Patterns of Gene Expression Differentially Regulated by Platelet-derived Growth Factor and Hypertrophic Stimuli in Vascular Smooth Muscle Cells

MARKERS FOR PHENOTYPIC MODULATION AND RESPONSE TO INJURY*

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In vascular smooth muscle cells (VSMC), platelet-derived growth factor (PDGF) suppresses expression of multiple smooth muscle contractile proteins, useful markers of differentiation. Conversely, hypertrophic agents induce expression of these genes. The goal of this study was to employ genomic approaches to identify classes of genes differentially regulated by PDGF and hypertrophic stimuli. Changes in gene expression were determined using Affymetrix RAE-230 GeneChips in rat aortic VSMC stimulated with PDGF. For comparison with a model hypertrophic stimulus, a microarray was performed with VSMC stably expressing constitutively active Gα16, which strongly induces smooth muscle marker expression. We identified 75 genes whose expression was increased by exposure to PDGF and decreased by expression of Gα16 and 97 genes whose expression was decreased by PDGF and increased by Gα16. These genes included many smooth muscle-specific proteins; several extracellular matrix, cytoskeletal, and chemotaxis-related proteins; cell signaling molecules; and transcription factors. Changes in gene expression for many of these were confirmed by PCR or immunoblotting. The contribution of signaling pathways activated by PDGF to the gene expression profile was examined in VSMC stably expressing gain-of-function H-Ras or myristoylated Akt. Among the genes that were confirmed to be differentially regulated were CCAAT/enhancer-binding protein δ, versican, and nexilin. All of these genes also had altered expression in injured aortas, consistent with a role for PDGF in the response of injured VSMC. These data indicate that genes that are differentially regulated by PDGF and hypertrophic stimuli may represent families of genes and potentially be biomarkers for vascular injury.

Vascular smooth muscle cells (VSMC) are the contractile component of blood vessels, and express a set of smooth muscle (SM)-specific genes, which are characteristic of their contractile, differentiated phenotype (1). In contrast to skeletal and cardiac myocytes, VSMC do not terminally differentiate, and they undergo phenotypic modulation in vivo and in vitro in response to environmental signals (2, 3). This process involves changes in gene expression, which convert these cells from a nonproliferative contractile phenotype to a proliferating synthetic one (2–5). Indeed, a well defined characteristic of vascular occlusive disease, arterial interventions in response to disease, and in vitro subculturing of SMC is the phenotypic modulation of SMC from a normally quiescent, contractile state to one of increased growth, migration, and matrix synthesis. Formation of neointima after vascular injury is largely a consequence of dedifferentiation, proliferation, and migration of medial SMC (6, 7). During injury to the blood vessels, PDGF assists this process by triggering phenotypic changes in VSMC, which are presumably mediated through changes in patterns of gene expression (8). In cultured VSMC, PDGF increases cell proliferation (9). In contrast, vasoconstrictors such as vasopressin (AVP) and angiotensin II promote increased contractility and induce hypertrophy (10–12). Previous studies from our laboratory have demonstrated that these two classes of agents have opposing effects on expression of smooth muscle markers (SM markers), specifically contractile proteins such as smooth muscle α-actin and SM22α (13–15), with AVP increasing and PDGF suppressing transcription of these genes.

The effects on SMC gene expression are mediated through distinct postreceptor signaling pathways. PDGF-induced suppression is mediated through activation of Ras and phosphatidylinositol 3-kinase/Akt pathways (14, 15), whereas AVP-mediated regulation of SM markers involves activation of heterotrimeric G proteins of the Gα family, leading to stimulation of c-Jun N-terminal kinase and p38 mitogen-activated protein kinases (12, 16). However, the phenotypic changes mediated by these agents are probably not exclusively mediated through changes in expression of contractile proteins but will instead be a consequence of more global changes in gene expression. Dedifferentiated VSMC have increased production of matrix proteins and metalloproteinases, which are critical for their proliferative and migratory properties (17, 18). Additionally, changes in the local environment of the cells or cell-matrix interactions can affect the phenotypic state of VSMC (19, 20). With the advent of microarray technology, it is feasible to

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The abbreviations used are: VSMC, vascular smooth muscle cells; SM, smooth muscle; AVP, arginine vasopressin; PDGF, platelet-derived growth factor; SRP, serum response factor; DAPI, 4,6-diamidino-2-phenylindole; SMC, smooth muscle cell(s); PBS, phosphate-buffered saline; C/EBPδ, CCAAT/enhancer-binding protein δ; Gro, growth-related oncogene 1; EST, expressed sequence tag.

