS animals exhibited a significantly elevated level of pERK in both the nucleus and cytoplasm when compared with those from LS controls. Based on the known involvement of ERK in proliferation and remodeling, these
data support a role for ERK in the altered signaling pathways that result from chronic hypertension.

cVSMCs of HS chronically hypertensive rats show marked proliferation compared with those of LS normotensive controls. The impact of hypertensive disease on cerebral artery gene expression was measured directly using immunohistochemical detection of the nuclear proliferation marker Ki-67 and by quantifying nuclei/arterial section. Arteries from HS animals displayed a significantly higher percentage of Ki-67-positive nuclei in both the smooth muscle and endothelial cell layers (Fig. 2). These data suggest that the elevated ERK activity corresponds with increased arterial cell proliferation in cerebral arteries from hypertensive animals.

Genes important for tissue remodeling are upregulated in cerebral arteries from hypertensive animals. The effect of hypertensive disease on cerebral artery gene expression was determined by microarray analysis using an Affymetrix RAE230A GeneChip array. Of the 15,923 genes represented on the microarray, 133 exhibited a significant 1.5-fold or greater change in expression (99 genes were upregulated and 34 were downregulated in HS compared with LS rats; GEO accession No. GSE5488). Rather than rank genes independently based on the log ratio change (M) or the P value (p), differential expression statistics were represented as a joint distribution using two-dimensional histograms represented by either a volcano plot [log2(M) vs. −log10(p), Fig. 3A] or log2(M) vs. standard deviation (Fig. 3B). This representation of the data allowed the assignment of volcano plot-motivated statistics to each probe set (p). Contours of p, that exclude 10% and 1% of the probe set were then used to define differential expression-based binary partitions. The p, thresh-
Fig. 3. Identification of genes of interest using database subsets. A: a volcano plot was created expressing $-\log_{10}$ of the $P$ value (p) on the y-axis versus the log$_2$ fold change (M) on the x-axis to reveal genes of interest in the upper quadrants for the complete probe set. Contours represent the probability density to highlight 10% (inner contour) and 1% (outer contour) of the probe sets. Genes of interest are identified and designated by colored circles: peristin (Postn; red), plasminogen activator inhibitor-1 (PAI-1; green), osteopontin (OPN; blue), and JunB (orange). B: regions describing the joint distribution of log$_2$ fold change (M) and standard deviation (STDEV) were obtained by explicitly estimating the probability density of the STDEV. Contour lines establish a boundary to highlight the 1% outer contour, and genes of interest are identified as in A (see METHODS for details). C: a volcano plot using the contours from A was overlaid with a subset from the rat genome database of genes associated with hypertension (red), Jun (blue), and Cac2-$\beta$ (purple). D: a probability density plot using the contours from B was overlaid with the subsets described in C. ApoE, apolipoprotein E.

old was supplemented with the condition $|\log_2 M| > 0.5$ and $P < 0.2$ to eliminate small values from the analysis.

Standouts in the analysis of the array data were several genes related to extracellular matrix and tissue remodeling including OPN (Spp1), plasminogen activator inhibitor-1 (PAI-1) (Serp1), bone-expressed sequence tag 5, and periostin (Fig. 3, A and B, and Table 2). Of note was the finding that guanylyl cyclase (Guacyl1a3), a major substrate for nitric oxide-mediated vasodilation, was downregulated in the cerebral arteries from HS rats. Several other forms of Guacyl1a3 were also significantly reduced, although to a lesser degree.

Fisher exact test was used to examine the hypothesis that genes specified by the transcriptional regulatory element and rat genome databases as related to hypertension and selected transcription factors are upregulated in arteries from Dahl HS animals (Fig. 3, C and D). Differential expression-based partitions of the probe sets at 1% and 10% revealed an overrepresentation of Jun-regulated genes and changes in CREB-regulated genes that approached significance (Table 3). CREB- and Jun-regulated genes overlapped for 10 probe sets, and hypertension- and Jun-regulated genes overlapped for two probe sets. With the use of the hypertension data set, the resulting volcano plot revealed an increase in apolipoprotein E (ApoE) that was not initially significant when analyzed among the entire data set, providing a potential link to genes involved in the pathological course of hypertensive disease.

