Articles in PresS. Physiol Genomics (May 4, 2004). 10.1152/physiolgenomics.00057.2004

Cardiac transcriptional response to acute and chronic angiotensin II treatments

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Keywords: hypertension, renin-angiotensin-aldosterone system (RAAS), angiotensin II (Ang II), microarray

ABSTRACT

Exposure of experimental animals to increased Angiotensin II (Ang II) induces hypertension associated with cardiac hypertrophy, inflammation, and myocardial necrosis and fibrosis. Some of the most effective anti-hypertensive treatments are those that antagonize Ang II. We investigated cardiac gene expression in response to acute (24 hour) and chronic (14 day) infusion of Ang II in mice; 24 hour treatment induces hypertension, and 14 day treatment induces hypertension and extensive cardiac hypertrophy and necrosis. For genes differentially expressed in response to Ang II treatment, we tested for significant regulation of Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Microarray Pathway Profiler (GenMAPP) databases as well as functional classes based on Gene Ontology (GO) terms. Both acute and chronic Ang II treatments resulted in decreased expression of mitochondrial metabolic genes, notably those for the electron transport chain and Krebs cycle; chronic Ang II treatment also resulted in decreased expression of genes involved in fatty acid metabolism. In contrast, genes involved in protein translation and ribosomal activity increased expression following both acute and chronic Ang II treatments. Some classes of genes showed differential response between acute and chronic Ang II treatments. Acute treatment increased expression of genes involved in oxidative stress and amino acid metabolism, whereas chronic treatments increased cytoskeletal and extracellular matrix genes, second messenger cascades responsive to Ang II, and amyloidosis genes. Although a functional linkage between Alzheimer's disease, hypertension, and high cholesterol has been previously documented in studies of brain tissue, this is the first demonstration of induction of Alzheimer's disease pathways by hypertension in heart tissue. This study provides the most comprehensive available survey of gene expression changes in response to acute and chronic Ang II treatment, verifying results from disparate studies, and suggests mechanisms that provide novel insight into the etiology of hypertensive heart disease and possible therapeutic interventions that may help to mitigate its effects.

INTRODUCTION

The renin-angiotensin-aldosterone system (RAAS) results in the formation of angiotensin II (Ang II) and is a major contributor to hypertension and the resulting target organ damage. Ang II is critical in stimulating physical and metabolic changes seen in hypertension, which is why the most effective anti-hypertensive treatments are those that antagonize the RAAS. Acute increase in Ang II levels elicits an immediate rise in blood pressure due to vasoconstriction and stimulation of other hormones, whereas chronic infusion of Ang II results in damage to vulnerable organs despite the tendency of blood pressure to return toward baseline. Although a 24 hour infusion may not produce visible pathological changes, it may alter the expression of genes in various tissues and was shown to produce evidence of myocardial cell damage (62). The earliest pathologic changes are seen after 2 to 3 days of infusion and consist of myocyte and renotubular cell necrosis with myocardial and renal scarring (25). Chronic infusions of 1-2 weeks duration lead to hypertrophy, necrosis and fibrosis of the myocardium (71).

In the heart, Ang II affects expression of a wide range of genes that underlie these varied physiological responses. Ang II increases the expression of regulatory, structural and cytokine genes that induce cardiac hypertrophy, extracellular matrix (ECM) formation, inflammation, and vascular remodeling and regulate blood pressure (11, 64). Ang II also increases reactive oxygen species and oxidative stress and depresses mitochondrial energy metabolism (55). Ang II up-regulates signaling pathways, including mitogen-activated protein kinase (MAPK), RhoA kinase, tumor necrosis factor beta (TGF beta), signal transducer and activator of transcription (STATs), and nuclear factor-kappaB (NF-kappaB) pathways.

Recent large outcome trials comparing antihypertensive agents have shown that drugs inhibiting the RAAS lead to end organ protection beyond that attributable to blood pressure lowering (13), thus corroborating the hypothesis that Ang II enhances cardiovascular tissue damage (24). However, little is known about the cellular and molecular alterations that precede and usher the development of visible

pathological changes in these tissues. In this study we conducted a comprehensive analysis of gene expression in mice subjected to acute (24 hour) and chronic (14 day) exposure to exogenous Ang II. The goal was to evaluate alterations in the expression of genes relevant to cellular integrity, trophic functions, and proliferation that might help us understand the mechanisms triggering the target organ damage seen in RAAS-associated hypertension. To this end, we analyzed regulation of GenMAPP and KEGG pathways and functional classes of genes based on GO term assignments. This information might be useful in the formulation of rational therapeutic interventions addressing specific mechanisms relevant to cardioprotection.

EXPERIMENTAL PROCEDURES

Animal handling

Acute or chronic Ang II infusion treatments were conducted using four groups of male 10-week old C57BL/6J mice obtained from the Jackson Laboratories (Bar Harbor, Maine). Animals were housed in the animal quarters with a 12-hour light/dark cycle, in pathogen free, temperature and humidity controlled room (22°C and 45-55% respectively) with food (Purina Rodent Chow 5002) and distilled water *ad libitum*.

Acute Ang II infusion: Two groups of mice were submitted to intravenous infusion of Ang II (N = 8) or saline (N = 7) for 24 hours while under constant direct (intra-arterial) blood pressure (BP) monitoring. Under anesthesia with intraperitoneal pentobarbital (50 mg/kg), the right iliac artery was catheterized using a modified polyethylene catheter PE-50 tubing flushed with approximately 50µl of 50 IU/ml heparin in 0.9 % saline. The right iliac vein was catheterized with silastic silicon tubing for drug administration. Both lines were exteriorized at the back of the neck and sealed with heat. After surgery, the mice were allowed a recovery period, and were housed overnight in separate cages with food and water.

Following a 24 hour recovery period, the two lines were unsealed and attached to a swivel. The arterial line was connected to a BP transducer, and mean BP was recorded with a computerized data-acquisition system (Power Lab/400, AD Instrument Pty Ltd, Castle hill, Australia). The venous line was connected to a Harvard infusion pump (Harvard Apparatus, Holliston, MA) for drug infusion. The baseline BP was recorded until it became stable. At this point, infusion of Ang II (30 ng/minute) or normal saline started and continued for a period of 24hours.

Chronic Ang II infusion: Two other groups of received Ang II (N = 9) or saline (N = 7) infusion, respectively, for 14 days via an osmotic minipump. The osmotic minipump (model 2002, Alzet Co., Colorado City, CO) was implanted subcutaneously, slightly posterior to the scapula under anesthesia with pentobarbital 50mg/kg ip. Ang II was dissolved in 0.5 mol/l NaCl and 1mmol/l acetic acid, at concentrations sufficient to allow an infusion rate of 40ng/min, known to produce hypertension. Control mice received saline solution via the osmotic minipump. Indirect systolic BP was monitored daily for 14 days by a noninvasive tail-cuff system (BP-2000, Visitech System, Apec, NC).

Tissue harvesting: At the end of treatment (2:00- 4:00 PM), the mice were euthanized with overdose pentobarbital and the hearts were quickly (<3 minutes) removed for mRNA preparation. The dissected tissues (<0.5 cm in any length) were submerged in approximately five volumes of RNAlater (Ambion, Austin, TX). Samples were kept at 4°C overnight and stored at –20°C individually. Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA), according to manufacturer specifications.

Microarray fabrication, RNA labeling and hybridization

Microarrays were constructed using the NIA 15k and BMAP mouse cDNA clone sets that together contain 27,010 clones representing approximately 22,000 unique transcript probes. PCR amplicons were prepared for printing as described previously (26). Following amplification and

purification, amplicons were resuspended at 100-200 nM in 50% DMSO and printed onto UltraGAPs aminosaline-coated slides (Corning Inc., Corning, NY) using an Intelligent Automation System (IAS) arrayer (Cambridge, MA). After printing, DNA was cross-linked to the slides by UV irradiation with a Stratalinker UV Crosslinker (Stratagene, La Jolla, CA) and stored in a vacuum chamber until used.

Detailed cDNA target preparation and hybridization protocols are available at http://pga.tigr.org/protocols.shtml. Briefly, cDNA was synthesized by random-primed reverse transcription in the presence aminoallyl dUTP using 10 μg of total RNA. Reaction products were purified and coupled to Cy3 or Cy5 NHS-ester (Amersham, Piscataway, NJ). The labeled cDNAs were purified, combined as appropriate for each hybridization, and lyophilized.

Slides were prehybridized in 1% Bovine Serum albumin in 5× SSC, 0.1% SDS for 45 minutes at 42^{o}C, after which the slides were washed and dried. Cy3 and Cy5 labeled cDNA was resuspended in 30ml of 50% formamide, 5×SSC, 0.1% SDS containing 0.5 μg mouse COT1-DNA, 1 μg poly-dA and hybridized to the microarray at 42^{o}C for 16 hours under glass coverslips. Following hybridization, slides were washed for 4 minutes at 42^{o}C in solution containing 1×SSC and 0.2% SDS, followed by a 4 minute wash of 0.1×SSC, 0.1% SDS at ambient temperature; then by two 2.5 minute washes of 0.1×SSC; at the ambient temperature. Slides were dried by centrifugation and scanned without delay at 10 μm resolution using an Axon 4000B scanner. Data were saved as 16-bit TIFF files and expression levels were extracted using TIGR Spotfinder (49).

Microarray experimental design and data analysis

Each experimental and control group consisted of 7 to 9 animals. RNA was combined to create two pools containing material from 3 to 5 randomly selected animals. To eliminate any possible bias in labeling or detection, paired dye-reversal hybridizations were performed for each comparison made. Experimental design incorporated both direct and reference comparisons (33). Direct comparisons hybridized RNA pools from experimental, Ang II -infused animals with matching control pools from

saline-infused animals. In reference hybridizations, each experimental and control RNA pool was compared to a common reference RNA (Stratagene Universal mouse reference). The expression level for each array element was derived from 8 data sources, two groups of pooled RNA, two direct (experimental vs. control) and two indirect (experimental vs. reference and control vs. reference) comparisons, each performed using dye-reversal labeling.

Prior to data analyses, signals were normalized using a locally weighted scatterplot smoothing regression (LOWESS) algorithm (18), implemented in the MIDAS software package (http://www.tigr.org/software/tm4; (49)) with the smoothing parameter set to 0.33 and flip dye consistency checking set to keep data with the range of ± 2.0SD. Data from both direct and indirect comparisons were used to compute comparisons of acute and chronic Ang II treatments. Analyses were performed only on genes with detectable hybridization signals in a minimum of 75% (6 of the 8) of the hybridizations, resulting in 23,510 array elements being included in the analysis. Data have been submitted to ArrayExpress with accession IDs E-TIGR-11 HTA1 and E-TIGR-12 HTA2.