19966 This paper is available on line at http://www.jbc.org
| GenBank™ Name | Description |
|---------------|-------------|
| AA892254 Add3 | Adducin 3, γ |
| AA894279 Caa2 | CCA2 protein |
| AF053312 Scya20 | Small inducible cytokine subfamily A20 |
| AF072929 Cspg2 | Chondroitin sulfate proteoglycan 2 (versican) |
| AF202115 Cp | Ceruloplasmin |
| AF245172 Gda | Guanine deaminase |
| AF411318 MT1a | Metallothionein |
| AI011713 | ESTs, moderately similar to I60307 β-galactosidase, α peptide |
| AI176126 | ESTs |
| AI233355 | ESTs |
| AI411366 | ESTs, weakly similar to mouse inward rectifier potassium channel 16 |
| AI548856 | Tug4 |
| AJ245974 | partial mRNA for BM1k MHC class Iβ antigen, strain SHR |
| AW319009 | ESTs, weakly similar to human fibronectin leucine-rich transmembrane protein 2 |
| AY902609 Pgy1 | P-glycoprotein/multidrug resistance 1 |
| BE113700 Prx2 | ESTs, highly similar to Prx-2, weakly similar to PMX1_MOUSE paired mesoderm homeobox protein 1 (PRX-1) |
| BE120953 LOC368009 | ESTs, weakly similar to T46612 multi-PDZ domain protein 1-rat |
| BF281299 | Odc1 |
| BF285344 | ESTs, highly similar to cardiac abnormality/abnormal facies (CATCH22), microdeletion syndrome |
| BF289368 | Lbp |
| BF400209 | ESTs, highly similar to hypothetical protein MGC13017 |
| BF417784 | Cebpd |
| BF419200 | C/EBPβ |
| BG374178 | ESTs, similar to mouse mast cell growth factor scf mRNA for stem cell factor KL-2 |
| BG375352 | ESTs, weakly similar to CAH5_RAT carbonic anhydrase VA (carbonate dehydratase VA) |
| BG378985 | Sdn5 |
| BG379294 | Glu1 |
| BG379680 | ESTs, weakly similar to PCO1_RAT procollagen C-protease enhancer protein precursor (type I procollagen COOH-terminal proteinase enhancer) (type 1 procollagen C-protease enhancer protein) |
| BG384599 | ESTs, Highly similar to 0812252A tubulin α |
| BG399592 | ESTs, similar to hypothetical protein DIERd497e |
| BG295559 | Mgst2 |
| BG299208 | Gda |
| BG299558 | ESTs, highly similar to A57501 uridine phosphorylase (EC 2.4.2.3) |
| BG296835 | ESTs, similar to CDK104 |
| BG303340 | ESTs, weakly similar to mouse lysoyl oxidase-like 4 |
| BG303596 | ESTs, similar to mouse elastin microfibril interfacer-1; weakly similar to 1917150A collagen:SUBUNIT IX ISOTYPE VIII |
| BM385331 | Mt2 |
| BM385924 | ESTs, similar to mouse annexin A6 |
| BM390600 | ESTs, similar to ATPase, vacuolar proton pump |
| NM_012580 | Hmxox1 |
| NM_012733 | Rbp1 |
| NM_012792 | Fmo1 |
| NM_012935 | Cryab |
| NM_012953 | Fosl1 |
| NM_013034 | Slc6a4 |
| NM_013151 | Plat |
| NM_016994 | C3 |
| NM_017074 | Cth |
| NM_017154 | Xdh |
| NM_017172 | Zfp681 |
| NM_017233 | Heme oxygenase |
| NM_017233 | Retinol-binding protein 1 |
| NM_017292 | Flavin-containing monoxygenase 1 |
| NM_012935 | Crystallin, α polypeptide 2 |
| NM_012953 | Fos-like antigen 1 |
| NM_013034 | Solute carrier family 6 (neurotransmitter transporter, serotonin), member 4 |
| NM_013151 | Plasminogen activator, tissue |
| NM_013854 | Gro1 |
| NM_013125 | Udgh |
| NM_013509 | Gsta1 |
| NM_013510 | Scya2 |
| NM_013511 | Spin2c |
| NM_013512 | Nb11 |
| NM_013513 | Slc29a1 |
| NM_013832 | Lgals3 |
| NM_013832 | Lectin, galactose binding, soluble 3 |
| NM_013832 | Cdo1 |
| NM_013832 | Cytoplasmic cysteine dioxygenase 1 |
| NM_013832 | Avpr1a |
| NM_013832 | Arginine vasopressin receptor 1A |
| NM_013832 | Rdc1 |
| NM_013832 | Chemokine orphan receptor 1 |
| NM_013832 | Enpp1 |
| NM_013832 | Ectonucleotide pyrophosphatase/phosphodiesterase 1 |
| NM_013832 | Timp1 |
| NM_013832 | Oxidized low density lipoprotein (lectin-like) receptor 1 |
| NM_013832 | Expi |
| NM_013832 | Extracellular proteinase inhibitor |
| NM_013832 | Gbp2 |
| NM_013832 | Guanylate-binding protein 2, interferon-inducible |
| NM_013832 | Mgst1 |
| NM_013832 | Microsomal glutathione S-transferase 1 |
| NM_013832 | Akr7a2 |
| NM_013832 | Aldo-keto reductase family 7, member A2 (aflatoxin aldehyde reductase) |
### TABLE II

Genes differentially regulated by exposure to PDGF and expression of G16Q212L: Genes that are decreased with PDGF and increased with Ga16 (97 genes)