Seven genes were selected for validation and further study based on their regulation by ERK signaling and evidence for their transcriptional regulation by ANG II (7). Of these, three were confirmed by RT-qPCR (OPN, PAI-1, and JunB), and the reduction in Guacyl1a3 approached significance (Table 4). Although the other selected genes were not validated, the trends in the data suggest that variability between samples precluded significance. The confidence in the changes associated with genes that did validate is strengthened by the fact that RT-qPCR was performed using RNA sample pools that were distinct from samples used in the array analysis.

**OPN protein expression is increased in cerebral arteries from hypertensive animals.** The confirmed extent of induction of OPN mRNA levels in response to hypertensive disease was particularly intriguing due to the previous implications that OPN regulates VSMC proliferation and migration and is over-expressed during postangioplasty restenosis (15, 35, 50). In addition, OPN is a matricellular gene that is known to be transcriptionally regulated by ANG II and ERK signaling (7). To determine whether the induction of OPN mRNA levels

Table 2. Microarray analysis using Affymetrix Rat Expression Set 230A to compare gene transcripts between LS and HS Dahl S rats

<table>
<thead>
<tr>
<th>Cluster ID</th>
<th>Gene ID</th>
<th>Gene Name</th>
<th>HS Fold Change Compared with LS Fold Change</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rn.8871</td>
<td>Spp1</td>
<td>OPN</td>
<td>+6.27</td>
<td>0.0142</td>
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<tr>
<td>Rn.29367</td>
<td>Serpine1</td>
<td>PAI-1</td>
<td>+4.31</td>
<td>0.0184</td>
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<td>Rn.93714</td>
<td>Jun</td>
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<td>0.0249</td>
</tr>
<tr>
<td>Rn.4166</td>
<td>Calm1</td>
<td>Calmodulin-1</td>
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<td>0.0328</td>
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<tr>
<td>Rn.11200</td>
<td>Edg2</td>
<td>LPAI1</td>
<td>+1.92</td>
<td>0.0196</td>
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<tr>
<td>Rn.15806</td>
<td>Junb</td>
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</tr>
<tr>
<td>Rn.13882</td>
<td>Best5</td>
<td>Best5</td>
<td>+3.50</td>
<td>0.047</td>
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<tr>
<td>Rn.1974</td>
<td>Guacyl1a3</td>
<td>Guanylate cyclase 1a3</td>
<td>-2.32</td>
<td>0.0190</td>
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<tr>
<td>Rn.30516</td>
<td>Postn</td>
<td>Periostin</td>
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<td>0.058</td>
</tr>
<tr>
<td>Rn.76589</td>
<td>Add3</td>
<td>Adducin3</td>
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<td>0.159</td>
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<tr>
<td>Rn.32351</td>
<td>Apoe</td>
<td>ApoE</td>
<td>+1.91</td>
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<tr>
<td>Rn.103750</td>
<td>Fos</td>
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<td>+1.27</td>
<td>0.683</td>
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</table>

Values are ±SE; *n = 3* independent assays using arteries from 3 rats/sample. HS (hypertensive) fold change compared with LS (normotensive control) fold change and $P$ value based on $t$-test as described in METHODS. The threshold was 1.5-fold change, and $P < 0.05$. Bottom: notable genes, $P > 0.05$. OPN, osteopontin; PAI-1, plasminogen activator inhibitor-1; LPAI1, type I lysophosphatidic acid receptor; Best5, bone-expressed sequence tag 5; ApoE, apolipoprotein E.

Table 3. Association of gene expression statistics using Fisher exact test

<table>
<thead>
<tr>
<th>Database</th>
<th>10%</th>
<th>1%</th>
</tr>
</thead>
<tbody>
<tr>
<td>CREB</td>
<td>0.089</td>
<td>0.11</td>
</tr>
<tr>
<td>Jun</td>
<td>0.67</td>
<td>0.040</td>
</tr>
<tr>
<td>Elk1</td>
<td>0.16</td>
<td>1.0</td>
</tr>
<tr>
<td>Arteriosclerosis</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Cardiovascular accident</td>
<td>0.20</td>
<td>1.0</td>
</tr>
<tr>
<td>Hypertension</td>
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<td>1.0</td>
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</table>

Evaluation was based on annotation-based partitions of the Affymetrix Rat Expression Set 230A probe sets using gene identities from the transcriptional regulatory element and rat genome databases as described in METHODS. Contoured $P$ values were determined using Fisher exact test. CREB, Ca$^{2+}$/cAMP response element binding protein.
correlated with an increase in OPN protein expression, immunohistochemistry was used to detect OPN in cerebral artery cross sections from HS and LS animals. Arteries from HS animals exhibited a significantly higher level of OPN protein staining throughout the cytoplasm of the smooth muscle cell layers (Fig. 4), suggesting that the induction of OPN at the mRNA level is translated to an induction of protein expression. Together, these data suggest that hypertensive disease affects cerebral arteries through enhanced ERK signaling and smooth muscle cell proliferation, which correlates with the induction of matricellular/proliferation-related genes, particularly OPN.

