Molecular basis for impaired collateral artery growth in the spontaneously hypertensive rat: insight from microarray analysis

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Abstract
Analysis of global gene expression in mesenteric control and collateral arteries was used to investigate potential molecules, pathways, and mechanisms responsible for impaired collateral growth in the Spontaneously Hypertensive Rat (SHR). A fundamental difference was observed in overall gene expression pattern in SHR versus Wistar Kyoto (WKY) collaterals; only 6% of genes altered in collaterals were similar between rat strains. Ingenuity Pathway Analysis (IPA) identified major differences between WKY and SHR in networks and biological functions related to cell growth and proliferation and gene expression. In SHR control arteries, several mechno-sensitive and redox-dependent transcription regulators were downregulated including JUN (\(\log_{2} 5.29, P = 0.02\)), EGR1 (\(\log_{2} 4.19, P = 0.01\)), and NFKB1 (\(\log_{2} 1.95, P = 0.04\)). Predicted binding sites for NFKB and AP-1 were present in genes altered in WKY but not SHR collaterals. Immunostaining showed increased NFkB nuclear translocation in collateral arteries of WKY and apocynin-treated SHR, but not in untreated SHR. siRNA for the p65 subunit suppressed collateral growth in WKY, confirming a functional role of NFkB. Canonical pathways identified by IPA in WKY but not SHR included nitric oxide and renin-angiotensin system signaling. The angiotensin type 1 receptor (AGTR1) exhibited upregulation in WKY collaterals, but downregulation in SHR; pharmacological blockade of AGTR1 with losartan prevented collateral luminal expansion in WKY. Together, these results suggest that collateral growth impairment results from an abnormality in a fundamental regulatory mechanism that occurs at a level between signal transduction and gene transcription and implicate redox-dependent modulation of mechno-sensitive transcription factors such as NFKB as a potential mechanism.

Introduction
An urgent medical need has been identified for novel therapies across the spectrum of peripheral arterial disease (PAD) (Gornik 2009), the prevalence of which is rising dramatically with increased occurrence of risk factors including aging, obesity, and hyperglycemia. These risk factors for PAD not only promote arterial disease, but also suppress the innate capacity for compensation to major arterial occlusion in humans and animal models...
(as reviewed by Ziegler et al. [2010]). Their presence may also suppress the efficacy of therapeutic interventions, including molecular and cell-based treatments (Kinnaird et al. 2008) and thus explain the failure of many preclinical studies performed in young healthy animals to predict clinical outcomes for PAD therapies. While potential mechanisms that mediate impaired compensation have been reviewed (Kinnaird et al. 2008), many of the studies supporting these potential mechanisms were focused on angiogenesis or tissue-cellular responses to severe ischemia or hypoxia and not the primary collateral arteries which experience increased blood flow and shear stress and comprise the primary site of vascular resistance and compensation subsequent to arterial occlusion in both humans and animal models of arterial insufficiency (as reviewed in Ziegler et al. [2010]). Consequently, the specific mechanisms mediating the impaired growth of collateral arteries in the peripheral circulation remain largely unknown.

The current study was undertaken to identify potential mechanisms responsible for impaired collateral growth in the Spontaneously Hypertensive Rat (SHR), an animal model widely utilized to assess vascular adaptations to peripheral arterial occlusion (Nelissen-Vrancken et al. 1992, 1993; Scheidegger et al. 1997; Emanuelli et al. 2001, 2002; Tamarat et al. 2002; Tuttle et al. 2002b; Srivastava et al. 2003; Iaccarino et al. 2005; Miller et al. 2007a; You et al. 2008; Matsumura et al. 2009). Microarray analysis of control and collateral mesenteric arteries was performed and the results demonstrate a fundamental difference in the overall collateral gene expression pattern in SHR versus normotensive control rats (Wistar Kyoto; WKY). Data mining revealed striking differences in the expression of molecules involved in the regulation of cell growth and proliferation and gene expression. Redox-dependent modulation of mechano-sensitive transcription factors is proposed as a potential mechanism that may explain, at least in part, the fundamental differences in collateral gene expression between WKY and SHR and the resultant impairment in SHR collateral growth.

Methods

General experimental approach

The mesenteric model of flow-mediated collateral growth (Unthank et al. 1996a,b) was used to assess mechanisms related to collateral growth and its impairment. The mesentery is part of the peripheral circulation and both acute and chronic intestinal occlusion occur clinically as a result of atherosclerotic lesions (Hirsch et al. 2006). This model allows similar ileal arteries representing normal flow control arteries and high flow collateral arteries to be evaluated in the same animal (within subject design). Additional advantages of the mesenteric model include a well-defined collateral path and known hemodynamic changes (Unthank et al. 1996a,b). The vessels are of a size that provide significant compensation as primary collaterals, can be easily dissected, and provide adequate RNA for analysis without pooling.

Animals, procedures, and treatments

Male WKY and SHR rats were obtained from Harlan (Indianapolis, IN) and studied at ~10 weeks of age. All procedures were approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee. The mesenteric model to induce collateral growth was created by ligation of sequential ileal arteries with special care to prevent angiogenesis from stretch or partial desiccation as previously described (Unthank et al. 1996a,b; Miller et al. 2007a).

For microarray analysis and real-time quantitative RT-PCR studies, control and collateral arteries were harvested 24 h postligation. This time was selected because minimal cellular remodeling/recruitment and luminal expansion have occurred and previous array and expression studies have shown significant mRNA alterations at 24 h after arterial occlusion (Lee et al. 2004; Prior et al. 2004). Prior to tissue harvesting, the caudal aorta was cannulated above the iliac bifurcation, and the renal arteries ligated as well as the abdominal aorta proximal to the superior mesenteric artery. Retrograde aortic perfusion of the mesenteric arteries and intestine was then performed with 30 mL of cold, phosphate-buffered saline followed by 10 mL of an RNA stabilization reagent (RNA later; Ambion, Austin, TX).

For immunostaining experiments, control and collateral arteries were harvested at 1–3 days postligation after perfusion with warm phosphate-buffered saline containing a dilator cocktail (0.1 mmol/L adenosine and 0.01 mmol/L sodium nitroprusside) followed by 10% neutral buffered zinc formalin containing the same dilators. Some SHR were treated with the antioxidant apocynin (3 mmol/L in drinking water) beginning 1 day before model creation and continuing until perfusion fixation at 3 days postligation. This dose restores both a normal redox status and collateral growth capacity in SHR mesenteric arteries (Miller et al. 2007a; Zhou et al. 2008).

In experiments to assess the importance of the angiotensin type 1 receptor (AGTR1) in successful collateral luminal expansion, luminal diameters were imaged with maximal dilation at the time of model creation and then 7 days later in rats with and without losartan pretreatment as previously described (Unthank et al. 1996a,b; Miller et al. 2007a).
To assess the potential role of NFκB in collateral growth, experiments were performed in which p65 or control siRNA were administered via tail vein using the hydrodynamic method as previously described (Modlinger et al. 2006; Palm et al. 2007). Briefly, p65 siRNA (ON-Target Plus SmartPool duplex, Thermo Scientific/Dharmacon, Pittsburgh, PA) or nonsense control siRNA (Thermo/Dharmacon; SmartPool universal siRNA control) was reconstituted with RNase-free water and diluted with Mirus Bio TransIT in vivo transfection agent. The siRNA was injected as a 6 mL bolus containing 50 and 25 μg, respectively, at 1 day prior to and 4 days after collateral model creation and luminal expansion assessed by diameter measurements as described above.