To identify genes whose expression differed significantly between acute and chronic Ang II treatments, we performed two-class unpaired SAM (significance analysis of microarrays) comparing expression in acute and chronic treatments. SAM implementation in TIGR MeV 2.2 (49) was based upon that of Tuscher (66) with the computed exchangeability factor s0 in the 5th percentile (72). For this analysis delta (δ) = 0.9, and PiHat ($\hat{\pi}$) = 0.4. Missing values were imputed using *k*-nearest neighbors with *k*=10 and 100 permutations; the median false discovery rate was set to 0%. The resulting list of significant genes was grouped into 4 groups using *k*-means Support, with 10 iterations and an 80% concurrence for genes to be included in each cluster.

To identify genes consistently up- or down-regulated in response to both acute and chronic Ang II treatments, the data were subjected to a one-sample T-test, with alpha set at 0.05. Of these 4026 genes that showed significant co-regulation by the one-sample T-test, only those (N = 3563) that showed no significant difference between acute and chronic treatments, as determined by SAM (false discovery rate

= 0) were used for subsequent analysis. Of these 3563 genes, 1301 were consistently up-regulated in both acute and chronic Ang II treatments, whereas 2262 were consistently down-regulated.

EASE (http://apps1.niaid.nih.gov/David, (28)) analysis was performed on significant genes identified by SAM and one-sample T-test as described above using TIGR Gene Index (TC) identifiers. EASE uses a Fisher Exact test to estimate significance for functional classes of genes in a significant subset relative to the representation on the array. In addition to testing Gene Ontology (GO; http://www.geneontology.org) terms for Biological Process, Cellular Component, and Molecular Function, EASE was used to identify significantly over-represented biological pathways, using both KEGG (http://www.genome.ad.jp/kegg) and GenMAPP (http://www.genmapp.org/MAPP_lists.html) pathways. For each cluster of genes, the significant GO terms identified by EASE were mapped to their respective GO Slim (http://www.ebi.ac.uk/proteome/index.html?http://www.ebi.ac.uk/proteome/goslim_terms.html) terms, allowing for functional groupings of these GO terms. GO Slim is a reduced set of 32 non-overlapping, higher level, biologically relevant GO terms that cover most aspects of the three GO ontologies of cellular component, biological process, and molecular function.

"Real-time" reverse transcription polymerase chain reaction (RT-PCR) analysis

RNA was collected from hearts of mice following the protocols detailed above, for acute Ang II (N = 4), acute saline (N = 2), chronic Ang II (N = 4), and chronic saline (N = 4) treatments. Single-stranded cDNAs were generated from reverse transcription of RNA samples using the Taqman Reverse Transcription Kit (Applied Biosystems, Foster City, CA) and were then subjected to PCR with SYBR Green (Applied Biosystems) as the detected fluourophore. Incorporation of the SYBR Green dye into the PCR products was monitored in real time with ABI Prism 7900HT Sequence Detection System, resulting in the calculation of threshold cycle (C_T) that defines the PCR cycle at which exponential growth of PCR products begins. The ROX (carboxy-X-rhodamine) passive reference dye was used to

factor in well and pipetting variability. Standard curves were determined for each RNA sample being analyzed using the 18S Ribosomal RNA (Universal 18S rRNA kit, Ambion, Austin, TX). The standard curves were normalized to each other through the 18S rRNA amplification and quantitation subsequently determined.

The expression levels of sixteen genes selected from the microarray analysis were verified by real-time RT-PCR, and one gene not on the array was also measured. Genes were selected among those most strongly up or down regulated among each cluster determined from MeV analysis. Six genes identified by microarray analysis as being up-regulated whose expression was measured by qRT-PCR included brain natriuretic peptide (BNP), Down Syndrome critical region 1 (DSCR1), osteoblast Specific Factor 2 (OSF2), Ras-related protein 2 (R-Ras2), serotonin receptor 1D (S1Dr), and uridine-cytidine kinase 2 (UCK2). Also measured by qRT-PCR was atrial natriuretic factor (ANF), which was not on the array but which has been previously shown to have similar expression profile to BNP. Ten down-regulated genes whose expression was measured by qRT-PCR included amyloid beta A4 precursor protein-binding family b member 1 (ABB1), apolipoprotein D (ApoD), glucose transporter 4 (GLUT4), glucose-regulated protein 78 kDa (Grp78 or BiP), Stress protein Herp, serotonin receptor 2 (SR5-HT2), thyrotroph embryonic factor (TEF), uncoupling protein 3 (UCP3), Urb protein, and an unknown protein. Forward and reverse primers used for the qRT-PCR reactions are provided in Table 1.

Ratios of acute Ang II treated/acute saline and chronic Ang II treated/chronic saline were calculated for each gene, resulting in two values for each gene, one acute and one chronic. These ratios were $\log_2$ transformed, to facilitate comparison to $\log_2$ transformed ratios determined from microarray hybridizations. Microarray data were regressed against qRT-PCR data (N = 32 from 16 genes) using linear regression to determine whether qRT-PCR validated microarray results. When a gene was represented by multiple elements on the array, the mean $\log_2$-transformed value was used in the regression analysis.

RESULTS AND DISCUSSION:

Physiological effects of Angiotensin II treatments

Ang II treatment increased systolic blood pressure and decreased heart rate. At the end of 24 hour acute Ang II treatment, mice had significantly elevated mean blood pressure (BP) relative to acute saline controls, 129.4 ± 1.8 mmHg versus 100.4 ± 2.3 mmHg, respectively ($p < 0.001$). Following 14 days of chronic treatment, BP was further elevated in Ang II-treated mice relative to saline controls, 150.3 ± 5.1 mmHg versus 107.6 ± 4.1 mmHg, respectively ($p < 0.001$). Because of high variability, heart rate was not significantly lowered in acute Ang II treated mice relative to saline-treated controls (566.4 ± 54.9 beats/min versus 667.2 ± 47.5 beats/min, respectively, p = NS). Chronic Ang II treatment, however, resulted in significantly lower heart rate (539.4 ± 14.1 beats/min for Ang II-treated mice versus 669.7 ± 23.5 beats/min for control , $p < 0.05$), indicating appropriate activation of baroreflexes. Despite these changes in BP and heart rate, there was no difference in heart weight between groups. In the acute experiment, heart weight was 111.5 ± 2.5 mg in Ang II-treated vs. 109.6 ± 8.1 mg in saline-treated mice. In the chronic experiment, heart weight was 108.5 ± 3.2 mg in the Ang II-treated vs. 109.6 ± 4.6 mg in the saline-treated mice.

Microarray data analysis

1705 array elements exhibited a differential response to either acute or chronic Ang II treatments; that is, these genes were differentially regulated from saline controls in response to only one of the two treatments, acute or chronic Ang II. Of the 1705 genes that responded only to one of the two treatments, the majority (1572) responded to acute (24 hour) Ang II treatment and only 133 responded only to chronic (14 day) Ang II treatment. Of the 1572 acutely-responsive genes, 701 were up-regulated and 871 were down-regulated in acute Ang II treatment in comparison to expression levels of acute

saline controls (Figure 1). Of the 133 genes that responded to chronic Ang II treatment, 100 were up-regulated and 33 were down-regulated relative to chronic saline controls (Figure 2).

An additional 1456 genes formed a separate cluster that showed *both* decreased expression in response to acute Ang II treatment and increased expression in response to chronic Ang II treatment (Figure 3). 3563 genes were similarly regulated in response to acute and chronic Ang II treatments (Figure 3); these genes were consistently either up or down-regulated in both acute and chronic Ang II treatments. 1301 genes increased expression in response to both acute and chronic Ang II treatment relative to saline controls, while 2262 were down-regulated following both acute and chronic Ang II treatments (Figure 3).

The grouping of genes by functional classes and pathways provides insight that is simply not possible by looking at particular gene lists. EASE is an algorithm that compares the representation of functional classes and pathways for genes in a differentially regulated subset to the entirety represented on the array. EASE performs a Fischer Exact Test to calculate the probability that the composition of the differentially expressed set occurred by chance. By using a tool such as EASE, we are able to gain greater biologically insight into the functional processes activated or repressed by various stimuli much more efficiently and reliably than we can by using list of differentially regulated genes. We characterize each cluster by the significant pathways and GO terms identified by EASE and also investigate the most strongly regulated genes within each cluster.

Acute responses

Acutely Up-Regulated Genes:

Using EASE, we discovered that the 701 genes with increased expression in response to acute Ang II treatment (Fig. 1) had a significant over-representation of ribosomal and translational processes. The significant GenMAPP and KEGG pathways identified by EASE in the acutely up-regulated group overlapped, with one significant GenMAPP pathway for ribosomal proteins and two significant KEGG

pathways, ribosome and translation. The GenMAPP pathway for nucleotide metabolism had a low (p = 0.06) but non-significant EASE score.

Gene Ontology class (GO term) assignments identified as being significantly over-represented among the acutely up-regulated genes agreed with GenMAPP and KEGG pathway analysis. Of the 54 significant GO terms identified by EASE for the 701 acutely genes, the largest group (29%) were for ribosomes and protein translation and synthesis, supporting the KEGG and GenMAPP pathway analysis. Significant cellular component GO terms indicated enrichment of genes specific to ribonuclear-protein complex and intracellular/cytosolic localizations (Figure 4). Other groups of GO terms for included peroxisome, nucleotide metabolism, and polyamine metabolism (see supplementary data). These findings suggest that increased Ang II levels initiate an immediate and significant transcriptional response, consistent with the role Ang II plays in signal transduction. However, this immediate response is mitigated over time and is no longer a primary response in chronically-exposed individuals.

Many of the genes most strongly up-regulated in acute Ang II treatment have been previously identified as responding either directly to Ang II or in response to cardiac injury, stress, and hypertension. For instance, RNA binding motif protein 3 (RBM3) was +2.4 fold up-regulated in response to acute Ang II treatment; RBM3 is a cold-stress induced RNA binding protein that affects post-transcriptional regulation of gene expression, possibly facilitating translation by binding 18 S ribosomal RNA (17). RBM3 is up-regulated in response to cytokines and may be associated with tissue growth and differentiation as it is also up-regulated in proliferative processes during hematopoesis (6). Also strongly up-regulated acutely (+1.7 fold) was angiomotin, a cell-surface protein that transduces angiostatin-induced inhibition of cell motility and induction of apoptosis (65).