| GenBank™ | Name | Description |
|----------|------|-------------|
| AA799392 | LOC246172 | ESTs, weakly similar to mouse sarcospan; weakly similar to mouse putative membrane protein (KRA) |
| AA799423 | Fh32 | Four and a half LIM domains 2 |
| AA800031 | Thbd | ESTs, similar to mouse thrombomodulin |
| AA818521 | LOC246172 | Nexilin |
| AA818521 | AA851369 | ESTs, similar to mouse solute carrier family 31, member 1 |
| AA851385 | AA859497 | ESTs, weakly similar to mouse isoleucin tRNA synthetase |
| AA866443 | AA866443 | ESTs, protease, serine 35 (Prss35) |
| AA926305 | AA926305 | ESTs, moderately similar to hypothetical protein FLJ11127, similar to mouse solute carrier family 6 |
| AA939694 | Myh11 | ESTs, similar to mouse Septin 8 |
| AA946388 | AA946388 | Myosin heavy chain 11 |
| AA969790 | AA969790 | ESTs, weakly similar to FMOD_RAT Fibromodulin precursor (FM), similar to osteoglycin |
| AB020967 | LOC246273 | Kinase, similar to tribbles homolog 3 (Drosophila) (Trib3) |
| AF228737 | Pfn2 | Profilin II |
| AI008883 | Fkh3 | ESTs, similar to mouse Forkhead homolog 3 (Fkh3) |
| AI009094 | AI044427 | ESTs, highly similar to dystrophia myotonica kinase, B15 |
| AI045848 | AI103572 | ESTs, weakly similar to pervin. Similar to mourse supervillin |
| AI104213 | AI117651 | ESTs, similar to mouse Ras homolog member E |
| AI117503 | AI117503 | ESTs, highly similar to DJB4_MOUSE DnaJ homolog subfamily B member 4 |
| AI175728 | AI176360 | ESTs, highly similar to palmdelphin |
| AI177028 | AI177028 | ESTs, weakly similar to mouse tubby like protein 4 (Tulp4) |
| AI180400 | AI180400 | ESTs, weakly similar to mouse retinoic acid-induced 14 |
| AI234852 | AI407483 | ESTs, weakly similar to human estrogen receptor-1 |
| AI234852 | AI407483 | ESTs, weakly similar to human estrogen receptor-1 |
| AI411574 | AI142159 | ESTs, similar to mouse insulin receptor |
| AI412159 | AI602501 | ESTs, highly similar to TES_MOUSE testin (TES1/TES2) |
| AI639342 | AI639342 | ESTs, weakly similar to PGS1_RAT Bone/cartilage proteoglycan I precursor (Biglycan) (PG-51) |
| AI639412 | AI640475 | ESTs, weakly similar to mouse CUG triplet repeat RNA binding protein 2 (Cugbp2) |
| AI651204 | AW253242 | ESTs, similar to maguin (membrane-associated guanylate kinase interacting protein-like) protein |
| AW253242 | Adarbl | ESTs, similar to mouse adenosine deaminase |
| AW522471 | AW522471 | ESTs, similar to mouse zinc finger DHHC domain |
| AW532489 | AW918352 | ESTs, similar to mouse kinesin heavy chain |
| AW918614 | BE095833 | ESTs, similar to spectrin 2 (Spnb2) |
| BE095833 | BE095833 | ESTs, weakly similar to LEC9_RAT galectin-9 (36-kDa β-galactoside-binding lectin) (urate transporter/channel) (UAT) |
| BE105712 | BE109102 | ESTs, similar to mouse CTP SYNTASE |
| BE111512 | BE111512 | ESTs, similar to mouse CTP SYNTASE |
| BF283150 | BF283150 | ESTs, similar to mouse phosphoribosyl pyrophosphate synthetase 2 (Prps2) |
| BF284817 | BF285649 | ESTs, ribosome-binding protein, p34 |
| BF288556 | BF416285 | ESTs, similar to disintegrin-like metalloprotease with thrombospondin like motive |
| BG374290 | BG374290 | Microtubule-associated protein 2 |
| BG377024 | BG377024 | ESTs, unknown serine-rich protein |
| BG380693 | BG380693 | ESTs, highly similar to P5CS_MOUSE δ 1-pyrroline-5-carboxylate synthetase (P5CS) |
| BG663107 | BG663107 | A kinase (PRKA) anchor protein (gravin) 12 |
| BG666221 | BG666221 | Thbd |
| BG666306 | BI274428 | ESTs, weakly similar to WASL_RAT neural Wiskott-Aldrich syndrome protein |
| BI274590 | BI274590 | ESTs, weakly similar to four and a half LIM domains 2 |
| BI276990 | BI276990 | ESTs, similar to mouse FL 1-LRR (leucine-rich repeat)-associated protein-1 |
| BI277545 | BI277545 | Rattus norvegicus type 2X myosin heavy chain (MYHC) mRNA, partial cds |
| BI279044 | BI279044 | ESTs, highly similar to MLRN_RAT myosin regulatory light chain 2, smooth muscle isoform (myosin RLC) |
| BI284461 | BI284461 | ESTs, similar to mousev-maf oncogene family protein K |
| BI284480 | BI284480 | ESTs, similar to scellin. Similar to LIM domain only 7 |
| BI286205 | BI286205 | ESTs, similar to mouse SM leiromodin 1 (Lmod1) |
| BI288556 | BI288556 | Nesprin-1 |
assess global changes in gene expression to begin to define the repertoire of proteins regulating SMC phenotype. For example, gene array analysis has been used to identify changes in gene expression in angiotensin II-stimulated SMC in the presence and absence of inhibitors of signaling pathways (22), between neointimal and aortic SMC (23). However, a comprehensive analysis of changes in gene expression induced by hypertrophic stimuli compared with PDGF has yet to be investigated.

The goal of this study, therefore, was to use microarray analysis to define subsets of genes that are regulated in opposing directions by PDGF compared with hypertrophic stimuli. By using cell lines stably expressing constitutively active forms of either H-Ras or Akt, downstream effectors of PDGF signaling, we were able to dissect out the contributions of these individual pathways to the regulation of specific genes. We report here a novel set of genes, which are involved in modulation of SMC phenotype during balloon injury.

**EXPERIMENTAL PROCEDURES**

**Materials**—Trypsin/EDTA and Eagle’s minimal essential medium were from Invitrogen. Antibodies to C/EBPβ, GKLF, and alkaline phosphatase-labeled IgGs were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibody to heme oxygenase-1 was from StressGen Biotech, antibody to nexilin was from BD Biosciences, antibody to ezrin was from Upstate Biotechnology, Inc. (Lake Placid, NY), and antibody to versican was from Affinity Bioreagents (Golden, CO).

**Cell Culture and Stable Transformations**—Rat aortic VSMC were isolated and cultured as previously described in detail (25). Cells (passages 4–12) were grown in Eagle’s minimal essential medium containing 1 mM l-glutamine, 2 ng/ml NaHCO₃, 100 international units/ml penicillin, 100 μg/ml streptomycin, and 10% (v/v) fetal calf serum at 37 °C in a humidified airCO₂ (19:1) atmosphere. For the stable transfections of VSMC with H-Ras, Myr-Akt, and Gαt₁α, cDNAs encoding either Akt containing the Src myristoylation sequence (15), the GTPase-deficient, constitutively active forms of the human T24 H-Ras with V12 (G12V) mutation (14), or GTPase-deficient Gαt₁α (αt₁αQ212L) (12) were packaged into a replication-defective retrovirus by the procedure described previously (26). Secreted retrovirus was supplemented with Polybrene (8 μg/ml), filtered (0.45 μm), and incubated with VSMC for 48 h. Cells expressing myristoylated Akt, H-Ras, or Gαt₁α were selected by culturing them in medium containing G418 (500 μg/ml). Individual clones were screened by immunoblotting with corresponding antibodies.

**Affymetrix Microarray Analysis**—VSMC stimulated with AVP or PDGF for 6 or 24 h and VSMC stably expressing H-Ras (14), Myr-Akt (15), or Gαt₁α (12) along with their control cells were subjected to total RNA isolation by using RNaseasy miniprep (Qiagen). Labeling of the probes for microarray was performed as described previously (27). Briefly, cDNA was synthesized by using the Superscript Choice System (Invitrogen) and purified by phenol/chloroform extraction and ethanol precipitation. In vitro transcription of labeled cRNA was performed from purified cDNA using a Bioarray high yield RNA transcript labeling kit (Enzo Diagnostics) in the presence of biotinylated UTP and CTP. Labeled cRNA was purified and then fragmented using fragmentation buffer (40 ×) Tris acetate, pH 8.1, 10 μM ROAc, 30 μM MgOAc). Hybridization of the probes to the Affymetrix RAE-230 rat Genechip was performed according to the manufacturer’s recommendations (Affymetrix, Santa Clara, CA) at the University of Colorado Health Sciences Center Microarray Core Facility. DNA chips were scanned (6-μm resolution) with an HP Gene Array scanner, and results were analyzed with GeneChip Suite 4.0 analysis software from Affymetrix and GeneSpring software from Silicon Genetics.