Global gene expression analysis

Microarray analysis of WKY and SHR mesenteric arteries was performed by the Indiana University Center for Medical Genomics (IUCMG) using the Affymetrix GeneChip Rat Genome U34A Array as previously described (Miller et al. 2007b) but with amplification (McClintick et al. 2003). Control and collateral arteries from each animal were labeled individually and hybridized to an array. No pooling was used. Data were analyzed using Affymetrix Microarray Suite 5.0 software (MAS5; Affymetrix Microarray Suite 5.0 User’s Guide, Santa Clara, CA). The experimental design for the microarray analysis is summarized in Figure 1. Same-animal control and collateral arteries were harvested from four WKY and four SHR. One WKY control sample had insufficient RNA and was excluded from analysis. A complete data set is available at the NCBI GEO database (http://www.ncbi.nlm.nih.gov/projects/geo) under accession GSE19524. Microarray data analyses were carried out using the Microarray Data Portal, a proprietary analytical and informatics algorithm developed by the IUCMG, and IPA (Ingenuity® Systems, www.ingenuity.com). To eliminate data from probe sets that are not reliably detected, only those probe sets identified as “present” by MAS5 in at least half of the arrays were analyzed (McClintick et al. 2003).

Real-time quantitative RT-PCR (qRT-PCR)

Total RNA was obtained from additional sets of animals and mesenteric artery mRNA expression assessed using real-time quantitative PCR as previously described in detail (Miller et al. 2007b). Aliquots (5.0 μL of 1:5–1:50 dilutions) of reverse transcription reactions (0.5 μg total RNA) were combined with the appropriate primers for targets or the beta-actin endogenous control (TaqMan® Gene Expression Assays; Applied Biosystems, Foster City, CA) in the presence of PCR reagents (QuantiTectTM Probe PCR Kit; Qiagen, Valencia, CA). Reactions were run in triplicate on an Applied Biosystems 7500 Real-Time PCR System using relative quantification (ddCt) with dual-labeled (FAM/MGB) probes as the product detection method. Standard cycling conditions (2-step PCR, 40 cycles) were used for all targets except AGTR1b (angiotensin II receptor, type 1b), which was modified to 45 cycles. Differences in PCR product yields between groups were determined by comparing the fold differences between target mRNA after normalization to beta-actin.

Immunohistochemistry

Immunostaining was performed using an immunoperoxidase technique on cross-sections of paraffin-embedded control and collateral vessels as previously described (Miller et al. 2007b). Briefly, for immunoperoxidase studies, slides from paraffin blocks were antigen retrieved using DAKO Target Retrieval solution (pH 6.0) to expose antigens masked by formalin. Endogenous biotin was blocked with avidin/biotin blocking system (DAKO) and endogenous peroxidase with 3% hydrogen peroxide. Antibodies were applied for 60 min at room temperature at a...
concentration determined by titering to eliminate nonspecific background staining. Slides were developed using DAKO’s EnVision+ Dual Link (DAKO North America Inc., Carpinteria, CA), HRP kit for rabbit or mouse primary antibodies in a DAKO Autostainer. The AGTR1 polyclonal primary antibody was generated from a peptide immunogen for the sequence LQLLKYIPPKAK SHSNLSTKMSTLSYRPSDNVSSSTK (Sigma Prestige Antibody HPA003596). The antibody for NFkB (nuclear factor kappa light-chain enhancer of activated B cells) was a mouse monoclonal against NFkB p65 (Santa Cruz, sc-8008). Quantitation was performed by CAL in a blinded manner by determining the percent of cells or nuclei with immunoreactivity within each wall layer (intima, media, and adventitia).

**Statistical and ontological analyses**

Two-way repeated measures analysis of variance (strain × vessel type) was used to assess statistical differences in the microarray experiments. For the microarray analysis, false discovery rates were calculated as previously described (Storey and Tibshirani 2003). Genes that had significant expression changes at $P \leq 0.05$ using log transformed signal values and fold changes $\geq 1.25$ were further analyzed with IPA. By associating these molecules with biological functions in the Ingenuity Knowledge Base, IPA functional analysis identified the biological functions most significant to the data set. Right-tailed Fisher’s exact test was used to calculate a $P$-value determining the probability that each biological function was due to chance alone. IPA canonical pathways analysis identified the pathways from the IPA library of canonical pathways that were most significant to the data set. Fisher’s exact test was used to calculate a $P$-value determining the probability that the association between the genes in the data set and the canonical pathway was explained by chance alone. Statistical significance of arterial diameter change and immunoreactivity results was assessed by two-way repeated measures analysis of variance with pairwise multiple comparison procedures performed with the Holm–Sidak method (Systat Software Inc., Sigmaplot for Windows v.11).

**Results**

**Differences between WKY and SHR collaterals**

A primary goal of the study was to identify potential molecules and discover related functions and pathways that might mediate the collateral growth impairment observed in SHR. To accomplish this objective in an unbiased manner, analysis of the microarray data was performed with IPA. The first objective was to determine the total number of molecules with altered expression between collateral and control arteries in SHR and WKY. The number of genes present on the microarray with adequate expression for analysis was 1795. IPA was used to determine the molecules with altered expression in collateral compared to control arteries (within subject design, $P \leq 0.05$, fold change $\geq 1.25\times$) that were both common and unique to WKY and SHR. There were a total of 125 and 111 genes with altered expression in WKY and SHR, respectively. Figure 2 reports the number of these molecules which were up- and downregulated, and shows that only 14 were common between WKY and SHR. These results indicate a fundamental difference between WKY and SHR in the global pattern of collateral gene expression. The potential impact of this difference between WKY and SHR was further assessed in IPA by analyses of networks, biological functions, and canonical pathways.

**Network analyses**

The five gene networks with the highest scores determined by IPA are shown in Table 1 for WKY and SHR. In WKY, the networks with the two highest scores were (1) Cellular Movement, Cellular Development, Cellular Growth and Proliferation, and (2) Gene Expression, Cell Death, and Hematological System Development and Function. We examined these two networks to further assess collateral expression differences between these two strains. As illustrated in Figure 2B and C, these two networks contained 18 and 17 molecules, respectively, with altered expression ($P \leq 0.5$ and fold change $\geq 1.25\times$) in WKY collaterals. Comparison to the molecules altered in the SHR collaterals for these two WKY networks revealed that only six molecules combined were common between strains (molecules identified by red plus signs in Figure 2B and C).