The significant increase (EASE, $p < 0.005$) of GO terms relating to polyamine metabolism and biosynthesis merits closer attention, as polyamines have been demonstrated to respond rapidly to Ang II treatment and are associated with cardiac hypertrophy. Ornithine decarboxylase (ODC) is the rate-limiting step in polyamine synthesis and an indicator of cell growth and differentiation. Both ODC and

polyamines are rapidly up-regulated in response to Ang II (30, 34), and over-expression of ODC in transgenic mice results in greatly enhanced cardiac hypertrophy following beta-adrenergic stimulation (53).

In our study, expression of genes relating to polyamine synthesis indicate a rapid increase in polyamine synthesis in response to acute Ang II, but this response and cellular polyamine levels return to basal in the chronic treatment. ODC, the rate-limiting step in polyamine synthesis, is regulated by the competing effects of ODC antizyme (OAZ), which binds to and degrades ODC, and antizyme inhibitor (OAZIN) which binds OAZ and thus stabilizes ODC. Increased cellular polyamine levels increase synthesis of OAZ, thus serving as a negative feedback loop on polyamine production. ODC was +2.0 fold up-regulated in the acute treatment but returned to basal expression in chronic Ang II treatment, indicating an acute increase in polyamine synthesis. OAZIN is strongly (+1.9 fold) and OAZ was weakly (+1.2 fold) up-regulated in response to acute Ang II treatment. The strong up-regulation of OAZIN supports increased polyamine synthesis in response to acute Ang II treatment (30, 34), and the weaker up-regulation of OAZ may be a response to increased polyamine levels.

Some of the most highly up-regulated genes in the heart following acute Ang II treatment regulate growth and development, including three growth inhibitors, Ras-related protein 2 (Rras2), neurite growth inhibitor (Nogo or RTN4), and thioredoxin. Rras2 had the greatest differential response of any gene in acute heart treatment, with more than four-fold up-regulation in response to acute Ang II treatment relative to saline controls. The oncogene Rras2 is a plasma membrane bound GTPase that transduces growth inhibitory signals and is expressed at highest levels in the heart (21). Nogo, also known as reticulon 4, is localized on the endoplasmic reticulum in central nervous system tissues and inhibits neurite outgrowth and was among the most highly expressed genes in response to acute Ang II treatment, with +3.0 fold increased expression. Despite the strong up-regulation of these growth inhibitors, several growth factors also had significantly increased expression: fibroblast growth factor

inducible protein 14 (FIN14) and fibroblast growth factor-related protein (FGF-12), and transforming growth factor beta (TGF beta) receptor type I were significantly increased in acute Ang II treatment.

Ang II induces oxidative stress and initiates a series of tissue responses that culminate in cardiac and renal tissue damage in chronic hypertension (5). Genes that protect against oxidative stress, such as thioredoxin and glutaredoxin, were up-regulated in acute heart treatment (+1.7 and +1.9 fold up-regulation, respectively, $p < 0.05$) but not in chronic Ang II treatments (+1.1 and -1.1 fold change, respectively, $p > 0.10$). Thioredoxin and glutaredoxin are redox-sensing, antioxidant molecules (54) that increase expression in damaged heart tissue. Thioredoxin concentrations in plasma and myocardium increase in heart failure and cardiomyopathy, reflecting oxidative stress (43), and glutaredoxin mediates hydrogen peroxide-induced decrease in mitochondrial respiration in rat cardiac tissue (48). Both molecules have additional growth-related functions; thioredoxin is a mediator of growth inhibition (27), and glutaredoxin facilitates growth-factor-induced polymerization and reorganization of filamentous actin by deglutathionylating globular actin (69). Skeletal muscle alpha-actin 1 was among the most strongly up regulated genes in acute Ang II treatment (+2.0 fold up-regulated), providing sufficient substrate for glutaredoxin-induced actin polymerization.

Other up-regulated oxidative stress-induced genes also demonstrated robust response to acute Ang II treatment. The transferrin receptor 1 (TfR1), which binds free iron ions and translocates them into the cell, was strongly up-regulated in response to acute Ang II treatment (+2.8 fold) but only moderately up-regulated in response to chronic Ang II treatment (+1.5 fold). Two superoxide dismutases (SOD) also showed differing expression patterns. Cytosolic Cu/Zn-SOD, which removes free radicals by forming hydrogen peroxide and oxygen was slightly up-regulated acutely (+1.2 fold) and down-regulated chronically (-1.2 fold), whereas mitochondrial SOD was strongly down-regulated in response to both acute (-2.3 fold) and chronic (-1.3 fold) Ang II treatments. Up-regulation of these oxidative stress responsive genes is of interest as reactive oxygen species and oxidative stress have been implicated in depression mitochondrial metabolism (38, 46, 47). Furthermore, Ang II has been

implicated in both generation of reactive oxygen species and in depression of mitochondrial metabolism (15, 51, 55).

Acutely Down-Regulated Genes:

Genes that were down-regulated in response to acute Ang II treatment were indicative of depression of mitochondrial metabolism. The strength and breadth of this response was reflected in both the pathway and GO term analyses. All four significant pathways for the 871 acutely down-regulated genes identified by EASE were related to mitochondrial energy metabolism: two GenMAPP pathways (the Krebs-TCA cycle and electron transport chain) and two KEGG pathways (energy metabolism and oxidative phosphorylation). Dysfunction of mitochondrial metabolism and the electron transport chain has been associated with heart failure (15), although the mechanism remains to be elucidated. Whereas acutely up-regulated genes were specific to the ribosome and cytosol, acutely down-regulated genes had significant cellular component GO terms for the mitochondria ($p < 0.0001$), extracellular matrix ($p < 0.001$), cytoplasmic microtubule ($p < 0.05$), and dystroglycan complex ($p < 0.05$). The dystroglycan complex connects the cytoskeleton to the extracellular matrix, thus stabilizing the sarcolemma. Disruption of the dystroglycan complex can lead to cardiac failure and necrosis (31). Almost half (46%) of the 62 significant GO terms identified by EASE for acutely down-regulated genes were energy pathway terms for carbohydrate or mitochondrial metabolism (Figure 1), including ten genes for H^+ transporter activity.

Critical genes regulating energy metabolism are down-regulated in failing human hearts, including uncoupling protein 3 (UCP3), glucose transporters 1 and 4 (GLUT1, GLUT4), and muscle carnitine palmitoyl transferase 1 (mCPT-1) (48). These genes were also down-regulated in response to Ang II treatments in the present study; mCPT-1, GLUT4, and GLUT8 were all strongly down-regulated in response to acute Ang II treatment (-2.2 fold, -3.0 fold, and -1.5 fold, respectively) but had little response to chronic Ang II treatment (-1.3 fold, -1.2 fold, and -1.1 fold, respectively). Expression of

GLUT4 decreases expression in aortic and cardiac arteries of rats with hypertension induced by treatment with deoxycorticosterone acetate (DOCA) and salt (4), implicating reduced glucose uptake and metabolism with contractile abnormalities in these animals. Further evidence that down-regulation of GLUT4 may be related to the cardiac remodeling is provided by GLUT4-null mice, which exhibit cardiac hypertrophy similar to that seen in hypertension and decreased fatty acid oxidation enzymes despite normal contractile properties, glucose transport and glycogen metabolism (57).

In contrast to mCPT-1 and glucose transporters, uncoupling proteins 2 and 3 (UCP2, UCP3) exhibited no significant change in expression in response to acute Ang II treatment (+1.1 and +1.0 fold) but were down-regulated chronically (-1.2 fold and -1.7 fold, respectively, $p < 0.05$). Chronic down-regulation of UCP3 may be related to a change of primary energy source to glucose from oxidation of fatty acids in response to sustained changes in cardiac workload seen in hypertension (60). Uncoupling proteins can be stimulated by cold stress, but UCP3 also regulates fatty acid metabolism and stimulates glucose uptake (10).

Genes for thyroid hormone receptors c-erbA alpha 1 and c-erbA alpha 2 were down-regulated in the acute Ang II treatment (-1.7 fold and -1.6 fold, respectively) but showed no change in expression in response to chronic Ang II infusion (+1.0 and -1.1 fold, respectively). Thus, acutely down-regulated genes had significant GO terms for thyroid hormone receptor activity, circulation, and regulation of heart, which supported the decreased in heart rate in Ang II treated mice. The known target genes were also down-regulated; thyroid hormone influences cardiac function by enhancing sarcoplasmic/endoplasmic reticulum calcium ATPase 2 (SERCA2) and co-regulating the insulin-responsive glucose transporter 4 (GLUT4) in the heart (16, 52), both of which were down-regulated in response to Ang II treatment.

Expression changes common to both acute and chronic responses

Acute and Chronic Up-Regulated Genes

Although twice as many genes were similarly regulated in response to both acute and chronic Ang II treatments as responded to only acute treatment, the significantly regulated pathways were similar in both groups. Of the 1301 genes up-regulated in response to both acute and chronic Ang II treatment, EASE analysis found significant KEGG and GenMAPP pathways for ribosome and translation ($p < 0.05$), as it had for acutely up-regulated genes. The ribosome pathway included 14 up-regulated ribosomal genes, and the translation pathway included three initiation factors (eIF1A, iIF2B, and eIF2 alpha Kinase 1), an elongation factor (eEF1 alpha 1), and two polyA binding proteins (PAB1 and PAIP1). Two GenMAPP pathways that were non-significant but still of interest were the G13 signaling and phosphatidylinositol pathways (p values of 0.11 and 0.12, respectively). The G13 signaling pathway activates RhoA, which regulates a signal transduction cascade linking G-coupled receptors to the actin cytoskeleton and initiates actin re-organization and polymerization, cell-cell adhesion, cytokinesis, and anti-apoptotic effects. The genes up-regulated in the G13 pathway in response to both acute and chronic Ang II treatment included RhoA, rhotekin, cofilin 2 (that causes disassembly of actin), cdc 42 (that stimulates calmodulin and causes actin polymerization), and PAK3 (that causes stress fiber disappearance).