ANG II exposure of cultured cVSMCs causes induction of hypertension target genes. The genes of focus, junB, PAI-1, and OPN, have been linked to ERK activation in other systems and have inducible activator protein-1 (AP-1) and/or CRE sites within their promoters. In addition, ANG II signaling has been linked to pathologies in the Dahl S hypertensive model and to ERK activation. A culture model of low-passage explanted cVSMCs was thus utilized to define the importance of ERK, JunB induction, and CREB activation in the regulation of junB, PAI-1, and OPN expression. These cells express the VSMC-specific marker calponin and exhibit the ANG II-mediated activation of ERK and CREB, as previously shown by our group and others in other cultured VSMC models (supplementary Fig. 2) (27, 38). A time course of ANG II treatment established an early transient induction of junB mRNA level and protein expression in cVSMCs (Fig. 5A). A delayed
induction of PAI-1 and OPN by ANG II was also observed, with significant increases in mRNA levels at 3 and 6 h (Fig. 5B). Of note, the induction of the OPN mRNA level was less than that observed in the Dahl S hypertensive model, likely reflective of higher basal OPN in the cVSMC model. Overall, these data show that ANG II signaling leads to the induction of the same target genes that are upregulated in the Dahl hypertensive model and that JunB upregulation by ANG II precedes the elevation of PAI-1 and OPN mRNA levels.

ANG II induction of junB, PAI-1, and OPN mRNA levels is reduced by MAPK inhibition. To determine a specific role for MAPKs in the ANG II-mediated response on gene targets, cVSMCs were pretreated with the selective MEK inhibitor U-0126 or the selective p38 inhibitor SB-203580 before ANG II treatments. MEK inhibition reduced the basal expression of junB mRNA and caused a significant inhibition of ANG II induction of both junB and PAI-1 mRNA levels (Fig. 6). In these experiments, ANG II caused an increase, although not significant, in OPN mRNA that was reduced by U-0126 and SB-203580 (Fig. 6C). A closer interpretation of the RT-qPCR data revealed a high basal expression of OPN in the presence of the DMSO vehicle, which has been observed previously in smooth muscle cells (15). Inhibition of p38 resulted in a trend toward reducing the level of ANG II induction of mRNA for all gene targets. MAPKs are thus important in ANG II signaling to gene targets related to hypertension, with a prominent role for ERK.

Silencing junB and CREB in cVSMCs using siRNA. To elucidate a role for JunB and CREB in the ANG II transcriptional regulation of gene targets (junB, PAI-1, and OPN), a siRNA transfection technique was developed for cVSMCs. Optimum transfection conditions were first determined using a positive control siRNA, targeting CypB, which achieved 70% knockdown of CypB mRNA (Fig. 7A). These conditions were used to selectively silence junB or CREB, using a nontargeting siRNA (si control) as a control for transfection. As shown in Fig. 7B, the transfection with siRNA targeting junB resulted in a significant 50% reduction in the ANG II-mediated junB transcriptional response at 30 min, which corresponded with a loss of JunB protein expression (Fig. 7C). CREB expression was not affected by ANG II treatment, but transfection with si CREB resulted in a considerable reduction in detectable CREB protein. The si CREB did not significantly inhibit the JunB transcription or its corresponding protein expression, although a slight decrease was consistently observed. Thus efficient and selective knockdown of junB and CREB can be achieved using transfection with siRNAs, and this experimental template was used to explore the effects of junB and CREB knockdown on the regulation of PAI-1 and OPN expression.