**Transient Transfection**—For transient transfections measuring promoter activity, plasmid encoding cytomegalovirus (CMV) promoter-driven luciferase reporter plasmid (PRL-Luc, Promega, Madison, WI) was co-transfected with a Renilla luciferase reporter plasmid (pRL-TK) at a ratio of 1:2. Forty-eight hours after transfection, VSMC were transiently transfected with AVP or PDGF for 6 or 24 h and VSMC stably expressing H-Ras (14), Myr-Akt (15), or Gαt₁α (12) along with their control cells were subjected to total RNA isolation by using RNaseasy miniprep (Qiagen). Labeling of the probes for microarray was performed as described previously (27). Briefly, cDNA was synthesized by using the Superscript Choice System (Invitrogen) and purified by phenol/chloroform extraction and ethanol precipitation. In vitro transcription of labeled cRNA was performed from purified cDNA using a Bioarray high yield RNA transcript labeling kit (Enzo Diagnostics) in the presence of biotinylated UTP and CTP. Labeled cRNA was purified and then fragmented using fragmentation buffer (40 ×) Tris acetate, pH 8.1, 10 μM ROAc, 30 μM MgOAc). Hybridization of the probes to the Affymetrix RAE-230 rat Genechip was performed according to the manufacturer’s recommendations (Affymetrix, Santa Clara, CA) at the University of Colorado Health Sciences Center Microarray Core Facility. DNA chips were scanned (6-μm resolution) with an HP Gene Array scanner, and results were analyzed with GeneChip Suite 4.0 analysis software from Affymetrix and GeneSpring software from Silicon Genetics.
Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 50 mM sodium fluoride, 2 mM EDTA 200 μM Na2VO4, and protease inhibitors (Sigma). Solubilized proteins were centrifuged at 14,000 g at 4 °C. Membranes were washed in TTBS, and bound proteins were visualized with alkaline phosphatase-coupled second-aries 12–16 h at 4 °C. Membranes were washed in TTBS, and bound

TABLE III
Regulation of smooth muscle-specific gene expression in VSMC

| GenBankk | Name        | Description                                      | 6 h   | 24 h   | H-Ras | Akt | G0/16 |
|----------|-------------|--------------------------------------------------|-------|-------|------|-----|-------|
| AA946388 | Myh11       | Myosin heavy chain 11                            | −1.9  | −2.3  | −1.9 | −3  | +2.6  |
| AF372126 | Tpm1        | Troponymisin 1, α                               | −2.3  | −5.7  | −2.5 |     |       |
| BI277545 | Myhc        | Myosin heavy chain type 2X                       | −4.3  | −3.5  | +20  |     |       |
| BI279044 | Myrlc       | Sim. myosin regulatory chain 2, SM isoform        | −2.8  | −7.8  | −3.7 | +3.2|       |
| BI292072 | Acta2       | Smooth muscle α-actin                            | −1.3  | −2   | 10.6 | −2  | +1.7  |
| BM392106 | Cald1       | Caldesmon 1                                      | −1.7  | −7   | −2   |     |       |
| NM-031549| Sm22        | Transgelin (smooth muscle 22 protein)             | −1.6  | −34  | −1.3 | +1.6|       |

Decreases are shown with minus signs, and increases are shown as plus signs. Values represent -fold changes over appropriate controls.

TABLE IV
Regulation of gene expression of several transcription factors in VSMC

| GenBankk | Name        | Description                                      | 6 h   | 24 h   | AVP  | H-Ras | Akt | G0/16 |
|----------|-------------|--------------------------------------------------|-------|-------|------|------|-----|-------|
| BA190034 | Isg12b      | ESTs, Isg12b, interferon, α-inducible protein 27-like | +2.5  | +52   | +7.5 | +3   |     |       |
| AI008883 | Fkh3        | ESTs, similar to mouse Forkhead homolog 3 (Fkh3)  | −3    | −5    | +1.7 |     |     |       |
| AI177425 | Tp53        | ESTs, similar to zinc finger protein ZNF216       | −1.7  |       |     |     |     |       |
| AI178527 | Dsb1        | ESTs, similar to mouse DEAD box polypeptide 21 (ddx21) | −1.6  | +5.7  | +2.8 |     |     |       |
| AI234852 | Sm22        | ESTs, similar to mouse retinoic acid induced 14   | −1.9  | −1.2  | +1.4 |     |     |       |
| AI579023 | HAC1        | ESTs, highly similar to nucleolar protein family A, member 1 | +2    | −2.8  | +1.7 | −5.7|     |       |
| BM387900 | Pxv2        | ESTs, homeobox protein MhoX (homeobox protein-2) (Rhox) | 2     | −8.6  |     |     |     |       |
| BP281131 | Gerp95      | GERp95                                           | −2.6  |       |     |     |     |       |
| BF145939 | cFos        | c-Fos                                             | −1.6  | −2.8  | +5.5 | −8.6| −5.7|       |
| BF184394 | Thrh        | Thyrotropin-releasing hormone receptor             | +2    |       |     |     |     |       |
| BF194200 | Cebpd       | C/EBP                                             | +2    | +1.1  | −3.2 | +2.5| −18  |       |
| BI282755 | Sla2a       | Solute carrier family 2, member 1                 | +1.7  | +2.6  |     |     |     |       |
| BI284904 | Grina       | NMDA receptor glutamate-binding chain             | +2    |       |     |     |     |       |
| BS251459 | Sordi       | Sorbilin dehydrogenase                           | +2.2  | −2.5  | −1.4 |     |     |       |
| BM385790 | GkLF        | ESTs, highly similar to Kruppel-like factor 4 (gut) and 2 (lung) | −2.2  | −2.6  |     |     |     |       |
| NM_012555 | Ets1        | Ets avian erythroblastic virus E2 oncogene homolog 1 | −2.6  | −1.7  | −1.4 | +2.8 | +1.9 |       |
| NM_012603 | Myc        | v-Myc avian myelocytomatosis viral oncogene homolog | −1.7  | +5.3  | +4.3 | +28  | −147 |       |
| NM_012733 | Rp1        | Retino-binding protein 1                          | +5.3  | +4.3  |     |     |     |       |
| NM_013055 | Zpk        | Zipper (leucine) protein kinase                   | +1.9  |       |     |     |     |       |
| NM_017172 | Zfp3611     | Zinc finger protein 36, C3H type-like 1           | −3.2  | +1.7  | −2.6 | +1.9 | −2.2 |       |
| NM_024127 | Gadd45a     | Growth arrest and DNA damage-inducible 45 α       | +2.8  | +6    | −2  |     |     |       |