In addition to the increased ribosome and translation activity identified by pathways analysis, EASE identified a significant increase in the expression of genes with cytoskeletal-associated GO terms. More than one-third (N = 25) of the 64 significant GO terms for genes up-regulated in response to both acute and chronic Ang II treatments were cytoskeletal, and the top ten most highly significant ($p < 0.0005$) terms were all cytoskeletal (Figure 3). The next largest group of GO terms was for the ribosome (N = 11); other significant GO terms included cell adhesion and motility, cell cycle and proliferation, and individual terms for calcium ion binding, Rho small molecule GTPase activity, steroid biosynthesis, and integrin-mediated signaling pathway. As mentioned above, Rho GTPase is an important second-messenger system in transducing Ang II effects on heart and other tissues. Integrins play a crucial role in cardiac hypertrophy, linking the extracellular matrix to the cytoskeleton and

transducing extracellular mechanical stress and chemical signals (6). The significant cellular component GO terms were primarily cytosolic terms for ribosome and microtubules, although there were also significant terms for lysosome and cell-substrate adhesion junctions.

Some of the genes most strongly up-regulated in response to both acute and chronic Ang II treatments have previously been documented to increase expression in response to hypertension or Ang II treatment (Table 2). The identification of significant regulation of these genes in our study is an indicator of the accuracy and reliability of our analysis. These include brain natriuretic peptide (BNP), metalloproteinase inhibitor 1 (TIMP1), Down syndrome critical region 1 (DSCR1), thrombospondin 1, osteopontin, heme oxygenase (HO) and MAP kinase phosphatases such as MAP kinase phosphatase 4 (MKP4). BNP is a ventricular cardiac hormone that antagonizes the RAAS, regulates fluid homeostasis, causes vasodilation, and has been implicated in ventricular remodeling (61); BNP levels in plasma and ventricle are increased following Ang II treatment (58) and in patients with congestive heart failure and myocardial infarction (61). Ang II treatment is known to induce genes associated with fibrosis, including TIMP1, thrombospondin 1, and osteopontin. TIMP1 is up-regulated in deteriorating heart failure as are matrix metalloproteinase 1 (MMP1) and interleukin-1beta (IL-1beta) and IL-6 (7). DSCR1 inhibits calcineurin, a calcium/calmodulin-dependent protein phosphotase that stimulates cardiac hypertrophy (39). Thrombospondin is an extracellular matrix protein that inhibits angiogenesis and increases in response to Ang II and hypertension. HO is a heme catabolizing protein that is up-regulated in the heart in response to Ang II (32).

Some other strongly up-regulated genes are novel and have not been previously associated with responses to Ang II treatment and hypertension (Table 2). These novel genes include TfR1, follistatin-like 3 (FSTL3), epithelial membrane protein 1 (EMP1), tumor necrosis factor receptor superfamily member Fn14, and uridine-cytidine kinase 2 (UCK2). Although TfR1 has not been directly implicated in responses to hypertension and Ang II treatment, Ang II treatment causes intracellular cardiac iron deposition that aggravates Ang II-induced fibrosis (32). As TfR1 mediates iron uptake, the increased

TfR1 expression we measured may underlie Ang II-induced iron deposition. FSTL3 is an extracellular matrix associated glycoprotein induced by TGF-beta that binds morphogens and growth factors and has increased expression during fetal rat heart development (37); TGF-beta is an important signaling pathway induced by Ang II in hypertension (36). EMP1 may be involved in cell proliferation and apoptosis (63), which are both major processes during Ang II-induced cardiac remodeling. Fn14 activation by TNF-like weak inducer of apoptosis (TWEAK) induces angiogenesis, apoptosis, and NF-kB activation (50), a key pathway in cardiac responses to Ang II (11). Although UCK2 increased expression in response to both acute and chronic Ang II treatments (2.2 fold), its increase was greater in response to the acute than chronic treatment (3.0 fold and 1.7 fold, respectively). UCK2 is a rate-limiting step in the salvage pathways of pyrimidine biosynthesis (59) and is elevated in pancreatic cancer tumors.

Acute and Chronic Down-Regulated Genes

Just as the significant pathways for acutely down-regulated genes were all metabolic, all ten significant GenMAPP and KEGG pathways for the 2262 genes down-regulated in response to both acute and chronic Ang II treatments were all for metabolic pathways as well. There was, however, a greater diversity of energy pathways represented in this larger group than was seen for those down-regulated only in acute. Again, there was substantial overlap in significant GenMAPP and KEGG pathways identified by EASE. The three GenMAPP pathways for down-regulated genes were mitochondrial fatty acid betaoxidation, fatty acid degradation, and Krebs-TCA cycle. KEGG pathways included fatty acid metabolism and citrate cycle (TCA cycle) as well as carbohydrate metabolism, propanoate metabolism, valine leucine and isoleucine degradation, oxidative phosphorylation, and energy metabolism. KEGG pathways for amino acid metabolism and pyruvate metabolism had low (p = 0.052 and 0.074, respectively) EASE scores, but were non-significant. As mentioned previously, Ang II treatment and sustained hypertension depress mitochondrial metabolism and trigger a switch of metabolic fuel utilization for cellular energy from fatty acid oxidation to glucose oxidation (60). These metabolic

changes are reflected in the depression of energy pathways associated with mitochondrial and fatty acid metabolism.

The 63 significant GO terms for the 2262 genes down-regulated in response to both acute and chronic exposure were dominated by metabolic terms (Figure 3). Consistently up-regulated genes were localized primarily to the ribosome and cytoskeleton (see above), and down-regulated genes were localized primarily to the mitochondria, as was also the case for genes responding only to the acute Ang II treatment. Metabolic terms predominated among the down-regulated genes, and there were few significant non-metabolic GO terms (see supplementary data). Although not significant ($p = 0.054$), GO terms for heme and porphyrin biosynthesis were among the non-metabolic GO terms with the lowest p-values and included the down-regulated heme biosynthetic pathway genes uroporphyrinogen III synthase, coporphyrinogen oxidase, and ferrochelatase. Porphyrin metabolism and heme biosynthesis pathways were not significantly down-regulated ($p = 0.15$ and 0.17, respectively) but were the non-metabolic pathways with the lowest *p*-values.

Some of the genes most strongly down-regulated in response to Ang II treatments have previously been identified in Ang II or hypertension studies, such as non-muscle myosin heavy chain (MHC), SERCA2, and estrogen receptors. Non-muscle myosin heavy chain is down-regulated by Ang II in the aortas of spontaneously hypertensive rats (23) . SERCA2 has an inverse relation with atrial natriuretic peptide (ANF) in failing human hearts (2). Both ANF and brain natriuretic peptide (BNP) are cardiac hormones with very similar expression patterns produced by the atrium and ventricle, respectively; BNP was the most highly expressed gene product in response to both acute and chronic Ang II treatments (+3.2 fold). Estrogen has been implicated both in remodeling of the heart and in regulating Ang II receptors (73), and both the estrogen receptor and estrogen-related receptor alpha (ERR1) were consistently down-regulated in response to Ang II treatment.

Many of the most strongly down-regulated genes have not previously been implicated in hypertension and responses to Ang II (Table 2), such as galectin-4, peroxisomal protein (PeP), isovaleryl

coA dehydrogenase, stat-like protein Fe65, the metabotropic glutamate receptor 1 (GRM1), and glutathione S-transferase mu2. Galectin-4 may be involved in cell adhesion, and PeP is a peroxisomal matrix protein that is involved with skeletal and cardiac muscle development. Down-regulation of stat-like protein Fe65 is consistent with its inhibition by the cytokine IL-1beta, which activates the NF-kappB pathway (74). Although neither the metabotropic glutamate receptor 1 nor glutathione S-transferase expression has been implicated in cardiac responses to hypertension or Ang II treatment, both genes showed significantly decreased expression in the hearts of rats chronically treated with nicotine (29); the authors speculated that chronic nicotine treatment affected cardiac function by down-regulating genes involved in energy metabolism, which is consistent with our findings.

Acutely Down-Regulated and Chronically Up-Regulated Genes

1456 genes shared an expression profile of being significantly down-regulated in response to acute Ang II treatment but up-regulated in response to chronic treatment. Pathway analysis for this group of genes differs from previously described clusters because EASE identified disparate GenMAPP and KEGG pathways. The two significant GenMAPP pathways identified by EASE were TGF Beta signaling and G Protein signaling, which have been established as Ang II signaling pathways (5). Two other low-scoring though non-significant GenMAPP pathways were also for second messenger signaling pathways: Wnt signaling and G Protein coupled receptors Class B secretin-like. In contrast, the two significant KEGG pathways were Alzheimer's disease and Neurodegenerative disorders pathways, and the two lowest scoring non-significant KEGG pathways were for Parkinson's disease and Huntington's disease.

The significant chronic up-regulation of genes in the Alzheimer's disease pathway is particularly interesting as both Alzheimer's disease plaques and heart disease co-occur. Heart disease and hypertension may be a forerunner to Alzheimer's disease, and Alzheimer's disease-like β-amyloid plaques are found in the brains of non-demented individuals with heart disease (56). Furthermore,

cardiac amyloidosis causes restrictive cardiomyopathy (3), a particularly lethal form of cardiomyopathy that does not respond to standard treatments. This study is the first report, to our knowledge, of up-regulation of the Alzheimer's disease pathway in heart tissue in response to hypertension induced by Ang II treatment. These results suggest investigation of therapeutics developed for the treatment of plaque formation in Alzheimer's may represent a viable alternative therapy for hypertension or cardiac amyloidosis. The inter-relation between heart disease and Alzheimer's and their treatment has already been demonstrated by the effects of statins, used to lower cholesterol and treat ischemic heart disease, on Alzheimer's disease. Although some statins appear to be protective against subsequent development of Alzheimer's disease, there are also indications that patients with Alzheimer's disease may be more susceptible to adverse effects of statins than are age-matched controls (1).

The significant cellular component GO terms for the 1456 genes down-regulated in acute and up-regulated in chronic Ang II treatments were all structural, relating to the extracellular matrix and basement membrane or to the cytoskeleton. Second messenger signal transduction cascades were also strongly represented among these chronically up-regulated genes; one quarter of the 61 significant GO terms were associated with second messenger signal transduction cascades and including G-protein coupled receptor proteins, regulation of JNK cascade, MAP-kinase scaffold protein activity, and semaphorin receptor activity. Non-significant but low-scoring signaling cascade terms included GTPase regulator activity ($p = 0.055$) and JAK-STAT cascade ($p = 0.068$). ECM and immune response terms were also well represented amongst the significant GO terms (see supplementary data).