**Biological functions**

Table 2 lists the five highest IPA rated biological functions within the molecular and cellular category for collateral artery gene expression in WKY and SHR. Not only are the top functions dissimilar for the WKY and SHR, but evaluation of the specific molecules altered within WKY and SHR collaterals display limited overlap or similarity for a given function. For example, of altered molecules in the functional groups of Cellular Growth and Proliferation and Cell Cycle (among top ranked for WKY and SHR, respectively, Table 2), only 1 molecule was common (PTPN2). In addition, Gene Expression,
Figure 2. Fundamental differences in collateral gene expression. (A) Venn diagram of number of genes with increased (↑) or decreased (↓) expression in collateral artery relative to same animal control artery for WKY and SHR ($P \leq 0.05$, fold change ≥1.25×). An IPA comparison analysis of the molecules with altered expression (125 in WKY, 111 in SHR) indicates 222 genes unique to either set, with only 14 common to both sets and demonstrating similar expression changes. The upregulated genes with common expression between WKY and SHR included AP1S1 (adaptor-related protein complex 1, sigma 1 subunit), C6orf115 (ABRA C-terminal like), LRRC59 (leucine rich repeat containing 59), and PTPN2 (protein tyrosine phosphatase, nonreceptor type 2); those downregulated were ATP1A2 (ATPase, Na+/K+ transporting, alpha 2 polypeptide), COX8A (cytochrome c oxidase subunit VIIIa (ubiquitous)), CST3 (cystatin C), ECH1 (enoyl CoA hydratase 1, peroxisomal), FUCA1 (fucosidase, alpha-L- 1, tissue), IDH2 (isocitrate dehydrogenase 2 (NADP+), mitochondrial), LDHB (lactate dehydrogenase B), PPA2 (pyrophosphatase (inorganic) 2), PPP1R1A (protein phosphatase 1, regulatory (inhibitor) subunit 1A), and ZFP36L1 (zinc finger protein 36, C3H type-like 1). The two IPA highest scored networks in WKY were Cellular Movement, Cellular Development, Cellular Growth and Proliferation shown in (B), and Gene Expression, Cell Death, Hematological System Development and Function depicted in (C). Molecules with significant up- and downregulation are identified with red and green shading, respectively. Comparison of these molecules between WKY and SHR demonstrated fundamental differences. For those genes in the Cellular Movement, Cellular Development, Cellular Growth and Proliferation, only AP1S1 (adaptor-related protein complex 1, sigma 1 subunit), ATP1A2, EEF2K (eukaryotic elongation factor-2 kinase), and CSRP2 (cysteine and glycine-rich protein 2) had altered expression in SHR (red +). Similarly, within the Gene Expression, Cell Death, Hematological System Development and Function network, only CLU (clusterin) and FUCA (fucosidase, alpha-L- 1, tissue) had altered collateral expression in SHR (red +). □ = cytokine, ○ = enzyme, ◇ = other, horizontal oval = transcription regulator, ▲ = phosphatase, ▼ = kinase, vertical oval = transmembrane receptor, trapezoid = transporter. Lines without arrows indicate binding, lines with arrows indicate stimulation, solid lines indicate direct interaction, and dashed lines indirect.
although not among the IPA highest ranked Bio Functions for either WKY or SHR, was included in the list of significantly altered molecular and cellular functions for both and included 39 molecules in WKY (39 subcategories with \( P \)-values from \( 3.43 \times 10^{-6} \) to \( 2.09 \times 10^{-3} \)) and eight in SHR (eight subcategories, \( P = 3.40 \times 10^{-5} \) to \( 1.27 \times 10^{-2} \)) without a single molecule in common.

**Canonical pathway analysis**

Based upon the functional analyses, canonical pathways within IPA were selected for the categories of Cardiovascular Signaling; Cell Cycle Regulation; Cellular Growth, Proliferation, and Development; and Transcriptional Regulation and further screened based upon molecular ratio \( \geq 0.03 \) and statistical significance at \( P \leq 0.01 \). The canonical pathways which met these criteria and were different between WKY and SHR are reported in Table 3. Significant alterations in WKY but not SHR were observed for signaling pathways associated with nitric oxide, the renin–angiotensin system (RAS), and transforming growth factor-beta (Fig. 3), all of which are known to have important roles in various types of arterial remodeling. In SHR, the canonical pathways of mitochondrial dysfunction and insulin receptor signaling were altered.

**Molecules with greatest fold changes**

Comparison of the molecules with the greatest fold changes were identified by IPA and further confirmed fundamental differences in gene expression between WKY and SHR collaterals. Table 4 reports the top ten up- and downregulated molecules in WKY and also shows the corresponding expression change, if any, in the SHR. Only one of the top 10 up- and downregulated molecules in WKY collaterals had significantly altered expression in the SHR while three of the top 10 upregulated molecules were altered in WKY while only three of

### Table 1. IPA highest ranked networks.

| Network ID | Associated network functions | Score |
|------------|------------------------------|-------|
| (A) WKY    |                              |       |
| 1          | Cellular Movement, Cellular Development, Cellular Growth and Proliferation | 34    |
| 2          | Gene Expression, Cell Death, Hematological System Development and Function | 31    |
| 3          | Gene Expression, Cancer, Small Molecule Biochemistry | 29    |
| 4          | Lipid Metabolism, Small Molecule Biochemistry, Vitamin and Mineral Metabolism | 26    |
| 5          | Gene Expression, Cell Death, Gastrointestinal Disease | 25    |
| (B) SHR    |                              |       |
| 1          | Cell-to-Cell Signaling and Interaction, Nervous System Development and Function, Cell Cycle | 42    |
| 2          | Cellular Movement, Cell Cycle, Cellular Growth and Proliferation | 37    |
| 3          | Cellular Development, Hepatic System Development and Function, Lipid Metabolism | 30    |
| 4          | Cellular Assembly and Organization, Cell Death, DNA Replication, Recombination, and Repair | 29    |
| 5          | Genetic Disorder, Neurological Disease, Skeletal and Muscular Disorders | 25    |

IPA, Ingenuity® pathway analysis; WKY, Wistar Kyoto; SHR, spontaneously hypertensive rat.

### Table 2. IPA highest ranked molecular and cellular functions.

| Name | \( P \)-value | # Molecules |
|------|---------------|-------------|
| (A) WKY Collateral vs. Control Arteries | | |
| Cellular Development | 1.96E-08–1.75E-03 | 50 |
| Cellular Growth and Proliferation | 1.96E-08–2.06E-03 | 54 |
| Cell-To-Cell Signaling and Interaction | 6.39E-08–2.06E-03 | 40 |
| Cell Death | 1.32E-07–2.09E-03 | 49 |
| Cellular Movement | 1.84E-07–2.09E-03 | 37 |
| (B) SHR Collateral vs. Control Arteries | | |
| Cellular Assembly and Organization | 2.07E-05–1.27E-02 | 19 |
| Free Radical Scavenging | 2.41E-05–8.06E-03 | 12 |
| Molecular Transport | 2.41E-05–1.27E-02 | 35 |
| Cell Cycle | 2.49E-05–1.27E-02 | 22 |
| Protein Synthesis | 2.71E-05–8.33E-04 | 14 |

IPA, Ingenuity® pathway analysis; WKY, Wistar Kyoto; SHR, spontaneously hypertensive rat.

- **Table 3.** Shows the canonical pathways that were significant between WKY and SHR.
- **Table 4.** Reports the top ten up- and downregulated molecules in WKY and their corresponding expression change in SHR.
- **Table 5.** Reveals that none of the top 10 upregulated molecules in WKY collaterals were altered in SHR while only three of the top 10 upregulated molecules in WKY collaterals were altered in SHR.
the greatest downregulated molecules exhibited decreased expression in WKY collaterals (ECH1, enoyl CoA hydratase 1, peroxisomal; ATP1A2, CST3, cystatin C). Growth arrest and DNA-damage-inducible α (GADD45α) was among the top 10 genes upregulated in SHR but not WKY collaterals.

### Differences between WKY and SHR control arteries

Next, comparisons between SHR and WKY control arteries were made to determine if additional insight might be obtained to explain the fundamental difference in collateral gene expression between these two rat strains. In SHR control arteries, 175 molecules had altered expression relative to WKY controls (≥1.25 fold change, P ≤ 0.05). The IPA highest rated networks, cellular and molecular biological functions, and molecules with great-

| IPA highest ranked canonical pathways | Analysis name | Ratio | P-value | Molecules |
|---------------------------------------|---------------|-------|---------|-----------|
| (A) Significantly altered in WKY but not SHR | Nitric Oxide Signaling in the Cardiovascular System | WKY | 0.050 | 0.000 | PRKAR2B, PLN, GUCY1A3, SLC7A1, ATP2A3 |
| | Nitric Oxide Signaling in the Cardiovascular System | SHR | 0.020 | 0.091 | ITPR3, ATP2A2 |
| | IL-1 Signaling | WKY | 0.047 | 0.001 | GNB1, FOS, JUN, PRKAR2B, ADCY6 |
| | IL-1 Signaling | SHR | 0.019 | 0.117 | GNG11, GNB2 |
| | TGF-β Signaling | WKY | 0.045 | 0.003 | FOS, JUN, TGFβ3, SERPINE1 |
| | TGF-β Signaling | SHR | NA | NA | |
| | Renin–Angiotensin Signaling | WKY | 0.040 | 0.001 | FOS, JUN, PRKAR2B, ADCY6, AGT |
| | Renin–Angiotensin Signaling | SHR | 0.016 | 0.154 | ITPR3, AGT |
| (B) Significantly altered in SHR but not WKY | Mitochondrial Dysfunction | SHR | 0.040 | 0.000 | NDUF5, XDH, COX8A, COX7A2L, APP, NDUF2, NDUF10 |
| | Mitochondrial Dysfunction | WKY | 0.017 | 0.079 | NDUF5, COX8A, TXNRD2 |
| | Insulin Receptor Signaling | SHR | 0.036 | 0.001 | RPS6KB1, BAD, EIF2B3, GSK3B, PPP1CA |
| | Insulin Receptor Signaling | WKY | 0.014 | 0.254 | PRKAR2B, EIF4EBP1 |