Decreases are shown with minus signs, and increases are shown as plus signs. Values represent -fold changes over appropriate controls.

RESULTS
Changes in Gene Expression Induced by PDGF in Rat VSMC—The goals of this study were to compare the effects of PDGF and hypertrophic stimuli on patterns of gene expression in VSMC. Microarray analysis was performed as described under "Experimental Procedures," using the Affymetrix Rat PDGF and hypertrophic stimuli on patterns of gene expression in VSMC. Microarray analysis was performed as described under “Experimental Procedures,” using the Affymetrix Rat
incubated for the same length of time in media containing 0.2% fetal calf serum. Stimulation with PDGF increased 215 (p < 0.0001) or 81 (2-fold) genes at 6 h and 56 (p < 0.0001) or 48 (2-fold) genes at 24 h compared with the appropriate control. Among these genes, only four were increased at both time points, whereas 17 (p < 0.0001) or 2 (2-fold) of the genes increased at 6 h, were down-regulated at 24 h of PDGF stimulation. In contrast, PDGF stimulation decreased 284 (p < 0.0001) or 106 (2-fold) genes at 6 h and 143 (p < 0.0001) or 44 genes (2-fold) genes after 24-h stimulation. 27 (p < 0.0001) or 106 (2-fold) genes at 6 h and 143 (p < 0.0001) or 44 genes (2-fold) genes at 24 h were induced by PDGF stimulation to the changes seen in the global gene expression profile induced by PDGF, we compared the increases and decreases induced by PDGF stimulation to the changes seen in VSMC stably expressing H-Ras (14) or constitutively active Myr-Akt (15) (see Supplementary Tables A and B). In general, VSMC stably expressing H-Ras (14) or constitutively active G\(_{\text{q}}\)-nulls/Akt pathways (14, 15). To investigate the contribution of these pathways to the changes in the global gene expression by activation of both the H-Ras and phosphatidylinositol 3-kinase/Akt pathways (14, 15). To investigate the contribution of these pathways to the changes induced by PDGF stimulation to the changes seen in VSMC stably expressing H-Ras (14) or constitutively active Myr-Akt pathways (14, 15). To investigate the contribution of these pathways to the changes in the global gene expression profile induced by PDGF, we compared the increases and decreases induced by PDGF stimulation to the changes seen in VSMC stably expressing H-Ras (14) or constitutively active Myr-Akt (15) (see Supplementary Tables A and B). In general, VSMC stably expressing H-Ras had similar changes as the ones stimulated with PDGF for 6 h. On the other hand, VSMC stably expressing Myr-Akt had the gene expression profile similar to the cells expressing PDGF for 24 h.

Changes in Gene Expression Induced by Hypertrophic Stimuli—We have previously demonstrated that AVP signaling in VSMC is mediated through coupling of the V1 receptor to G\(_{\alpha_q}\), a member of the family of trimeric G proteins. This was assessed by transfection of constitutively active G-proteins, in which the intrinsic GTPase activity has been abolished by a point mutation. Since the constitutively active form of G\(_{\alpha_q}\) (\(\alpha_q\)Q299L) was cytosolic in VSMC, expression of another member of the G\(_{\alpha}\) family, G\(_{\alpha_{16}}\) (\(\alpha_{16}\)Q212L) was used instead (12). Stable expression of G\(_{\alpha_{16}}\) in VSMC gives a stronger, more consistent hypertrophic response compared with VSMC exposed to vasoconstrictors such as AVP (12). To compare the effects of PDGF and its downstream signaling pathways with changes in gene expression induced by hypertrophic stimuli, we examined VSMC stimulated with AVP for 6 or 24 h, and VSMC stably expressing constitutively active G\(_{\alpha_{16}}\) (G\(_{\alpha_{16}}\)Q212L). Similar to the study by Campos et al. (21) with angiotensin II, VSMC stimulated with AVP for either 6 or 24 h induced minimal changes in gene expression. AVP stimulation increased 11 (p < 0.0001; 3 of 4) or 3 (2-fold; 3 of 4) of the genes that were decreased by 24-h PDGF stimulation. However, of the genes increased by PDGF, only two (p < 0.0001; 3 of 4) were decreased by AVP. Whereas expression of G\(_{\alpha_{16}}\) caused large scale changes in gene expression, we sought to focus on genes that were differentially regulated by G\(_{\alpha_{16}}\) compared with PDGF. Tables I and II are lists of genes, regulated in opposing directions by PDGF at either time point compared with G\(_{\alpha_{16}}\). We identified 75 genes whose expression was increased by PDGF at either time point and decreased by expression of G\(_{\alpha_{16}}\) (Table I) as well as 97 genes whose expression was decreased by PDGF at either time point and increased by expression of G\(_{\alpha_{16}}\) (Table II).