Chronic responses

The majority (N = 100) of the 133 genes that responded only to chronic Ang II treatment were up-regulated and were comprised primarily of extracellular matrix (ECM) components (Figure 2), reflecting the changes in the cellular structure in the heart required by a long exposure to a stressor such as elevated Ang II. These increases in ECM are expected as one of the primary effects of sustained Ang

II treatment is cardiomyocyte hypertrophy, vascular damage, fibrosis, and thickening and stiffening of the extracellular matrix (5, 45). The majority of the 24 significant GO terms were for structural (extracellular matrix or cytoplasmic microfibrils) or immune response, with the remainder relating to calcium ion binding (Fig. 2). The significant cellular component GO terms were extracellular matrix (ECM), collagen, and microfibril. The only significant pathway identified by EASE analysis for the 100 chronically up-regulated genes was inflammatory response (GenMAPP, $p \leq 0.0001$).

ECM and structural genes dominated the list of genes up-regulated in response to chronic Ang II treatment. The gene most strongly up-regulated in response to chronic Ang II treatment, however, was osteoblast-specific factor 2 (OSF2). OSF2 is regulated by mitogen-activated protein kinase (MAPK) (72), which is up-regulated in response to chronic Ang II treatment (5). When comparing gene expression of hearts from mice with early and late stage heart failure, OSF2 expression is significantly higher in advanced heart failure (9), supporting our findings of increased expression in response to chronic Ang II treatment relative to acute Ang II treatment (+5.3 fold versus +1.0 fold, respectively, $p < 0.05$). A wide range of collagens, which comprise the ECM, were also up-regulated chronically, including collagens type I alpha 1 and alpha 2, type II alpha 1, type III alpha 1, type IV alpha 1 and alpha 2, and type XVI alpha 1. Other ECM and structural genes up-regulated in response to chronic Ang II treatment included alpha-actinin 4, myofibril-associated glycoprotein 2, fibronectin, osteonectin, follistatin-like protein 1 (FSTL1), and two matrix metalloproteinases (MMP): MMP2 (gelatinase A) and MMP14.

Chronic Ang II treatment also resulted in increased expression of genes for growth factors that have previously been shown to change expression in response to Ang II or hypertension. The role of growth factors in hypertension is critical as cardiomyocytes are terminally differentiated and cannot respond to increased physical demand by cell proliferation but only by hypertrophy (45). Chronic Ang II treatment resulted in +1.6 fold increased expression of insulin-like growth factor I (IGF-I), an Ang II – induced growth factor that stimulates cardiac hypertrophy (12). Two growth inhibitors, pigment

epithelial-derived factor (PEDF) and cyclin-dependent kinase inhibitor 1 (p21; CDKI1), also increased in expression in response to chronic Ang II treatment, +1.6 fold and +2.6 fold, respectively. PEDF is a potent inhibitor of angiogenesis that can block tumor growth (70), and p21 inhibits Ang II-induced cardiac hypertrophy (45).

Whereas acute Ang II treatment resulted in down-regulation of thyroid hormone receptors associated with slowing of heart rate, chronic Ang II treatment resulted in differential expression of cardiac dopaminergic and serotonergic receptors that regulate vasodilation and vasoconstriction. The observed changes in expression of these receptors were consistent with increased vasodilation and were only observed in response to the chronic Ang II treatment. Expression of D4 and $5HT_{1D}$ receptors increased +1.5-fold and +1.9-fold respectively, in response to chronic Ang II treatment, while $5HT_2$ receptor expression decreased -1.6-fold. Stimulation of dopamine D4 and serotonin $5HT_{1D}$ receptors cause vasodilation (22, 67), whereas stimulation of serotonin $5HT_2$ receptors results in vasoconstriction and augmentation of other vasoconstrictors such as Ang II (22, 67). Agonists for $5\text{-}HT_{1A}$ receptors and antagonists for $5\text{-}HT_{2B}$ receptors serve as effective anti-hypertensive treatments (14). Increased expression of $5\text{-}HT_{2B}$ receptors is associated with ventricular hypertrophy(42), whereas $5\text{-}HT_{2B}$ knockout mice have ventricular hypotrophy and structural and metabolic abnormalities (41). Thus, it has been suggested that serotonin, via $5\text{-}HT_{2B}$ receptors, may be involved in regulating mitochondrial function and protecting cardiomyocytes from apoptosis (40). It remains to be determined whether these changes in expression of the serotonergic receptors were in response to chronic elevated Ang II treatment itself or to the sustained hypertension resulting from the chronic treatments.

The most highly expressed gene in the heart following chronic Ang II treatment not related to the ECM or previously associated with Ang II was Apolipoprotein D (ApoD). ApoD is a lipocalin that is usually expressed in the liver and pancreas and is associated with diabetes mellitus, hyperinsulinemia, and obesity. Recent clinical work supports a link between Ang II, hypertension, and diabetes; Ang II blockade by losartan decreases blood pressure and also decreases the probability of new-onset diabetes

during treatment (19). In our study, ApoD was down-regulated (-1.5 fold) in response to acute Ang II treatment and strongly up-regulated (+1.5 fold) in response to chronic Ang II treatments. Although ApoD has not been directly associated with hypertension, it has been linked to the metabolic syndrome X, a cluster of co-occurring health problems which include obesity, hypertension, hyperlipidemia, non-insulin-dependent diabetes mellitus, and ischemic heart disease (20, 68). Thus, ApoD may be part of a generalized response in the heart to damaging stressors or may be causatively linked to the heart pathology that develops in the metabolic syndrome X. Increased ApoD expression in the brain is also seen in Alzheimer's disease (8). These findings suggest that cardiac ApoD expression may be a good marker for heart disease and also suggest that Ang II may play a causative role in generating a wide range of hypertensive-associated disease than had been previously thought and that Ang II antagonists may have a place in managing these diseases.

Due to the small number of genes (29) down-regulated in response to chronic Ang II treatment, no significant pathways or GO terms were identified. Many of the down-regulated genes were of unknown function and even fewer have established regulation by Ang II or hypertension; the exception being $5HT_2$, as discussed above.

Two genes known to be induced by cold exposure, cold-inducible RNA-binding protein (CIRP) and UCP3, were significantly down-regulated in chronic Ang II treatment. UCP3 down-regulation is characteristic of failing and hypertensive hearts (48, 60). CIRP suppresses fibroblast growth (44), thus down-regulation of CIRP in response to chronic Ang II treatment may allow myocardial growth. Two other cold-induced genes, RBM3 and UCP2, also showed moderate down-regulation in chronic Ang II treatment. It is unlikely that the down-regulation of these four hypothermia-induced genes in the heart was a result of a change in ambient temperature, as all saline and Ang II treated mice were held in the same facility simultaneously.

qRT-PCR expression validation

Measurements of gene expression from qRT-PCR comparing Ang II treatment to its saline control supported microarray results (Fig. 5), with R = 0.63 and R^2 of 0.39 (N = 32). For most of the genes, the direction (up or down-regulation) and strength of the microarray results agreed with the qRT-PCR results (Fig. 5). The strongest up-regulation measured by microarray resulted in strong up-regulation, as determined by qRT-PCR. UCK2, R-Ras2, and DSCR1 all showed strong up-regulation in response to acute Ang II treatment and lesser or no response to chronic Ang II treatment. In contrast, both methods showed OSF2 to have the greatest up-regulation following chronic Ang II treatment. BNP was up-regulated in response to both acute and chronic treatment, though the microarray data shows a stronger response to acute. BNP has a very similar expression profile to ANF, which was not on our array; qRT-PCR measurements of ANF showed very similar findings to those measured for BNP, with $\log_2$ value of 1.8 for acute and 0.7 for chronic Ang II treatments. Both methods found differential expression for ApoD (down in acute, up in chronic) and for the 5-HT 2 receptor (up in acute, down in chronic).

SUMMARY:

This study has allowed the most comprehensive survey to date of the effects of exposure to increased levels of Angiotensin II and the concomitant hypertension, monitoring expression patterns for 23,510 genes in response to acute (24 hour) and chronic (14 day) Ang II treatments. Our analysis has validated the regulation of many individual genes and pathways that have previously been identified in disparate studies while placing the expression of each into a more holistic context. In addition, we have identified important new classes of Ang II-responsive genes that provide new insight to possible mechanisms of hypertension-induced cardiomyopathies.

One of the most significant responses to hypertension induced by Ang II treatment is down-regulation of energy pathways. Acute (24 hour) treatment resulted in down-regulation of mitochondrial

metabolism, specifically the mitochondrial electron transport chain and Kreb-TCA cycle. Chronic Ang II treatment depressed genes involved in both mitochondrial energy metabolism and fatty acid oxidation. Ang II and chronic overload are known to depress cardiac mitochondrial metabolism and promote the switch from fatty acids to glucose as energy substrates (46, 60), although the mechanism whereby Ang II transduces these effects remains unknown (15). It has been suggested, however, that the increase in reactive oxygen species in response to Ang II and in heart failure may induce these metabolic changes as well as hypertrophy, fibrosis, and apoptosis characteristic of chronic Ang II treatment or hypertension (5, 55).

Acute Ang II treatment caused an immediate increase expression of genes for ribosomes, growth regulation, and oxidative stress response. Sustained treatment resulted in a continuation of increased ribosomes and translation, but also an increase in cytoskeletal and extracellular matrix genes, and a host of second messenger signaling systems known to transduce the effects of Ang II including the JNK cascade, MAPK, NF-kappaB, TGF-beta, JAK-STAT, Wnt signaling, and Rho GTPase. Also increased in response to both acute and chronic Ang II treatments were genes known to increase expression in response to Ang II treatment and hypertension, such as BNP, thrombospondin, osteospondin, and heme oxygenase. Chronic Ang II treatment saw a significant increase in a number of genes associated with amyloidosis, which can cause a particularly lethal form of cardiomyopathy (35). Genes that increased expression only in response to chronic Ang II treatment included a host of ECM and structural genes and growth factors. The regulation of these genes helps to explain the morphological changes observed in hypertensive hearts.

This study suggests two areas that may prove fruitful for treatment of hypertension, other than treatments focusing on inhibiting the RAAS: serotonergic regulation of the heart and treatment for neurodegenerative diseases, such as Alzheimer's disease. Monoamine receptors that regulate vasomotor tone, notably dopamine D4, serotonin $5HT_{1D}$, and $5HT_2$ receptors, were differentially expressed in response to chronic Ang II treatment, presumably allowing for increased vasodilation (22).