JunB and CREB have selective roles in the ANG II-mediated regulation of PAI-1 and OPN gene expression. Although silencing of junB transcript had no effect on the induction of PAI-1 by ANG II, silencing CREB transcript prevented the PAI-1 response. CREB is thus important in the ANG II-mediated signaling that results in PAI-1 transcription (Fig. 8A). PAI-1 protein levels were not assessed due to lack of suitable Western blot antibodies. Conversely, si junB significantly reduced both the basal and ANG II-stimulated transcription of OPN, whereas knockdown of CREB resulted in an unexpected induction of OPN mRNA and protein (Fig. 8, B and C).

Although both JunB and CREB are regulated through ERK signaling, the data presented here suggest that they have opposing roles related to PAI-1 and OPN gene expression, which may be important with respect to signaling during the...
development of vascular pathologies as a result of hypertension.

DISCUSSION

Chronic hypertensive disease leads to pathological changes in the arterial wall (1), and the goal of this study was to better define the alterations in signaling pathways and gene expression that underlie these pathologies in cerebral arteries. Here we present the first evidence that salt-induced malignant hypertensive disease in Dahl S animals results in the chronic activation of ERK and increased proliferation of cerebral artery smooth muscle cells. These changes correlate with increased mRNA levels of multiple genes known to be regulated by ERK signaling, most notably the AP-1 transcript junB and the matricellular genes OPN and PAI-1. These findings were extended using a culture model of cVSMCs, revealing parallels between genes altered in chronic hypertension and genes responsive to ANG II signaling. Results also establish, for the first time, selective roles for JunB and CREB in the induction of OPN and PAI-1 by ANG II.

ERK has been associated with altered gene expression in several models of vascular disease (7, 38). Our data showing elevated ERK activation in cerebral arteries from Dahl S hypertensive animals corroborate an earlier study in the same model linking the chronic activation of ERK with glomerular...
injury in Dahl S rats (18). In cultured VSMCs, ERK phosphorylation induced by mechanical injury leads to the overexpression of OPN protein, and ANG II induces PAI-1 expression in a ERK-dependent manner (8, 31). Furthermore, OPN and PAI-1 were identified in a matricellular gene cluster as highly ANG II responsive (7). Our data thus support the findings of others, implicating an important role for altered signaling among ANG II, ERK, and the expression of OPN and PAI-1.

Both OPN and PAI-1 have been previously implicated as participants in vascular remodeling. OPN is a marker of proliferation in rat VSMCs, and its expression in cultured VSMCs correlates with the downregulation of contractile proteins, suggesting a switch from the contractile to the proliferative phenotype (15, 17, 50, 56). OPN plays a role in coronary artery postangioplasty restenosis through its chemotactic properties, where it mediates smooth muscle cell extracellular matrix invasion of the intimal layer (35). Moreover, OPN has been referred to as a “delayed early gene” because of its role in cell proliferation following mitogenic stimulation in arterial smooth muscle cells (15).

PAI-1 is a potent chemotactic molecule and promotes migration through its interactions with the LDL receptor-related protein and is the primary physiological antagonist to both tissue and urokinase plasminogen activators (14). Increased levels of PAI-1 have also been detected in vascular lesions induced by atherosclerosis or balloon catheter injury (11, 46). PAI-1 promotes proliferation and protects arterial smooth muscle cells from apoptosis, thus there is an increased likelihood of hyperplasia following angioplasty when PAI-1 expression is abnormally elevated (9). Our findings extend these conclusions to cerebral arteries, supporting a role for OPN and PAI-1 in the proliferative changes observed in cerebral arteries of hypertensive rats.

Although not observed previously in cerebral arteries, an induction of OPN and PAI-1 has been observed in other models of hypertensive disease. An array analysis to detect changes in genes from the left ventricle of the heart in the spontaneously hypertensive rat (SHR) model found an overexpression of OPN, PAI-1, fibronectin-1, ApoE, and cathepsin K (45). Upregulation of OPN and PAI-1 was also detected in kidneys from stroke-prone SHR with malignant hypertension (33). PAI-1 is elevated in the plasma of chronically hypertensive patients, and populations expressing the 4G/4G PAI-1 promoter polymorphism, which confers increased basal PAI-1 transcription, exhibit an increased risk of developing arterial hypertension (29). These data suggest that our observed changes in cerebral artery gene expression are not restricted to the etiology of the hypertensive disease or to arterial tissue and that the Dahl model may be useful for deciphering the consequences of hypertension on cerebral artery function in human disease.