Characterization and Verification of the Genes—As expected, a number of the genes differentially regulated by PDGF and G\(_{\alpha_{16}}\) included contractile proteins, which have been used as markers for differentiated VSMC. These included SM22a, SM-myosin heavy chain, tropomyosin 1\(_{\alpha}\), and caldesmon 1, which was only down-regulated by expression of H-Ras. In addition to SM-specific genes, a number of transcription factors were differentially regulated by PDGF and G\(_{\alpha_{16}}\). These included C/EBP\(\delta\), retinol-binding protein-1, and Prx-2, which were induced by PDGF in an Akt-dependent manner, and decreased by expression of G\(_{\alpha_{16}}\) (Table IV). As verification of the microarray data, we observed increased expression of C/EBP\(\delta\) by Western blotting in VSMC stimulated with PDGF for 6, 24, or 48 h as well as VSMC stably expressing H-Ras, and Myr-Akt, compared with their appropriate controls (Fig. 1A). In contrast, but consistent with its role in opposing PDGF stimulation, expression of G\(_{\alpha_{16}}\) decreased the expression of C/EBP\(\delta\) (Fig. 1B). To determine a functional link between increased C/EBP\(\delta\) expression and regulation of SMC-specific gene expression, the effects of overexpressing C/EBP\(\delta\) on SM \(\alpha\)-actin promoter activity were determined. Overexpression of C/EBP\(\delta\) decreased basal SM \(\alpha\)-actin promoter activity and markedly inhibited induction seen with AVP in VSMC (Fig. 1C), suggesting its involvement in regulation of SMC-specific gene expression. In contrast to C/EBP\(\delta\), PDGF stimulation
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### TABLE V

| GenBank™ Name | Description | 6 h | 24 h | H-Ras | Akt | Go_{16} |
|---------------|-------------|-----|------|-------|-----|---------|
| AA800031 PhI2 | Four and a half LIM domain 2 | -2  | -4.3 | +1.5  | +1.7|
| AA851304 Vl12 | Villin2 (Ezrin) | -1.7 | +4.6 | -2.2  |
| AA894279 Add3 | Adducin 3, gamma | +2.8 | +6   | -4    |
| AA047385 Myh11 | Myosin heavy chain 11 | -1.9 | -1.7 | -1.9  | -3  | +2.6   |
| AF053361 Tpm3 | Nonmuscle tropomyosin 3, γ | +1.4 | -1.9 | -1.9  |
| AF056034 LOC246172 | Nexilin | -2.5 | -2.3 | -12   | -4.6| +3.2   |
| AF072892 Csgp2 | Versican (chondroitin sulfate proteoglycan 2) | +1.7 | +7   | +5.3  | -4  |
| AF084544 Tpm1 | Tropomyosin 1, α | -2.3 | -5.7 | -2.5  |
| AI204123 Roc1 | ESTs, similar to mouse Rho-associated coiled-forming kinase-1 | -1.4 | -1.6 | -1.7  | +1.5|
| AI230238 Col10a1 | Collagen type X α1 | -2  | -3.2 | -4    | +1.9|
| BI277745 MyhC | Myosin heavy chain type 2X | -4.3 | -3.5 | +20   |
| BI326356 Phi1 | Four and a half LIM domains 1 (skeletal) | -1.3 | -1.6 | -11   | +1.7|
| BM392106 Cald1 | Caldesmon 1 | -1.7 | -7   |
| NM_016994 C3 | Complement component 3 | +3  | -64  | +5.7  | -274|
| NM_024400 Adamts1 | ADAMTS-1 (a disintegrin and metalloproteinase with thrombospondin motifs 1) | -3  | -24  |
| NM_031549 Sm22 | Transgelin (SM 22 protein) | -1.6 | -34  | -1.7  | +1.6|
| NM_053819 Tmp1 | Tissue inhibitor of metalloproteinase 1-collagenase | +1.7 | +1.7 |
| NM_153577 Expi | Extracellular proteinase inhibitor | +21  | -100 |

Decreases are shown with minus signs, and increases are shown with plus signs. The values represent -fold changes over appropriate controls.

#### FIG. 2

**Regulation of cytoskeletal proteins in VSMC.** A, VSMC were serum-restricted for 24 h and stimulated with PDGF (20 ng/ml) for the indicated lengths of time, or VSMC stably expressing Myr-Akt or H-Ras were serum-restricted for 24 h. Cell extracts were prepared and immunoblotted with antibodies specific for nexilin and ezrin. B, VSMC were serum-restricted for 24 h and stimulated with AVP (10^{-3} M) for the indicated lengths of time or VSMC stably expressing Go_{16} were serum-restricted for 24 h, and extracts were immunoblotted with nexilin and ezrin.

Additional comments: Decreased expression of transcription factors, such as G(L)KLF, c-Fos, c-Myc, and Forkhead homolog 3 (Table IV). Consistent with the array data, Western blotting showed that GKL (KLF4) protein expression was decreased at 24 h of PDGF stimulation and was also decreased in cells expressing Myr-Akt or H-Ras (Fig. 1A), whereas expression of Go_{16} had the opposite effect, resulting in increased expression of GKL (Fig. 1B).

PDGF and Go_{16} had opposing effects on the expression levels of several cytoskeletal proteins (Table V). Expression of nexilin, an F-actin-binding protein, was decreased at both 6 and 24 h of PDGF stimulation as well as by expression of H-Ras or Myr-Akt, whereas its expression was increased with Go_{16}. Western blotting verified the array data showing decreased protein expression of nexilin following 24 or 48 h of PDGF stimulation, as well as decreased expression in cells expressing H-Ras and Myr-Akt compared with the appropriate empty vector controls (Fig. 2A). Although not determined to be statistically significant by microarray analysis, Western analysis showed that expression of nexilin was increased by AVP at all time points examined, as well as in VSMC expressing Go_{16} (Fig. 2B). In addition to nexilin, PDGF and H-Ras decreased and Go_{16} increased expression of Rho-associated kinase-1 and four and a half LIM domain proteins 1 and 2 (Table V). Interestingly, although array analysis showed decreased expression of ezrin, another actin-binding protein, by 24-h PDGF treatment, Western blotting showed that PDGF increased protein levels of Ezrin at all time points examined (Fig. 2A). Consistent with the array data, Western analysis also showed increased protein levels of ezrin by constitutive expression of H-Ras and Myr-Akt but decreased levels by constitutive expression of Go_{16} (Fig. 2, A and B).