Pharmacological regulation of serotonin is as effective treatment for hypertension as are traditional RAAS antagonists (14), and $5HT_{2B}$ receptors have been implicated in regulation of cardiac hypertrophy, regulation of mitochondrial metabolism, and protection against apoptosis (40, 42). The identification of ApoD and other genes involved in plaque formation and the identification of significant regulation of pathways previously associated with the development of neurodegenerative diseases including Alzheimer's suggests that drugs developed for treatment of Alzheimer's may prove efficacious for cardiac amyloidosis. In addition to nine genes in the KEGG Alzheimer's disease pathway that increased expression in chronic Ang II treatments, ApoD also exhibited increased expression in response to chronic Ang II treatments. Although it has not been previously linked with hypertension and responses to Ang II, ApoD is associated with the metabolic syndrome X and may contribute to amyloidosis (8, 68).

The causal relationship between activation of the RAAS and increased hypertensive tissue damage of end organs was proposed long ago on the basis of experimental animal studies and clinical observations (24). Over the years it was proven by numerous clinical outcome trials, where antihypertensive therapies blocking the RAAS have resulted in relative protection from end organ damage and type 2 diabetes mellitus compared to therapies that lower blood pressure to the same extent by different approaches (13). A large body of literature has attempted to elucidate the biochemical, cellular and molecular mechanisms underlying these differences. The current findings may provide explanations for some of these mechanisms and point the direction for future research.

Acknowledgements

This work was supported by NIH grants U01 HL66580-01 (JQ) and U01 HL66617-01 (HG).

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Figures:

Figure 1. Genes differentially expressed in the heart in response to acute (24 hour) but not chronic (14 day) Ang II treatments (fold change relative to expression levels of matching saline controls). Top panel: genes up-regulated (blue, N = 701) and down-regulated (white, N = 871) in response to acute Ang II treatment. Data for each gene in each treatment is derived from four measurements: both direct and indirect comparisons, each with dye-reversed replicates.

Bottom panel: pie charts of functional groupings of significant GO terms for each cluster (left, up-regulated acute cluster, N = 54 GO terms; right down-regulated acute cluster N = 62 GO terms).

Figure 2. Genes differentially expressed in the heart in response to chronic (14 day) but not acute (24 hour) Ang II treatments (fold change relative to expression levels of matching saline controls). Top panel: genes up-regulated (grey, N = 100) and down-regulated (white, N = 33) in response to chronic Ang II treatment. Data for each gene in each treatment is derived from four measurements: both direct and indirect comparisons, each with dye-reversed replicates.

Bottom panel: pie chart of functional groupings of significant GO terms for the chronic up-regulated cluster (N = 24 GO terms).

Figure 3. Genes consistently up-regulated in both acute and chronic Ang II treatments (grey, N = 1302), consistently down-regulated in both acute and chronic Ang II treatment (white, N = 2262), and genes down-regulated in acute and up-regulated in chronic Ang II treatments (stippled, N = 1456). Top panel: mean values for acute and chronic responses (fold change) for each group. Data for each gene in each treatment is derived from four measurements: both direct and indirect comparisons, each with dye-reversed replicates.

Bottom panel: pie charts of functional groupings of significant GO terms for each cluster; left, up-regulated acute & chronic cluster, N = 64 GO terms; middle down-regulated acute & chronic cluster N = 63 GO terms; right down-regulated acute and up-regulated chronic cluster, N = 60 GO terms.

Figure 4. Heat map of $\log_{10}$ transformed *p*-values from EASE analysis of GO terms. GO terms were clustered by Support Trees Clustering in MeV.

Figure 5. Comparison of expression values for 16 genes from qRT-PCT and microarray hybridization. Ratio of expression for each gene in response to Ang II treatment to its matching control was $\log_2$ transformed for qRT-PCR and microarray data. Each gene had two pairs of values, one for acute treatment and one for chronic treatment. When a gene had multiple spots on the array, the mean value of the $\log_2$ ratios was used. Top panel: regression of data from microarray hybridization against qRT-PCR, $p < 0.0001$. Bar graphs present direct comparison of $\log_2$ transformed results from microarray hybridization and qRT-PCR for seven genes. Open bars represent qRT-PCR values and dark bars represent values from microarray hybridizations.

Table 1: Quantitative real-time PCR primers.

Gene Symbol	GenBank Accession #	TIGR ID#	Primer	Sequence
ABB1	AI840092	TC1030032	forward	GAATTCCCAGCACCAAAGAA
			reverse	CGGCTACTGGAAGACAGGAC
ApoD	AI837042	TC804512	forward	ATTTCTTTGCTTTGCGTTCC
			reverse	ACTTTCCATGAGTCCCCTCC
BNP	AW541489	TC1015293	forward	TGCTTTGGGCACAAGATAGA
			reverse	AGACCCAGGCAGAGTCAGAA
DSCR1	AI846152	TC975094	forward	GGTGAATCAGAGCATGTGGA
			reverse	ACACACACGATGACTGGGAA
GLUT4	AW549761	TC919901	forward	AGAGCGCATCAGTCTCCATT
			reverse	CGCCCTTAGTTGGTCAGAAG
Grp78	AA407780	TC873610	forward	TCAGATCTTCTCCACGGCTT
			reverse	AGCAGGAGGAATTCCAGTCA
Herp	AI835088	TC836145	forward	GACCCCAACAATAACCTCCA
			reverse	AGCCATGCTGTGCTCATAAA
OSF2	AI849775	TC1020953	forward	GCCAATCTTCAAGCAAGTCC
			reverse	CCTCAGTTCCTACCCCACAG
R-Ras2	AW546427	TC1004309	forward	TTTCCAGTGGCCAAAATACC
			reverse	CAAGTGCAGAACCAGTCCAA
S1Dr	AI853647	TC1052189	forward	GCATCCTAGAACGCAAGAGG
			reverse	AAAGAAAGGCAACCAGCAGA
SR5-HT2	AU015268	TC979176	forward	GGAATGGCTTGTCCCATAGA
			reverse	ATGGATGCGTGTCCAGTTTT
TEF	AI850638	TC973964	forward	AGAGGGGCCTGTAAGAGGAG
			reverse	CCAACACCAAAAGACAGCAA
UCK2	AW543081	TC1039945	forward	CCCTTGGAACCTGTGTTGTT
			reverse	CGGCAAAGGAGACAGAGAAG
UCP3	BE448207	TC986522	forward	TGAAGACTTGCTCCCCAGTT
			reverse	TCGGATCTTTAGGCTCTCCA
URB	AW552006	TC1040543	forward	TTTGGGTGTTGGAGAGGAAG
			reverse	ATCTTTGACCAAGTGGGCTG
ANF	not on array	TC854609	forward	CCGAAGATAACAGCCAAGGA
			reverse	ACAGTGGCAATGTGACCAAG
unknown	AI845730	TC1024235	forward	CATGCCCAGCTAAATCCACT
			reverse	TGAAAGGTCTCATTTCCCGT

Table 2: The top 15 genes that increase (top panel) or decrease (bottom panel) expression in response to both Acute and Chronic Ang II treatments. Degrees of freedom (df) and p values from two-tailed, one-sample T-test (null hypothesis $\log_2$ ratio = 0).

Genbank#	TC#	gene name	df	P value	fold Δ
AW541489	TC1015293	brain natriuretic peptide (BNP)	5	0.014	3.2
AI842847	TC876109	metalloproteinase inhibitor 1 precursor (TIMP1)	6	0.001	2.5
AU018180 AW543081 AI850362	TC1039945	uridine-cytidine kinase 2 (UCK 2)	6	0.006	2.3
C87282 AI853558	TC876184	tumor necrosis factor receptor (Fn14; TWEAKR)	6	0.003	2.3
AW552170 AI846152	TC975094	Down syndrome critical region 1 (DSCR1)	7	0.001	2.2
AI846661	TC814595	dimethylarginine dimethylaminohydrolase 1 (DDAH1)	7	0.042	2.2
AI838607	TC841569	thrombospondin 1 (THBS1)	7	< 0.001	2.2
AU040277	TC824477	phosphoserine aminotransferase (PSAT)	7	0.030	2.1
AI838613 BE380760	TC847447	epithelial membrane protein 1 (EMP1)	7	< 0.001	2.0
AI850990 AU042170	TC818943	heat-stable antigen-related protein (HSA-C)	7	0.024	1.9
AA408815	TC899372	follistatin-related protein 3 (FSTL3)	6	< 0.001	1.9
AI852203	TC919767	KIAA1126 protein	7	0.001	1.8
AI841289	TC805059	dnaK-type molecular chaperone hsp70	7	< 0.001	1.8
AI837099	TC886927	phosphatidic acid phosphatase type 2c	7	0.029	1.8
AW549480	TC848494	versican; chondroitin sulfate proteoglycan 2 (CSPG2)	7	< 0.001	1.8

C76710	TC892948	galectin-4 (LGALS4)	7	< 0.001	-1.9
AI849056	TC819381	KIAA1470 protein	5	0.039	-1.9
AI847556	TC819798	hypothetical protein	7	0.003	-1.8
AI846720	TC815890	nonmuscle myosin heavy chain	7	0.003	-1.8
AI836530	TC847613	protein STRAIT11499 homolog	7	< 0.001	-1.8
AI835654	TC935982	hypothetical protein	7	0.034	-1.7
AW556555	TC821074	peroxisomal protein (PeP)	6	0.006	-1.7
AW549803	TC876331	similar to putative protein	5	0.028	-1.7
AI847059	TC799169	protein kinase BRPK	6	0.001	-1.7
AI840092	TC1030032	amyloid beta A4 precursor protein-binding family B member 1	7	0.001	-1.6
BE456547	TC805214	forkhead box protein O3A	7	0.009	-1.6
AI849471	TC892436	branched chain ketoacid dehydrogenase E1 alpha	7	0.002	-1.6
AI841295	TC915425	homolog to glutathione S-transferase	5	0.003	-1.6
AI850932	TC935622	isovaleryl coenzyme A dehydrogenase (IVD)	7	0.002	-1.6
AI854716	TC809393	sarcoplasmic/endoplasmic reticulum calcium ATPase 2	7	0.004	-1.6

Figure 1.