In this study, several AP-1 family member transcripts were variably elevated in cerebral arteries from hypertensive animals including c-jun and c-fos; however, junB transcript was consistently upregulated. JunB has been shown to dimerize and transactivate AP-1 genes but can also act as a negative regulator of c-Jun for some genes (10, 37). In cultured VSMCs, JunB has been detected in active AP-1 complexes in cells responding to receptor tyrosine kinase and G protein-coupled receptor agonists, and its induction is related to the production of reactive oxygen species (41). These findings together with our findings that Jun- and CREB-regulated genes are overrepresented in cerebral arteries from hypertensive animals indicate that signaling through AP-1 and CRE promoter elements may be particularly relevant in the arterial remodeling response.

The early onset and transient nature of JunB induction by ANG II in cultured VSMCs is consistent with the activation of other AP-1 family members responding to mitogen stimulation (26). The delayed stable induction of OPN and PAI-1 transcript by ANG II also agrees with previous findings that prolonged ERK leads to their induction (8, 15). Persistent upregulation of junB in malignant hypertension may thus indicate a defect in the regulation of junB that contributes to remodeling and involves OPN and PAI-1 expression.

Our RNA silencing experiments indicate that JunB is important for the ANG II-mediated upregulation of OPN and that CREB is not required for junB induction but acts as a negative regulator of OPN expression. Although the junB promoter contains a half-site CRE, it also contains both AP-1 and serum response element sites that likely compensate for the CRE through their stimulation by ANG II in our model (28, 53). Two AP-1 sites and a CRE have been identified within the OPN promoter, and a mutational analysis suggests that the AP-1 sites are the most critical (23). The CREB findings were at first unexpected since others have found that dominant-negative CREB reduces OPN expression, and CREB has been associated with c-Fos binding to AP-1 sites on the OPN promoter (23). The differences may be agonist specific, and because ANG II initiates transactivation through both CREB and AP-1, our data suggest that not only AP-1 activity predominates in induction of OPN but that CREB activity interferes with the AP-1 induction.

Contrary to the OPN results, silencing of junB had no effect on the ANG II-mediated induction of PAI-1 mRNA levels, whereas silencing of CREB prevented PAI-1 induction. The PAI-1 promoter contains at least two AP-1 sites, and the CREB target database identifies two half-site CREs. AP-1 has been previously shown in fibroblasts to be important in PAI-1 induction through ERK (25), thus the lack of effect of silencing junB in our studies suggests an alternate AP-1 member may be important for PAI-1 induction by ANG II or that the AP-1 effect is cell-type specific. Although implicated by promoter elements, our data are the first to define a specific role for CREB in PAI-1 induction by ANG II. Taken together, our data support a model for gene expression in cerebral arteries following salt-induced hypertensive disease that includes the upregulation of matricellular genes that are universally regu-

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**Fig. 9.** Proposed model for regulation of gene expression in cerebral arteries responding to hypertension. Dysregulated ANG II signaling activates a signaling cascade that includes activation of ERK and selective regulation of gene expression through JunB and CREB.
lated by ANG II and ERK and selectively regulated by JunB and CREB (Fig. 9).

The question arises as to whether the upregulated genes in the hypertensive model contribute to the development of arterial remodeling or are a compensatory healing response to arterial injury. The genes pursued in our study have been linked to both induction of hypertrophy and hyperplasia as well as to healing responses. One possibility is that the vessel wall becomes locked into an endless cycle of mechanical, oxidative, and inflammatory stresses, all linked to compensatory proliferation. Defining a specific role for the genes identified in each of these processes will be facilitated using selective knockdown models to dissect the role of these genes in the context of a hypertensive environment.

This study is the first to use microarray analysis to examine gene expression in intact cerebral vessels from Dahl S malignant hypertensive animals. Our results substantiate previous data and offer new findings related to changes in gene expression induced by hypertension. These data also illustrate how array analyses, especially when evaluated using the Fisher exact test, can be useful tools for assessing sets of genes altered in disease. Our data using cultured VSMCs to elucidate a role for JunB and CREB in the regulation of PAI-1 and OPN give us greater insight into the molecular mechanisms that underlie the overexpression of these genes. In addition to revealing a role for JunB and CREB in the development of hypertensive disease, these results will serve as a springboard of information to explore other sets of genes that contribute to the manifestations of cardiovascular disease in the Dahl S and other models of hypertension.

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