ADCY6, adenylate cyclase 6; AGT, angiotensinogen; AGTR1, angiotensin II receptor, type 1; APP, amyloid beta (A4) precursor protein; ATP2A2, ATPase, Ca++ transporting, cardiac muscle, slow twitch 2; ATP2A3, ATPase, Ca++ transporting, ubiquitous; BAD, BCL2-associated agonist of cell death; COX7A2L, cytochrome c oxidase subunit VIIa polypeptide 2 like; COX8A, cytochrome c oxidase subunit VIIa; EIF2B3, eukaryotic translation initiation factor 2B, subunit 3; EIF4EBP1, eukaryotic translation initiation factor 4E-binding protein 1; FOS, FBJ osteosarcoma oncogene; GNB1, guanine nucleotide-binding protein (G protein), beta polypeptide 1; GNB2, guanine nucleotide-binding protein (G protein), beta polypeptide 2; GNG11, guanine nucleotide-binding protein (G protein), gamma 11; GSK3B, glycogen synthase kinase 3 beta; GUCY1A3, guanylate cyclase 1, soluble, alpha 3; ITPR3, inositol 1,4,5-trisphosphate receptor, type 3; JUN, jun proto-oncogene; NDUF5, NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 10; NDUF5, NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 2; NDUF5, NADH dehydrogenase (ubiquinone) Fe-S protein 5; NDUFS7, NADH dehydrogenase (ubiquinone) Fe-S protein 7; PLN, phospholamban; PPP1CA, protein phosphatase 1, catalytic subunit, alpha isoform; PRKAR2B, protein kinase, cAMP dependent regulatory, type II beta; PRKAR2B, protein kinase, cAMP dependent regulatory, type II beta; PRKAR2B, protein kinase, cAMP dependent regulatory, type II beta; PRKAR2B, protein kinase, cAMP dependent regulatory, type II beta; PRKAR2B, protein kinase, cAMP dependent regulatory, type II beta; PRKAR2B, protein kinase, cAMP dependent regulatory, type II beta; PTPN1, protein tyrosine phosphatase, nonreceptor type 1A; RP56KB1, ribosomal protein S6 kinase, polypeptide 1; SERPINE1, serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1; SLC7A1, solute carrier family 7 (cationic amino acid transporter, y+ system), member 1; TGFβ3, transforming growth factor, beta 3; TXNRD2, thioredoxin reductase 2; XDH, xanthine dehydrogenase; IPA, Ingenuity Pathway Analysis; WKY, Wistar Kyoto; SHR, spontaneously hypertensive rat; IL, interleukin; TGF, transforming growth factor.
### Table 4. Molecules with greatest fold changes in collateral expression in WKY with comparison to SHR.

| WKY fold change | WKY P-value | SHR fold change | SHR P-value | Symbol  | Entrez gene name |
|-----------------|-------------|-----------------|-------------|---------|------------------|
| 3.97            | 0.002       | 1.72            | 0.413       | ESM1    | Endothelial cell-specific molecule 1 |
| 2.84            | 0.012       | −1.00           | 0.989       | COL12A1 | Collagen, type XII, alpha 1         |
| 2.65            | 0.010       | 1.22            | 0.505       | SLC7A1  | Solute carrier family 7 (cationic amino acid transporter, y+ system), member 1 |
| 2.48            | 0.019       | 1.41            | 0.422       | IL1B    | Interleukin 1, beta                 |
| 2.42            | 0.035       | 1.70            | 0.174       | DIO3    | Deiodinase, iodothyronine, type III |
| 2.37            | 0.010       | −0.98           | 0.925       | ACTA1   | Actin, alpha 1, skeletal muscle     |
| 2.28            | 0.031       | −1.01           | 0.984       | CRYAB   | Crystallin, alpha 8                 |
| 2.25            | 0.002       | −1.05           | 0.899       | IDI1    | Isopentenyl-diphosphate delta-isomerase 1 |
| 2.25            | 0.007       | −1.79           | 0.123       | TGBF3   | Transforming growth factor, beta 3  |
| 2.19            | 0.041       | 1.31            | 0.419       | CYP51A1 | Cytochrome P450, family 51, subfamily A, polypeptide 1 |
| −6.45           | 0.039       | −1.27           | 0.595       | AGT     | Angiotensinogen (serpin peptidase inhibitor, clade A, member 8) |
| −4.08           | 0.016       | −2.61           | 0.053       | SULT1A1 | Sulfoconjugate transferase family, cytosolic, 1A, phenol-preferring, member 1 |
| −3.44           | 0.048       | −1.32           | 0.243       | TNXA    | Tenascin XA                          |
| −2.93           | 0.049       | −2.08           | 0.679       | JUN     | Jun proto-oncogene                   |
| −2.86           | 0.000       | −1.97           | 0.128       | GSTT2   | Glutathione S-transferase theta 2    |
| −2.84           | 0.010       | −2.21           | 0.034       | ATP1A2  | ATPase, Na+/K+ transporting, alpha 2 polypeptide |
| −2.74           | 0.044       | −1.65           | 0.113       | EPHX1   | Epoxide hydrolase 1, microsomal (seronbiotic) |
| −2.68           | 0.001       | −2.32           | 0.623       | FMOD    | Fibromodulin                         |
| −2.49           | 0.029       | −1.86           | 0.182       | EEF2K   | Eukaryotic elongation factor-2 kinase |
| −2.48           | 0.025       | −0.68           | 0.831       | CX3CL1  | Chemokine (C-X3-C motif) ligand 1    |

IPA, Ingenuity® pathway analysis; WKY, Wistar Kyoto; SHR, spontaneously hypertensive rat.