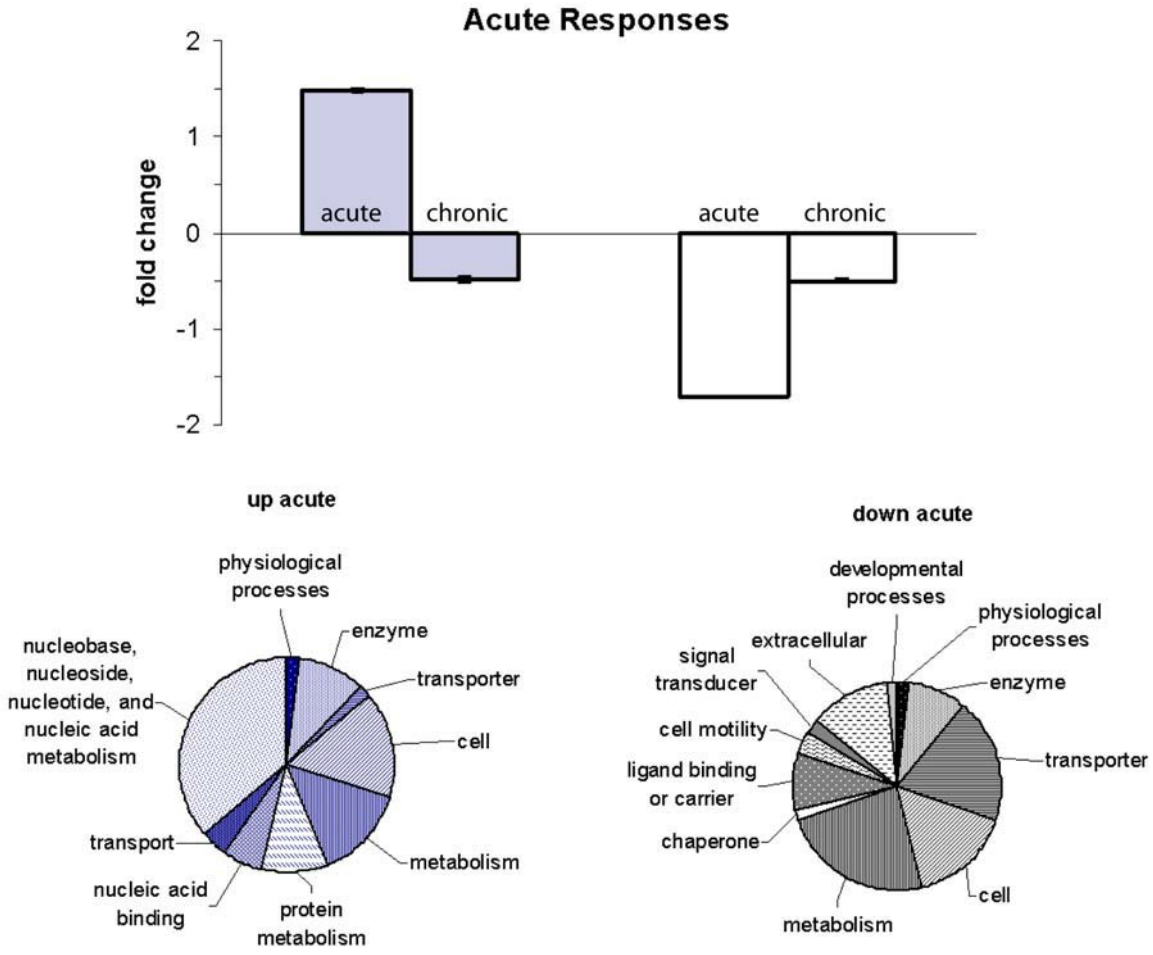

Acute Responses
fold change
2
1
0
-1
-2
acute
chronic
acute
chronic
up acute
physiological processes
enzyme
transporter
cell
metabolism
protein metabolism
nucleic acid binding
transport
nucleobase, nucleoside, nucleotide, and nucleic acid metabolism
down acute
developmental processes
physiological processes
enzyme
transporter
cell
metabolism
chaperone
ligand binding or carrier
cell motility
signal transducer
extracellular

Figure 2.

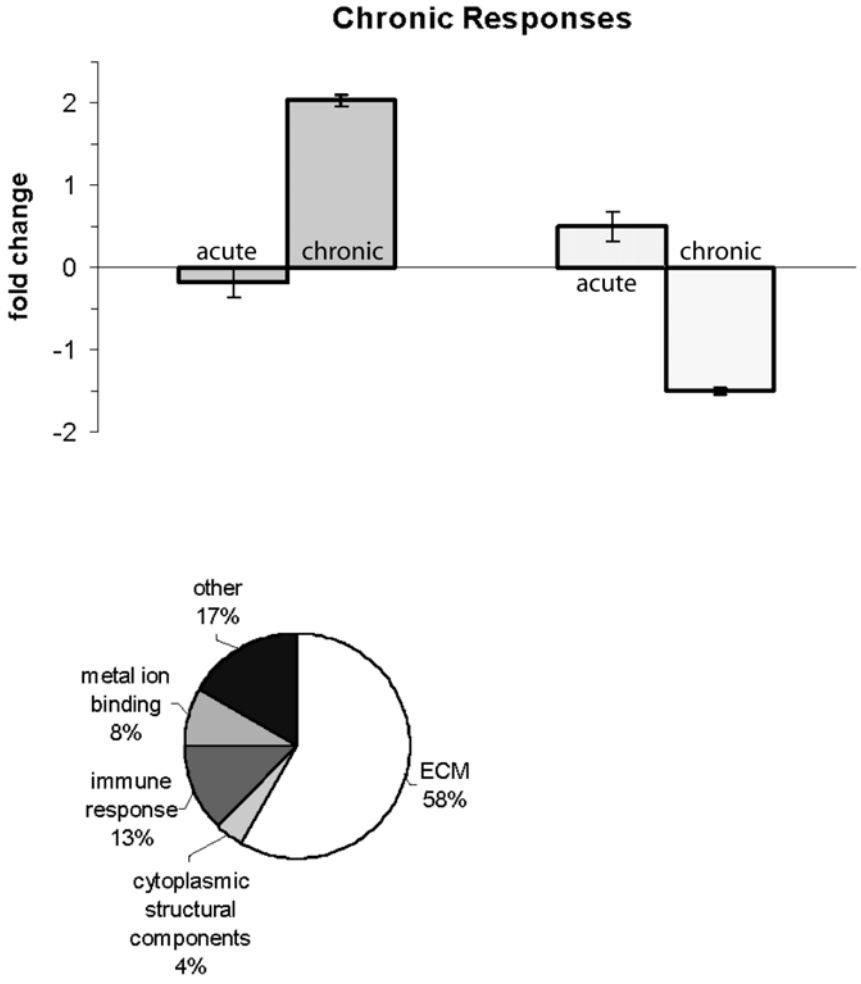
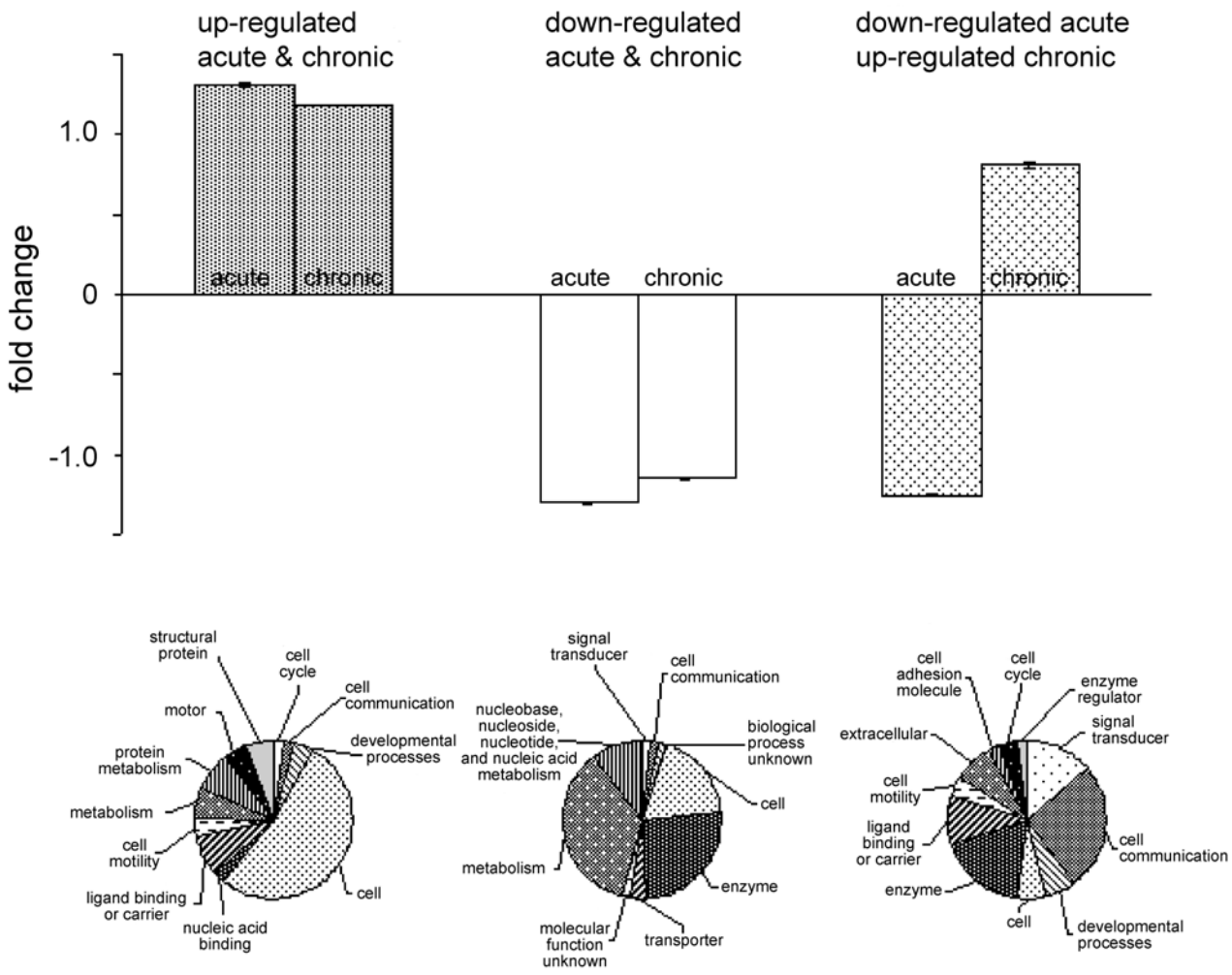

Chronic Responses
fold change
2
1
0
-1
-2
acute
chronic
acute
chronic
other
17%
metal ion
binding
8%
immune
response
13%
cytoplasmic
structural
components
4%
ECM
58%

Figure 3.