Additionally, PDGF and Go_{16} regulated the expression of several extracellular matrix proteins, proteoglycans, and matrix enzymes (Table V). Expression of collagen type X α1, previously shown to be associated with cell hypertrophy (29, 30), was decreased by 6 and 24 h of PDGF stimulation, as well as by expression of Myr-Akt, but was increased by 24 h of AVP stimulation as well as by expression of Go_{16}. Real time quantitative PCR for ColXα1, used to verify the array results, showed that PDGF and Akt suppressed, whereas AVP and Go_{16} increased, ColXα1 mRNA levels (Fig. 3A). No change was observed in clones expressing H-Ras as predicted by array data. In contrast, versican, an extracellular matrix proteoglycan of blood vessels, was increased following 24 h of PDGF and by constitutive activation of both H-Ras and Akt but decreased by constitutive Go_{16}; real time quantitative PCR was used to confirm the array data (Fig. 3B). Although it was not predicted, both 6- and 24-h AVP stimulation decreased the mRNA levels of versican in VSMC (Fig. 3B).

In addition to its reported effects on VSMC phenotypic modulation, PDGF is a strong mitogen inducing proliferation of cultured VSMC. Several proliferation-associated genes were identified from the analysis of the microarray data (Table VI). One of these, growth-related oncogene-1 (Gro), a chemotaxant chemokine, was increased following 6- and 24-h stimulation with PDGF. Real time quantitative PCR of Gro verified the effect of PDGF on mRNA expression, showing that PDGF stimulation increased the mRNA levels of Gro in an Akt-mediated manner (Fig. 4). Expression of Go_{16} decreased Gro mRNA levels.
FIG. 3. Regulation of extracellular matrix protein expression in VSMC. A, VSMC were serum-restricted for 24 h and stimulated with PDGF (20 ng/ml) or AVP (10^{-6} M) for the indicated lengths of time, or VSMC stably expressing Myr-Akt, H-Ras, or Go_{16} were serum-restricted for 24 h and then total RNA isolated. Real time quantitative PCR for collagen type X was performed as described under “Experimental Procedures.” Ribosomal RNA was used for the normalization of cDNA from each sample. Results represent the mean and S.E. of two independent samples performed in at least triplicate. B, real time quantitative PCR for versican was performed as described under “Experimental Procedures.” Ribosomal RNA was used for the normalization of cDNA from each sample. Results represent the mean and S.E. of two independent samples performed in at least triplicate.

TABLE VI
Regulation of gene expression of several proliferation-related proteins in VSMC

| GenBank™ Name | Description | 6 h | 24 h | AVP | H-Ras | Akt | Go_{16} |
|---------------|-------------|-----|------|-----|-------|-----|---------|
| AA651304      | Vill2       | -1.7| +4.6 | -2.2|
| AA99254       | Sod2        | +1.9| -2.5 | +3.5 | -9   |
| AA946156      | Igf1        | -2.6| +3.7 | -17  |
| AF072892      | Cspg2       | +1.7| +7   | +5.3 | -4   |
| AF084544      | Cp          | +1.9| +9   | -10   |
| AF202115      | Odc1        | +1.6| +2.2 | +1.7  |
| AI230048      | Dbp         | +0.7| +6.7 | +2.8  |
| BF419200      | Cebp        | +2  | +1.1 | -3.2 | +2.5 |
| NM_012551     | Egr1        | -1.6| +1.9 | +1.6  |
| NM_012580     | Hmox1       | +2.2| +6   | -2.5  |
| NM_012603     | Myc         | -1.7| -1.4 | -2.8 | +1.9  |
| NM_012620     | Pau1        | -7  | +5.3 | -42  | -3.5  |
| NM_013037     | Itil1       | -2.2| +1.4 | -5   | +2.6 | -4     |
| NM_013147     | Hmgyr       | -2.2| -3.7 | -4.6 | +1.6  |
| NM_013174     | Tgfb3       | -2.6| -3.7 | -4.6 | +1.6  |
| NM_022381     | Pena        | +1.6| +1.5 | +3   |
| NM_024127     | Gadd45a     | +2.8| +6   | -2.2  |
| NM_030845     | Gro         | +6  | +2.8 | +39  | -169  |
| NM_031515     | Kras2       | -1.6|       |       |
| NM_031532     | Lgals3      | +1.7|       |       |
| NM_133306     | Otr1        | +2.8| -9.8 | +6.5 | +3   |
| NM_053819     | Timp1       | +1.7| +1.7 |       |
| NM_053974     | Eif4e       | -1.7|       |       |
| U05989        | Pawr        | -2.3| -3   | -1.7 | +1.4 |
Correlation of in Vitro Expression Patterns of Select Gene Products with in Vivo Expression Patterns in Injured Blood Vessels—Vascular injury-induced VSMC phenotypic modulation is associated with the down-regulation of SMC-specific marker genes, such as SM α-actin and SM22α, and contributes to neointima formation. Immunoﬂuorescent staining was used on tissue sections from sham-injured, 7-day, and 4-week post-ballooning-injured rat thoracic aortas to determine whether the changes in gene expression observed in cultured SMC in response to PDGF and/or hypertrophic stimuli correlate with in vivo changes in SMC phenotype. We found barely detectable VSMC expression of C/EBPβ in sham-injured vessels but increased accumulation of C/EBPβ in the nuclei of neointimal VSMC at 5 days after injury (Fig. 5A). By 4 weeks postinjury, C/EBPβ was restricted to the most luminal neointimal VSMC. Consistent with a functional role in suppressing α-actin promoter activity (Fig. 1C), high in vivo levels of C/EBPβ were associated with low levels of α-actin expression in neointimal VSMC (Fig. 5A). A similar, but cytosolic in vivo expression pattern was observed for versican (Fig. 5B), another gene identiﬁed as being up-regulated by PDGF in an H-Ras- and Akt-dependent manner. Versican was only detectable in the endothelium of sham-injured vessels, but was signiﬁcantly up-regulated in the developing neointima at 7 days postinjury. In contrast, nexilin, down-regulated by PDGF, H-Ras, and Akt and up-regulated by AVP and Go16, was abundantly expressed in sham-injured vessels but signiﬁcantly down-regulated in injured aortas (Fig. 5C).