### Table 5. Molecules with greatest fold changes in collateral expression in SHR with comparison to WKY.

| SHR fold change | SHR P-value | WKY fold change | WKY P-value | Symbol  | Entrez gene name |
|-----------------|-------------|-----------------|-------------|---------|------------------|
| 3.40            | 0.049       | 1.38            | 0.822       | CYP4A14 | Cytochrome P450, family 4, subfamily a, polypeptide 14 |
| 1.91            | 0.011       | 1.10            | 0.650       | AKAP11  | A kinase (PRKA) anchor protein 11 |
| 1.91            | 0.034       | 1.16            | 0.773       | GADD45A | Growth arrest and DNA-damage-inducible, alpha |
| 1.89            | 0.047       | 1.72            | 0.172       | EIF2B3  | Eukaryotic translation initiation factor 2b, subunit 3 gamma, 58 kDa |
| 1.80            | 0.048       | −1.14           | 0.773       | NAPA    | N-ethylmaleimide-sensitive factor attachment protein, alpha |
| 1.73            | 0.030       | 1.57            | 0.164       | LOC68240| Similar to NMDA receptor regulated 1-like |
| 1.72            | 0.015       | 1.05            | 0.833       | MRPL24  | Mitochondrial ribosomal protein L24 |
| 1.71            | 0.018       | −0.95           | 0.888       | ACSL4   | Acyl-CoA synthetase long-chain family member 4 |
| 1.68            | 0.037       | 1.05            | 0.876       | GSK3B   | Glycogen synthase kinase 3 beta |
| 1.68            | 0.018       | −1.03           | 0.878       | GFM1    | G elongation factor, mitochondrial 1 |
| −2.93           | 0.006       | −1.58           | 0.638       | THY1    | Thy-1 cell surface antigen |
| −2.41           | 0.017       | −2.23           | 0.018       | ECH1    | Enoyl CoA hydratase 1, peroxisomal |
| −2.31           | 0.015       | −1.11           | 0.750       | HSD3B7  | Hydroxy-delta-5-sterol dehydrogenase, 3 beta- and steroid delta-isomerase 7 |
| −2.21           | 0.034       | −2.84           | 0.010       | ATP1A2  | ATPase, Na+/K+ transporting, alpha 2 polypeptide |
| −2.06           | 0.047       | −1.14           | 0.658       | IRF3    | Interferon regulatory factor 3 |
| −1.99           | 0.006       | −1.07           | 0.768       | PTPRA   | Protein tyrosine phosphatase, receptor type, A |
| −1.88           | 0.010       | 1.09            | 0.679       | SLC6A6  | Solute carrier family 6 (neurotransmitter transporter, taurine), member 6 |
| −1.86           | 0.005       | −1.13           | 0.214       | ITGA7   | Integrin, alpha 7 |
| −1.83           | 0.038       | −1.98           | 0.000       | CST3    | Cystatin C |
| −1.83           | 0.015       | −1.54           | 0.055       | IVD     | Isovaleryl-CoA dehydrogenase |

IPA, Ingenuity® pathway analysis; WKY, Wistar Kyoto; SHR, spontaneously hypertensive rat.
est fold changes are reported in Table 6. Cell growth, cell cycle, cell proliferation, and gene expression were biological processes included within the highest rated gene networks and molecular and cellular functions (Table 6A and B). Molecules with the greatest upregulation in SHR relative to WKY were CD74 (HLA class II histocompatibility antigen gamma chain) and HLA-DR (IK cytokine, downregulator of HLA II) (Table 6C). CX3CL1 was the molecule with the greatest downregulation. Six of the 10 molecules with the greatest degree of downregulation in SHR control arteries were molecules involved in transcriptional regulation, including JUN, EGR1 (early growth response 1), and EGR2 (early growth response 2). In addition to these molecules with the greatest fold changes, several other molecules of potential significance in terms of impacting flow-mediated outward remodeling also exhibited altered expression. As can be seen in the online database, these include CYBA (cytochrome b-245, alpha polypeptide or p22phox), an NAD(P)H oxidase component, and NFKB1 (nuclear factor of kappa light polypeptide gene enhancer in B cells 1) which gives rise to p105 and the DNA-binding component of NFkB, p50. CYBA expression was increased 3.2 × (P = 0.027) in SHR; NFKB1 was downregulated 1.95 × (P = 0.044).

Table 6. Summary of IPA analysis for SHR versus WKY control arteries.

(A) Highest ranked networks

| Network ID | Associated network functions | Score |
|------------|------------------------------|-------|
| 1          | Cell Death, Gene Expression, Cellular Growth and Proliferation | 35    |
| 2          | Antigen Presentation, Cell-To-Cell Signaling and Interaction, Hematological System Development and Function | 29    |
| 3          | Inflammatory Response, Cell Death, Cell-To-Cell Signaling and Interaction | 26    |
| 4          | PostTranslational Modification, Protein Folding, Protein Degradation | 26    |
| 5          | Protein Synthesis, Cell Cycle, RNA PostTranscriptional Modification | 24    |

(B) Top biological functions: molecular and cellular functions

| Name                              | P-value | # Molecules |
|-----------------------------------|---------|-------------|
| Cell Cycle                        | 2.29E-06-2.05E-02 | 19          |
| Cell Death                        | 2.29E-06-1.88E-02 | 30          |
| Cellular Development              | 2.29E-06-1.99E-02 | 26          |
| Gene Expression                   | 1.90E-05-2.11E-02 | 28          |
| Cellular Growth and Proliferation | 4.75E-05-1.99E-02 | 34          |

(C) Molecules with greatest fold changes in SHR vs. WKY control arteries

| Fold change | P-value | Symbol | Entrez gene name | Description |
|-------------|---------|--------|------------------|-------------|
| 12.34       | 0.006   | CD74   | CD74 molecule, major histocompatibility complex, class II invariant chain |
| 12.27       | 0.002   | HLA-DRA| IK cytokine, downregulator of HLA II |
| 12.15       | 0.033   | APOD   | Apolipoprotein D |
| 5.94        | 0.004   | FCGR2A | Fc fragment of IgG, low affinity Ila, receptor (CD32) |
| 5.36        | 0.003   | MAF    | V-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian) |
| 5.33        | 0.007   | THBS4  | Thrombospondin 4 |
| 4.94        | 0.028   | DMBT1  | Deleted in malignant brain tumors 1 |
| 4.77        | 0.002   | ENTPD2 | Ectonucleoside triphosphate diphosphohydrolase 2 |
| 4.45        | 0.012   | FMO1   | Flavin-containing monoxygenase 1 |
| 4.45        | 0.029   | LYZ    | Lysozyme |
| −11.24      | 0.031   | CX3CL1 | Chemokine (C-X3-C motif) ligand 1 |
| −9.87       | 0.009   | NR4A3  | Nuclear receptor subfamily 4, group A, member 3 |
| −6.59       | 0.007   | VCAM1  | Vascular cell adhesion molecule 1 |
| −5.47       | 0.002   | KLF6   | Kruppel-like factor 6 |
| −5.37       | 0.024   | DRD1   | Dopamine receptor D1 |
| −5.17       | 0.023   | JUN    | Jun proto-oncogene |
| −4.08       | 0.001   | IRF1   | Interferon regulatory factor 1 |
| −4.05       | 0.012   | EGR1   | Early growth response 1 |
| −3.97       | 0.001   | DUSP1  | Dual specificity phosphatase 1 |
| −3.70       | 0.002   | EGR2   | Early growth response 2 |

IPA, Ingenuity pathway analysis; WKY, Wistar Kyoto; SHR, spontaneously hypertensive rat.
Transcription factor binding sites

The IPA analyses described above implicated potential differences in transcription factor expression and transcriptional regulation between SHR and WKY in collateral and control arteries. Therefore, Motif Modeler (Liu et al. 2006) was used to identify predicted transcription factor binding sites based on the molecules whose gene expression was altered in the collateral arteries. Potential binding sites were found for 19 potential transcription factors in WKY and SHR. Remarkably, there was no overlap in the predicted transcription factors between WKY and SHR (Table 7). Several of the identified binding sites in WKY collaterals were for transcription factors known to be sensitive to mechanical stimulation (shear stress and/or circumferential wall stress) or redox status, including at least three known to be influenced by both mechanical stimuli and reactive oxygen or nitrogen species (NFkB [Allen and Tresini 2000; Davis et al. 2004; Lan et al. 1994], AP-1 [activator protein 1] [Lan et al. 1994; Allen and Tresini 2000], and NF-E2 [nuclear factor, erythroid derived 2] [Dai et al. 2007; Warabi et al. 2007]).

Assessment of the potential functional significance of microarray results

A pathway list was created in IPA which included molecules selected from those with the greatest fold changes.