up-regulated
acute & chronic
down-regulated
acute & chronic
down-regulated acute
up-regulated chronic
fold change
1.0
0
-1.0
acute
chronic
acute
chronic
acute
chronic
structural protein
cell cycle
cell communication
motor
developmental processes
protein metabolism
metabolism
cell motility
ligand binding or carrier
nucleic acid binding
cell
signal transducer
cell communication
nucleobase, nucleoside, nucleotide, and nucleic acid metabolism
biological process unknown
cell
metabolism
enzyme
molecular function unknown
transporter
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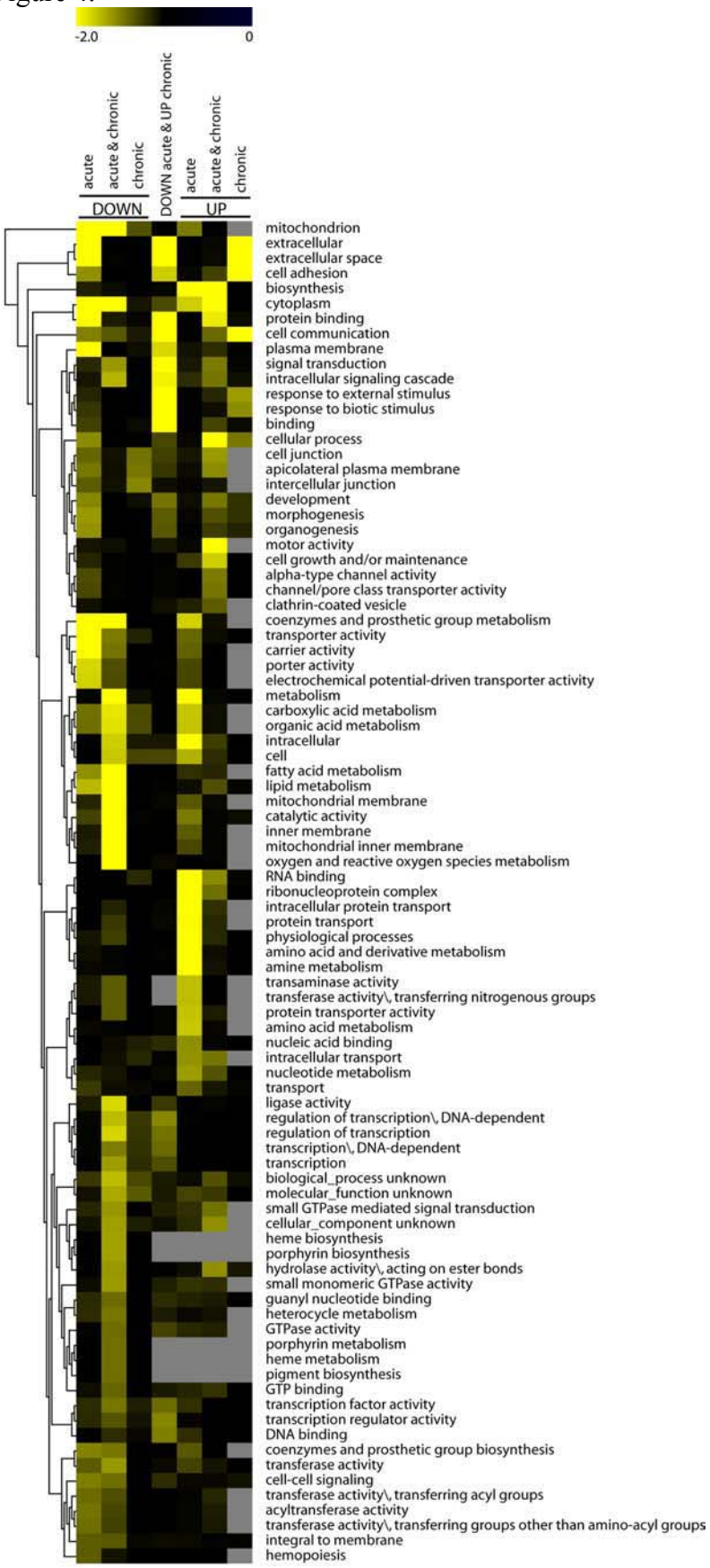
cell cycle
enzyme regulator
signal transducer
extracellular
cell motility
ligand binding or carrier
cell communication
enzyme
cell
developmental processes

Figure 4.


-2.0
0
acute
acute & chronic
chronic
DOWN acute & UP chronic
acute
acute & chronic
chronic
DOWN
UP
mitochondrion
extracellular
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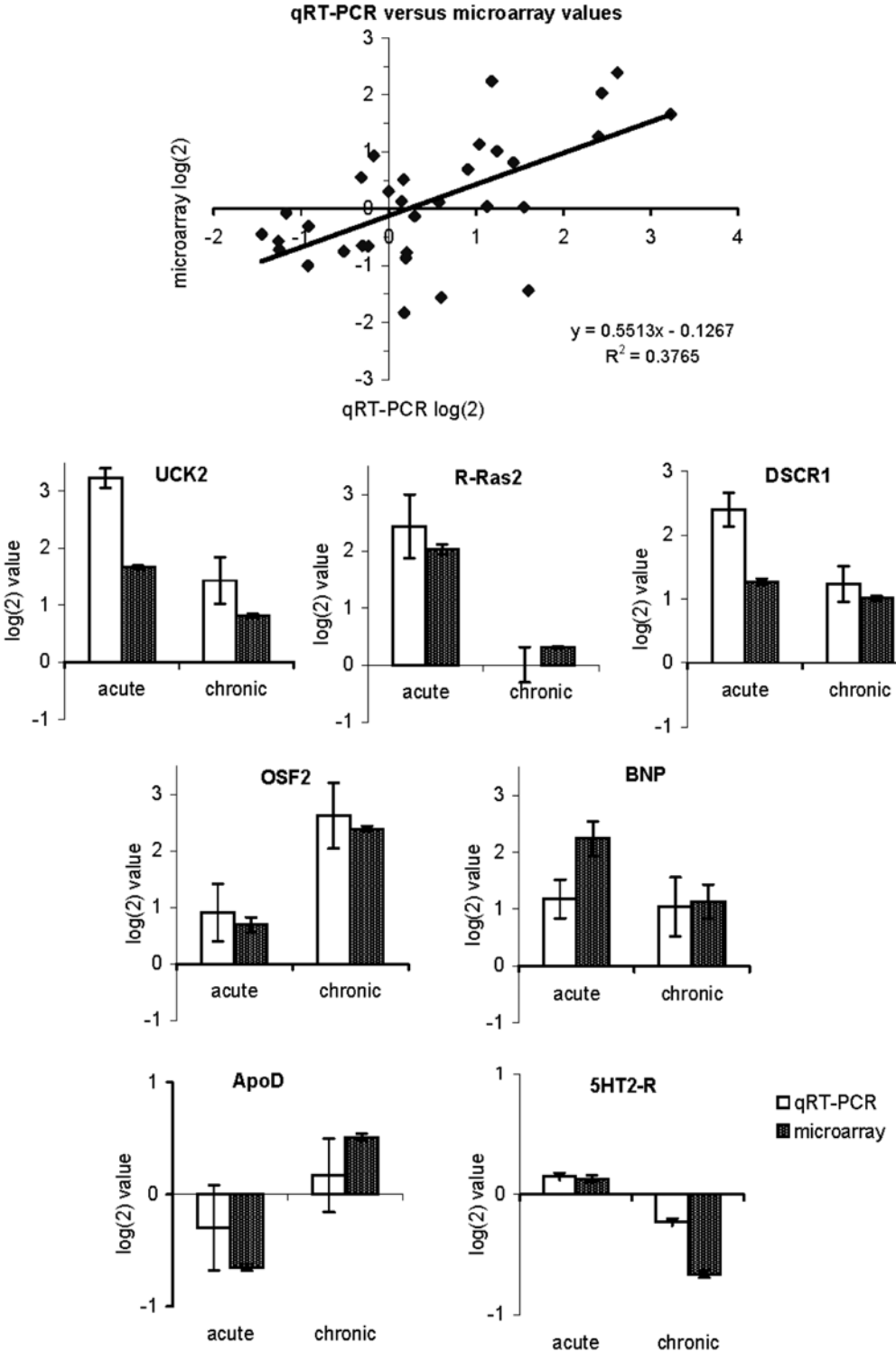
cell adhesion
biosynthesis
cytoplasm
protein binding
cell communication
plasma membrane
signal transduction
intracellular signaling cascade
response to external stimulus
response to biotic stimulus
binding
cellular process
cell junction
apicolateral plasma membrane
intercellular junction
development
morphogenesis
organogenesis
motor activity
cell growth and/or maintenance
alpha-type channel activity
channel/pore class transporter activity
clathrin-coated vesicle
coenzymes and prosthetic group metabolism
transporter activity
carrier activity
porter activity
electrochemical potential-driven transporter activity
metabolism
carboxylic acid metabolism
organic acid metabolism
intracellular
cell
fatty acid metabolism
lipid metabolism
mitochondrial membrane
catalytic activity
inner membrane
mitochondrial inner membrane
oxygen and reactive oxygen species metabolism
RNA binding
ribonucleoprotein complex
intracellular protein transport
protein transport
physiological processes
amino acid and derivative metabolism
amine metabolism
transaminase activity
transferase activity\, transferring nitrogenous groups
protein transporter activity
amino acid metabolism
nucleic acid binding
intracellular transport
nucleotide metabolism
transport
ligase activity
regulation of transcription\, DNA-dependent
regulation of transcription
transcription\, DNA-dependent
transcription
biological_process unknown
molecular_function unknown
small GTPase mediated signal transduction
cellular_component unknown
heme biosynthesis
porphyrin biosynthesis
hydrolase activity\, acting on ester bonds
small monomeric GTPase activity
guanyl nucleotide binding
heterocycle metabolism
GTPase activity
porphyrin metabolism
heme metabolism
pigment biosynthesis
GTP binding
transcription factor activity
transcription regulator activity
DNA binding
coenzymes and prosthetic group biosynthesis
transferase activity
cell-cell signaling
transferase activity\, transferring acyl groups
acyltransferase activity
transferase activity\, transferring groups other than amino-acyl groups
integral to membrane
hemopoiesis

Figure 5.


qRT-PCR versus microarray values
microarray log(2)
qRT-PCR log(2)
y = 0.5513x - 0.1267
R2 = 0.3765
UCK2
R-Ras2
DSCR1
OSF2
BNP
ApoD
5HT2-R
log(2) value
acute
chronic
qRT-PCR
microarray