**DISCUSSION**

Plasticity of VSMC resulting in phenotypic modulation is important in the physiologic and pathophysiologic state of the vascular wall. During the early stages of development or following injury to the blood vessels, VSMC present a dedifferentiated phenotype, which is characterized by low expression levels of SM-specific genes, including SM α-actin, SM22α, calponin, and SM-myosin heavy chain (31, 32). Previously, our laboratory and others showed that changes in SM-specific gene expression in cultures of adult VSMC could be modulated by hormones and growth factors (13–16, 33, 34). In general, PDGF suppressed expression of these proteins, whereas hypertrophic stimuli, such as AVP, increased expression. Although changes in contractile proteins have been widely used to deﬁne VSMC phenotype, the functional responses to these speciﬁc agonists will undoubtedly be a consequence of changes in expression of broad families of genes. The advent of microarray technology allows us to begin to define these changes, with the ultimate goal of deﬁning critical events that impact on the physiologic properties of these cells. Since PDGF and hypertrophic stimuli have many similar effects on VSMC, including mobilization of intracellular Ca2+, activation of MAP kinase family members, and increases in protein synthesis, it is likely that there will be signiﬁcant numbers of genes whose expression will be similarly regulated by both classes of agents. However, in this study, we focused on identifying genes whose expression was regulated in opposing directions by PDGF and hypertrophic stimuli. We hypothesized that these genes would be markers of phenotypic modulation and would be altered during vascular injury.

As a validation of our approach, we identiﬁed a number of contractile proteins, which were differentially regulated by the two classes of stimuli, many of which have previously been reported. The majority of these genes contain multiple CArG boxes in their promoter regions, and expression is likely to be controlled through SRF-dependent pathways (see Ref. 35). In addition, we identiﬁed a large number of differentially regulated genes that have not been reported to be controlled through SRF-dependent pathways. Many of these encode proteins functionally involved in cytoskeletal and cell-matrix interactions (see Tables V and VI). Nexilin, which is down-regulated by PDGF, is an F-actin-binding protein localized to cell-matrix junctions (36) and may be involved in focal contacts. Adducin 3 γ is a cytoskeletal assembly protein up-regulated by PDGF and down-regulated by hypertrophic stimuli, involved in actin-driven migration (37). Another cytoskeletal organizer protein ezrin was up-regulated by PDGF and down-regulated by Go16. A reduction in the expression of nexilin combined with increases in ezrin and adducin would be predicted to increase cell migration, consistent with the effects of PDGF. We also demonstrated that levels of nexilin are decreased in blood vessels after injury. This novel ﬁnding suggests a critical connection between PDGF-induced gene expression and neointimal formation.

The opposing effects of PDGF and hypertrophic stimuli on cytoskeletal related proteins are likely to lead to changes of F-actin/G-actin ratio in VSMC. Numerous studies have demonstrated that alterations in F-actin/G-actin are critical in controlling SRF-dependent genes (38–41). This is mediated at least in part through translocation of coactivators of the myocardin/MKL family (reviewed in Ref. 42). Our data show that
Rho-associated kinase-1, a downstream effector of RhoA, was down-regulated by PDGF and up-regulated in response to expression of Go_{19}. Decreased ROCK expression would be predicted to inhibit RhoA signaling and block SRF-dependent transcription (43) and induction of SM-specific genes in VSMC (44).

Alterations in expression of matrix-related proteins were also observed. Expression of collagen type X \( \alpha_1 \) was decreased by PDGF and strongly up-regulated by AVP stimulation, correlating with a hypertrophic response (30). Versican (CSPG2), an extracellular matrix proteoglycan, was up-regulated by PDGF and down-regulated by Go_{19} and AVP. Versican is secreted by VSMC in normal blood vessels, and its expression is increased in atherosclerosis and restenosis and in cells isolated from neointima (24, 45, 46). Coordinated down-regulation of hypertrophy-related matrix proteins (ColX\( \alpha_1 \)) and up-regulation of migratory and proliferative matrix proteins (versican) could lead to a PDGF-mediated dedifferentiated, migratory...
phenotype of VSMC, an important step in neointima formation. A change in the expression of a transcription factor can regulate the transcriptional activity of several genes through cascades of transcriptional regulations. Increased expression of C/EBPβ, observed in our experiments, has been correlated with increased PDGFα receptor expression and vascular remodeling (47). Overexpression using molecular transfection approaches demonstrated that C/EBPβ decreased SM-specific gene expression (Fig. 1C), and increased expression was observed in neointima formation in vivo (Fig. 5A). C/EBPβ could induce the transcription of a number of other genes involved in SMC phenotypic modulation. Preliminary analysis of the promoter region of human versican (accession number U15963) showed several putative C/EBPβ binding sites (48), suggesting C/EBPβ-dependent up-regulation of versican.

Activation of the PDGF receptor leads to the engagement of multiple downstream signaling cascades. By correlating changes in gene expression in response to PDGF with changes induced by expression of either gain-of-function H-Ras or Akt, it appears that these effectors have distinct roles in mediating the response to PDGF. Activation of H-Ras was more critical for the early changes in gene expression mediated by PDGF, whereas activation of Akt was better correlated with changes observed at longer time points (Table VII). We would therefore propose that PDGF rapidly activates the Ras pathway, which is critical for initial changes in gene expression. This is followed by a more sustained activation of the phosphatidylinositol 3-kinase/Akt pathway (15), which is a major determinant of the delayed changes in gene expression. It should be noted that expression of either H-Ras or Myr-Akt altered expression of several genes not shown to be regulated by PDGF. We attribute this to the fact that these cells are expressing constitutively active forms of these enzymes, whereas PDGF stimulation leads to a transient activation of these pathways. Furthermore, there were significant numbers of genes whose expression was altered by PDGF but not by either H-Ras or Akt. This would indicate that PDGF engages additional effectors, as has been well demonstrated. In particular, we have not examined the role of phospholipase Cγ in this study.

Phenotypic modulation of VSMC by environmental factors requires coordinated action of common signaling pathways leading to large scale changes in gene expression, which ultimately result in the distinct biology of VSMC in disease states. The balance between mitogenic and hypertrophic stimuli leading to specific gene expression profiles determines the physiological or pathologic states of the arterial vessels. We therefore propose that genes differentially regulated by these two classes of stimuli provide potential biomarkers for the phenotypic state of VSMC both in vitro and in vivo. The data presented here will increase our understanding of the molecular pathways mediating this response in normal development and in pathophysiologic states.

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Patterns of Gene Expression Differentially Regulated by Platelet-derived Growth Factor and Hypertrophic Stimuli in Vascular Smooth Muscle Cells: MARKERS FOR PHENOTYPIC MODULATION AND RESPONSE TO INJURY
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