Table 7. Number of altered genes with predicted transcription factor binding sites in WKY and SHR.

| Transcription factor | WKY # | SHR # | Transcription factor | WKY # | SHR # |
|----------------------|-------|-------|----------------------|-------|-------|
| GATA-1               | 24    | 0     | AML-1a               | 0     | 54    |
| Zic1                 | 24    | 0     | AML1                 | 0     | 54    |
| PR                   | 23    | 0     | Pax-2                | 0     | 24    |
| NF-E2                | 22    | 0     | IRF-1                | 0     | 20    |
| AP-1                 | 22    | 0     | MyoD                 | 0     | 19    |
| STAT3                | 22    | 0     | PPARγ:RXR-α         | 0     | 19    |
| NF-kappaB (p65)      | 21    | 0     | NERF1a               | 0     | 18    |
| Ik-1                 | 20    | 0     | Myogenin             | 0     | 18    |
| Zic2                 | 20    | 0     | E2A                  | 0     | 18    |
| GR                   | 20    | 0     | TATA                 | 0     | 17    |
| PBX                  | 19    | 0     | E12                  | 0     | 17    |
| YY1                  | 18    | 0     | SMAD-4               | 0     | 17    |
| CDP                  | 13    | 0     | PEBP                 | 0     | 15    |
| SOX-9                | 13    | 0     | SF-1                 | 0     | 14    |
| Crx                  | 13    | 0     | T3R                  | 0     | 14    |
| ELF-1                | 13    | 0     | ERR alpha            | 0     | 13    |
| HNF4, COUP           | 12    | 0     | MEIS1A:HOXA9         | 0     | 12    |
| SREBP-1              | 11    | 0     | CDP CR1              | 0     | 11    |
| OCT1                 | 7     | 0     | Zic3                 | 0     | 11    |

Symbol of transcription factor or regulatory-binding site followed by full name and the TFBS accession number in brackets. AML1, acute myeloid leukemia 1 (also known as runt-related transcription factor 1, Runx1) [M00751]; AML-1a, acute myeloid leukemia 1a, transcription factor encoded by AML1 [M00271]; AP-1, activator protein 1 [M00174, M00188, M00925, M00926]; CDP, CCAAT displacement protein/Cut homeobox [M00095]; CDP CR1, Cut repeat 1 of CDP [M00104]; Crx, cone-rod homeobox [M00623]; E12, E box protein E12 [M00693]; E2A, E2A immunoglobulin enhancer-binding factors E12/E47 (also known as TCF3, transcription factor 3 [M00973]; ELF-1, E74-Like Factor 1 (Ets Domain Transcription Factor) [M00746]; ERR alpha, estrogen-related receptor, alpha [M00511]; GATA-1, GATA-binding protein 1 [M00062]; GR, Glucocorticoid receptor (also known as nuclear receptor subfamily 3, group C, member 1, NR3c1) [M00955]; HNF4, hepatocyte nuclear factor 4, alpha [M00967]; Ik-1, IKF1-IKAROS family zinc finger 1 [M00086]; IRF-1, interferon regulatory factor 1 [M00062]; MEIS1A:HOXA9, homeobox A9 [M00420]; MyoD, myogenic differentiation 1 [M00001, M00184]; myogenin [M00712]; NF-E2, nuclear factor, erythroid derived 2 [M0037]; NERF1a, E74-like factor 2 (ets domain transcription factor) [M00531]; NF-kappaB (p65), nuclear factor of kappa light polypeptide gene enhancer in B cells [M00052]; OCT1, organic cation transporter 1, POU class 2 homeobox 1 [M00135]; PEBP, phosphatidylethanolamine-binding protein [M00984]; PPARγ, peroxisome proliferator-activated receptor alpha [M00518]; SF-1, splice site 1 [M00727]; SMAD-4, SMAD family member 4 [M00733]; SOX-9, SRY (sex determining region Y)-box 9 [M00410]; SREBP-1, Sterol regulatory element-binding transcription factor 1 [M00220]; T3R, Thyroid hormone receptors [M00963]; TATA, TATA box [M00216]; YY1, YY1 transcription factor [M00059]; Zic1, Zic family member 1 [M00448]; Zic2, Zic family member 2 [M00449]; Zic3, Zic family member 3 [M00450]. WKY, Wistar Kyoto; SHR, spontaneously hypertensive rat.
Figure 4. IPA constructed network of key molecules selected from those with the greatest fold changes (Esm1, CX3CL1, CD74, HLA-DR, TGFβ3, ILβ1, GADD45α, SLC7A1), members of highest scored Canonical pathways (eNOS, SLC7A1, AGT, AT1R, TGFβ3), and the NAD(P)H oxidase component, CYBA. Altered regulation of several of these molecules was confirmed by RT-PCR (Table 8). IPA generated connections between the molecules and overlaid specific functions.

Table 8. Comparison of microarray and RT-PCR analyses for selected genes important in arterial remodeling.

|               | Microarray | RT-PCR |
|---------------|------------|---------|
|               | WKY        | SHR     | WKY    | SHR    |
| (A) Control artery relative expression |
| Cyba          | 393 ± 167  | 1104 ± 169† | 0.928 ± 0.095 | 2.140 ± 0.495† |
| (B) Collateral/control ratio |
| Agt           | 0.159 ± 0.106* | 0.674 ± 0.415 | 0.401 ± 0.088* | 0.563 ± 0.288 |
| AGTR1b        | 0.463 ± 0.208 | 0.482 ± 0.198* | 3.018 ± 0.703*‡ | 0.425 ± 0.156† |
| CX3CL1        | 0.564 ± 0.376* | 0.645 ± 0.394 | 0.533 ± 0.102* | 0.479 ± 0.062* |
| ESM1          | 3.99 ± 1.298* | 1.81 ± 0.545 | 4.758 ± 1.159* | 2.169 ± 0.356* |

Data are given as relative expression for cyba in control arteries and for collateral/control ratios for the remaining molecules. Results between Microarray and RT-PCR were similar except for AGTR1b in WKY where expression was increased rather than decreased, (N ≥ 4; *, †, ‡: P ≤ 0.05 Collateral vs. Control, SHR vs. WKY, qRT-PCR vs. Microarray). Cyba, cytochrome b-245, alpha polypeptide, p22-phox; Agt, angiotensinogen; AGTR1b, angiotensin II receptor, type 1b; CX3CL1, chemokine (C-X3-C motif) ligand 1; ESM1, endothelial cell-specific molecule 1. WKY, Wistar Kyoto; SHR, spontaneously hypertensive rat.

(ESM1, endothelial specific molecule 1; CX3CL1; CD74; HLA-DR; TGFβ3; ILβ1; GADD45α; SLC7A1), members of the highest ranked Canonical pathways (eNOS, endothelial nitric oxide synthase; SLC7A1; AGT, angiotensinogen; AGTR1b, TGFβ3), and the NAD(P)H oxidase component, CYBA. The interconnecting pathway and biological functions generated by IPA from this list are
shown in Figure 4. Altered regulation of several of these was confirmed by RT-PCR (Table 8).

NFXB was central to the generated pathway and linked to the major functions of gene expression, cell growth and proliferation, and cell cycling. It was included within the list of transcription factors with predicted binding sites in genes altered in WKY but not SHR collaterals that were sensitive to both mechanical stimuli and redox status. To assess its potential role, NFXB immunoreactivity and nuclear localization were assessed in control and collateral arteries in SHR and WKY rats. Representative arterial cross-sections are shown in Figure 5A. While there was a remarkable increase in the percent of nuclei with immunoreactivity in the intima, media, and adventitia of WKY collaterals relative to same animal control arteries, no such increase was observed in the SHR collaterals (Fig. 5B). As changes in redox status have been reported to both activate and inhibit NFXB (Allen and Tresini 2000; Grumbach et al. 2005), additional SHR rats were treated with the antioxidant apocynin which restores a normal redox status.

Figure 5. Nuclear localization of NF-κB during successful and impaired collateral growth. (A) Representative images showing NF-κB immunoreactivity (brown) in collateral arteries 3 days after arterial ligation in WKY, SHR, and apocynin pretreated SHR (SHR+Apo). Nuclear localization is apparent especially within the intima of WKY and SHR+Apo, as indicated by arrows, but not in SHR. (B) Analysis of the percentage of cells with immunoreactivity in each wall layer indicates a statistical increase in all wall layers of collaterals from WKY and SHR+Apo relative to same animal controls, (n ≥ 3, *P ≤ 0.001). (C) Inhibition of p65 expression suppressed collateral growth. Paired comparisons of control and collateral arteries before and 7 days after arterial ligation demonstrated significant collateral enlargement in WKY administered a control nonsense siRNA (*P ≤ 0.001, n = 4) but not in WKY pretreated with siRNA to p65 (P ≤ 0.001 vs. nonsense siRNA collateral, n = 3). No effect of p65 siRNA was observed on the diameters of control arteries.
in SHR mesenteric arteries (Zhou et al. 2008). When arterial ligation was performed in these animals, nuclear localization occurred within the collaterals similar to that observed within the WKY rats (Fig. 5A and B). To further assess the role for NFkB in collateral growth, we assessed the effect of inhibiting p65 expression with siRNA. The results demonstrated that WKY receiving p65 siRNA had significantly suppressed collateral growth compared with rats receiving a control (nonsense) siRNA (Fig. 5C).

One deviation of the RT-PCR results from the microarray analysis was the increased rather than decreased expression of the AGTR1b in WKY. Because of the potentially important role of the RAS and especially AGTR1 in arterial remodeling, additional studies were performed to assess the role of the AGTR1 in collateral growth. Immunostaining for the AGTR1 showed a remarkable increase throughout the arterial wall of WKY but not SHR collaterals (Fig. 6A and B). A functional role for the AGTR1 in successful collateral growth was assessed in additional experiments in which a group of WKY received losartan to suppress AGTR1 activation. These results demonstrated that collateral diameter enlargement was prevented by pretreatment with losartan (P = 0.215), which prevents collateral growth in WKY. No effect of losartan was observed on the diameters of control arteries, consequently the open bars are not apparent on the graph (n = 5). The combined data suggest that the upregulation and activation of the AGTR1 is not only correlated with, but is also required for collateral growth in the young normotensive WKY.

**Discussion**

To our knowledge, this is the first microarray study to compare primary collateral arteries from animals with and without known cardiovascular risk factors. Comparisons between the normotensive WKY and the SHR (1) demonstrate profound differences in the overall collateral...
gene expression, (2) suggest redox-dependent transcriptional regulation as a fundamental underlying cause, and (3) identify important biological functions and molecular pathways that may mediate the impaired remodeling of collateral arteries in the presence of cardiovascular risk factors. Advantages and limitations of the model and approach and the potential significance of these observations are considered below.

**Advantages and limitations of the model and approach**

In the present study, we focused on differences in gene expression between mesenteric collateral arteries in WKY which exhibit significant growth and in SHR collaterals which do not enlarge (Tuttle et al. 2002b; Miller et al. 2007a). The SHR strain was selected because it has multiple vascular risk factors including essential hypertension, metabolic abnormalities, and abnormal redox status (Okamoto and Aoki 1963; Potenza et al. 2005; Zhou et al. 2008) and is documented to have impaired compensation to arterial occlusion in the peripheral circulation including the mesentery and hindlimb (Nelissen-Vrancken et al. 1992, 1993; Scheidegger et al. 1997; Emanuelli et al. 2001, 2002; Tamarat et al. 2002; Tuttle et al. 2002b; Srivastava et al. 2003; Iaccarino et al. 2005; Miller et al. 2007a; You et al. 2008; Matsumura et al. 2009). We chose to use the mesenteric model of collateral growth because it has a simple, well-defined collateral pathway that facilitates identification and isolation of the primary collaterals and controls. In addition, vessels from one rat contain sufficient DNA for microarray analysis with amplification. To our knowledge, these are the model and strain combinations that are best characterized in terms of collateral artery blood flow, wall shear rate, and periarterial NO and $H_2O_2$ concentrations (Unthank et al. 1996a,b; Tuttle et al. 2001, 2002b; Zhou et al. 2008). Blood flow and wall shear rate increase in collaterals of both strains to approximately the same degree (Tuttle et al. 2002b). While both flow-mediated collateral growth and NO production are fully suppressed in the SHR mesenteric arteries, both are completely restored by antioxidants (Tuttle et al. 2002b; Miller et al. 2007a; Zhou et al. 2008).

In contrast, in more complex tissues there are multiple potential collaterals which experience unknown hemodynamic and redox changes and enlarge to different degrees, or even regress (Longland 1953; Herzog et al. 2002; Distasi et al. 2009). In addition, collateral vessels in other organs are embedded within the parenchymal tissue and it can be difficult to both identify and isolate them. While it is possible that responses in mesenteric arteries may not represent completely what occurs in other organs, flow-mediated remodeling is a universal phenomenon within the arterial tree (reviewed by Unthank et al. [2011]) and available studies indicate similarities in cellular proliferation, matrix activation, and gene expression and impairment by risk factors associated with oxidative stress (Masuda et al. 1989; Unthank et al. 1996a,b; Miyashiro et al. 1997; Tuttle et al. 2001, 2002b; Sho et al. 2002, 2003; Xu et al. 2002; Haas et al. 2007).

Differences between strains in mechanical stimuli associated with blood flow and pressure would certainly influence gene expression in control arteries as well as flow-mediated collateral growth and gene expression. However, we have previously observed the increases in collateral flow and wall shear rate in this model to be similar between these two strains (Tuttle et al. 2002b). We have also demonstrated that the impairment of collateral growth in the SHR is independent of hypertension; the effect of agents on reversal of collateral growth impairment in SHR is more dependent upon antioxidant than antihypertensive properties (Miller et al. 2007a). Thus, we do not consider differences in pressure or collateral blood flow to be the primary cause of impaired collateral growth or abnormal gene expression in the SHR.

It is important to note that flow-mediated remodeling is mediated by multiple, complex mechanisms and the specific mechanisms responsible for impairment of flow-mediated remodeling may differ depending upon specific risk factors present (Kinnaird et al. 2008) and genetic background (Hochberg et al. 2002; Sheridan et al. 2007; Ceyhan et al. 2012). In this regard, while the WKY is typically used as the normotensive control for the SHR, genetic differences exist between these two inbred strains. Consequently, differences in collateral growth capacity and gene expression could result from the genetic differences between these strains. Thus, these results in SHR may not be representative for other conditions or rat strains including other models of hypertension. However, aging results in a similar impairment of flow-mediated remodeling (Miyashiro et al. 1997; Tuttle et al. 2002a) where a fundamentally different expression pattern in collaterals is observed at the protein level (Tuttle et al. 2002a). Thus, our current and previous (Tuttle et al. 2002a) studies suggest that abnormal transcriptional or translational mechanisms are involved in the impairment of collateral growth that may be associated with risk factors that include hypertension and aging.

Interpretation of microarray analysis of heterogeneous tissues can be difficult and has significant limitations. Expression changes which occur in one cell type may not be apparent if similar changes do not occur in other cell types or may be masked by changes in the opposite direction. Assessment of potential networks and pathways and interconnections can lead to incorrect conclusions because expression changes may be occurring in different cells. Also,
differences in the baseline state can confound conclusions. In the current study, the samples were a heterogeneous mixture of the various cells normally present in the arterial wall. It is likely that differences in relative proportions existed between control arteries of WKY and SHR. However, our within-subject design comparing same animal collaterals to controls provided some control for such baseline genetic differences in the control arteries. Our network analyses are presented primarily to emphasize the overall difference in the gene expression patterns and indicate potential significance. The pathways are presented as areas where further investigation is warranted. Indeed, previous microarray studies in a similar tissue type (Dai and Faber 2010) and even more diverse tissues/organs (Lee et al. 2004; Packham et al. 2009) have demonstrated the ability of heterogeneous tissues to identify specific molecules, functional groups, and pathways which mediate collateral growth.

**Fundamental difference in gene expression patterns**

**Potential cause of impaired collateral growth**

The enlargement of preexisting arteries which form alternative or collateral perfusion pathways and experience increased blood flow and shear stress after arterial occlusion is a complex process involving many coordinated cellular and molecular processes (Wahlberg 2003; Simons 2005). Changes in gene and protein expression of molecules regulating cell cycling, growth, development, and migration are associated with successful collateral growth (Lee et al. 2004; Dai and Faber 2010). Dai and Faber demonstrated that hindlimb collaterals from eNOS null mice exhibited reduced collateral growth and cellular proliferation and reduced expression of the majority of the upregulated cell cycle genes (Dai and Faber 2010). Our data in SHR with abnormal mesenteric NO regulation (Zhou et al. 2008) and impaired collateral growth (Tuttle et al. 2002b) are consistent with this observation, but indicate a potentially more global and fundamental difference in gene expression. While a similar number of genes had altered expression in collaterals of WKY and SHR, there was very little similarity or overlap in the specific genes altered (Fig. 2, Tables 4 and 5). This impacted not only groups of genes associated with cell cycling and proliferation but essentially every network, cellular, and molecular function, and canonical pathway examined (Fig. 2, Tables 1–3). Such global differences in gene expression are consistent with our previous proteomic study in aged rats (Tuttle et al. 2002a) and suggest that collateral growth impairment results from an abnormality in a fundamental process or regulatory mechanism that occurs at a level between signal transduction and gene transcription.

**Potentially important molecules and canonical pathways**

The comparison of global gene expression changes between WKY and SHR control and collateral arteries reveals many differences that may be of importance in advancing our understanding of the promotion and impairment of flow-mediated outward remodeling. It is not possible to discuss all of these within the scope of this paper. Altered molecules that may influence vascular growth, cell proliferation, and inflammation include CX3CL1 (fractalkine, neurotactin) (Green et al. 2006; Ryu et al. 2008; Borghese and Clanchy 2011), ESM1 (endocan) (Bechard et al. 2001; Béchard et al. 2001; Aitkenhead et al. 2002; Sarrazin et al. 2006; Rennel et al. 2007), CD74, HLA-DR (Schirmer et al. 2009; Borghese and Clanchy 2011; Fan et al. 2011), and GADD45A (Zhan et al. 1994; Bruemmer et al. 2005). Potentially relevant canonical pathways include mitochondrial dysfunction and signaling pathways involving nitric oxide (NO), TGFβ, and the renin–angiotensin system. Mitochondrial dysfunction may occur in the SHR (Graham et al. 2009; Piotrzkowski et al. 2009) and has been linked to impaired vascular compensation for repetitive ischemia and reperfusion in the heart of obese Zucker rats (Pung et al. 2012). While the specific enzymatic and cellular sources of NO remain controversial (Dai and Faber 2010; Troidl et al. 2010), the majority of available evidence supports a substantial role for NO in flow-mediated remodeling and collateral growth. Abnormal NO signaling is observed (Maffei et al. 2002; Zhou et al. 2008), and flow-mediated, NO dilation is impaired (Qui et al. 1994; Matrougui et al. 1997) in SHR mesenteric arteries. Consistent with these observations, our work has shown that in vivo NO production is not increased with flow in SHR mesenteric arteries but that basal NO levels are greatly elevated by abnormally high concentrations of hydrogen peroxide (Zhou et al. 2008). The latter observation is consistent with reports of increased eNOS activity and/or elevated NO production in SHR (Kelm et al. 1995; Nava et al. 1995, 1998; Qiu et al. 1998; Briones et al. 2000; Maffei et al. 2002). While eNOS and iNOS expression and inhibition have been the primary end points evaluated in NO-dependent collateral growth, the abnormal regulation discussed above and the altered expression of multiple molecules within the cardiovascular NO signaling pathway of WKY (Fig. 3) suggest that a more comprehensive analysis is needed to understand the role of this important signaling pathway in collateral growth.

The renin–angiotensin system may be involved in both the promotion and impairment of vascular compensation to arterial occlusion (Silvestre et al. 2002; Tamarat et al.
translocation (Fig. 5), and collateral growth capacity in the SHR (Miller et al. 2007a). The specific mechanisms responsible for the inhibition of NFkB nuclear translocation in response to elevated shear stress in the SHR warrant more investigation and may involve NO-dependent mechanisms such as p50 nitrosylation (Matthews et al. 1996; Grumbach et al. 2005), downregulation of NFkB1 and therefore p50, or inhibition by elevated H2O2 as observed in human T cells (Flescher et al. 1998) at concentrations similar to those we have measured in the SHR mesenteric arteries (Zhou et al. 2008). Such inhibition of activation and/or decreased expression of NFkB components could mediate, at least in part, the abnormal expression pattern we observed in SHR collaterals in this study as well as the impairment of outward remodeling and cellular proliferation (Tuttle et al. 2002b). Regardless of the specific molecules involved, the present data support the hypothesis that chronic redox changes in the vasculature alter the transcriptional regulation that occurs in response to mechanical stimuli such as shear stress in collateral arteries subsequent to arterial occlusion.

**Conclusion and Clinical Significance**

As reviewed by Ziegler et al. (2010), clinical observations indicate a remarkable capacity for vascular compensation to peripheral arterial occlusion which is profoundly compromised in the presence of vascular disease risk factors. The flow-mediated dilation and enlargement of preexisting small arteries provide the primary compensation to focal arterial occlusion in rodent and human limbs (reviewed by Ziegler et al. [2010]). The enlargement of these vessels is impaired in the presence of vascular risk factors, but the specific mechanisms responsible are unknown and represent important targets for novel therapies. Such therapies to promote vascular growth and function are needed to prevent the progression of peripheral arterial disease (Gornik 2009). Significant evidence has accumulated that oxidative stress and a proinflammatory state are associated with impaired flow-mediated remodeling and collateral growth in animals and humans (Vita et al. 2008; Ziegler et al. 2010) and that antioxidant therapy reverses this impairment. Our results support the hypothesis that abnormal transcription factor activation and gene expression mediate, at least in part, the impairment of collateral growth in the SHR. Based upon our recent observations of abnormal H2O2 and NO in SHR and aged collaterals (Zhou et al. 2008, 2009), and the abnormal collateral gene (current study) and protein (Tuttle et al. 2002a) expression, we further hypothesize that redox status modulates shear-mediated gene expression in collateral arteries. Thus, therapies to correct an imbalance between reactive nitro-
gen and oxygen species in the presence of vascular disease risk factors could be important either as primary or adjuvant treatment to enhance collateral development.

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Author Contributions

Conception and experimental design: J. L. U., J. N. M., S. J. M. Conducted various experiments and data collection: J. L. U., J. N. M., C. A. L., M. R. D., S. J. M. Data analysis and interpretation: J. L. U., J. N. M., C. A. L., L. L., M. R. D., S. J. M. Preparation of data for presentation: J. L. U., C. A. L., M. R. D., S. J. M. Preparation of manuscript draft: J. L. U., S. J. M., C. A. L., M. R. D. Critical review and revision and final approval of manuscript: J. L. U., J. N. M., C. A. L., L. L., M. R. D., S. J. M.

Conflict of Interest

None declared.

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