Microarray Analysis of Altered Gene Expression in Kidneys of Adult Spontaneously Hypertensive Rats

Ja-Ryong Koo, MD[*]
Kai Hui Liang, MD[†]
Nosratola D Vaziri, MD[†]

[*]*Department of Internal Medicine, Hallym University Hospital, Chunchon, South Korea*
[†]*Division of Nephrology and Hypertension, Departments of Medicine, Physiology and Biophysics, University of California, Irvine, Irvine, California*

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ABSTRACT

The spontaneously hypertensive rat (SHR) is used as an animal model of essential hypertension in humans. As with human essential hypertension, a polygenic etiology has been considered in the pathophysiology of hypertension and the associated metabolic syndrome in SHR. Given the critical role of the kidney in the regulation of blood pressure, we used DNA microarray analysis to explore differentially expressed genes in kidneys between adult SHR and Wistar-Kyoto (WKY) rats. In addition, real-time polymerase chain reaction (RT-PCR) was used to quantify mRNA expression of several selected genes. Among 8,799 known genes and expressed sequence tag (EST) clusters of Affymetrix Rat Genome U34A arrays, we identified 74 differentially expressed transcripts, of which 43 were up-regulated and 31 were down-regulated in SHR. Genes with known function were grouped into functional categories including those related to lipid and glucose metabolism, insulin resistance, signal transduction and intracellular trafficking, antioxidant and anti-inflammatory defense, drug metabolism and regulation of gene transcription. Real time RT-PCR results independently confirmed the microarray results for four down-regulated genes (glutamylcysteine gamma synthetase light chain [GCGS}, glutathione S-transferase [GST], heme oxygenase 3 [HO3], phosphoinositide 3-kinase p85 alpha subunit [PI3K]) and one up-regulated gene (stearoyl-CoA desaturase 2). These findings point to dysregulation of genes involved in lipid and glucose metabolism, insulin signal transduction pathway (down-regulated PI3K), antioxidant defense system (down-regulated GCGS, GST and HO3), and anti-inflammatory defense system (downregulation of CD59) in adult hypertensive SHR. Further studies are planned in young prehypertensive SHR in order to dissect the primary abnormalities from those caused by the associated hypertension.

INTRODUCTION

Spontaneously hypertensive rat (SHR) was first described by Okamoto and Aorki,[1] who had undertaken a selective brother-sister inbreeding of Wistar-Kyoto (WKY) rats with an outbred WKY male and a female rat with slightly elevated blood pressure. SHR is commonly used as an animal model to explore the pathogenesis and management of essential hypertension in humans. As with human essential hypertension, the precise genetic basis of hypertension in SHR remains largely unclear. However, a polygenic etiology has been considered.[2,3] Hypertension in both humans and in SHR is frequently accompanied by a wide range of metabolic disorders, including dyslipidemia, insulin resistance, oxidative stress, endothelial dysfunction, and increased propensity for renal and cardiovascular complications.[3-8] The constellation of these hemodynamic and metabolic disorders is referred to as metabolic syndrome X and points to its polygenic nature.

The availability of microarray technology has provided the opportunity to explore the differential global expression of many genes between the SHR and their normotensive WKY counterparts from which the SHR originated. Kidney plays a central role in the regulation of blood pressure and the pathogenesis of hypertension.[9] In view of these considerations, we sought to compare global gene expressions in the kidney between gender- and age-matched adult SHR and WKY rats.

METHODS

Animals

Asymptomatic 8-week-old female SHR and age-matched female WKY rats (Harlan Sprague Dawley, Indianapolis, IN) were housed in a temperature-(21°C) and light-controlled environment (12:12-h light-dark cycle) and were provided food and water ad libitum. Three animals were included in each group. The experimental protocol was approved by the University of California Irvine (UCI) Animal Welfare Committees. Hypertension in SHR was confirmed by tail-cuff measurement of blood pressure. The animals were observed for 14-weeks, at which point they were anesthetized with intraperitoneal injections of pentobarbital, 50 mg/Kg, and killed by exsanguination using cardiac puncture. Kidneys were immediately harvested, cleaned, and promptly frozen in liquid nitrogen. The samples were then stored at -70°C until they were processed.

RNA Processing

Kidneys were homogenized on ice in TRIzol reagents (GIBCO BRL, Gaithersburg, MD, by a Polytron homogenizer (Kinematica, Lucerne, Switzerland). Cellular debris was removed by centrifugation for 5 min at 12,000 g, and total RNA was isolated using the TRIzol protocol (GIBCO BRL). After the ethanol precipitation step, an additional cleanup step was performed using Qiagen RNeasy (Qiagen, Santa Clarita, Calif).

Microarray Analysis

Reverse and in vitro transcription, hybridization, scanning, and preliminary analysis of samples were performed by UCI DNA MicroArray facility (Irvine, Calif).

Reverse Transcription

In the study, 5 µg of total RNA were converted into double-stranded cDNA (ds-cDNA) using a cDNA synthesis kit (Superscript Choice System, GIBCO-BRL) with an oligo (dT) 24 primer containing a T7 RNA polymerase promoter on its 3' end (Genset, La Jolla, Calif). After second strand synthesis, the reaction mixture was extracted with phenol-

chloroform-isoamyl alcohol and the ds-cDNA was recovered by ethanol precipitation.

In vitro Transcription

After second-strand synthesis, labeled cRNA was generated from the cDNA sample by an in vitro transcription reaction supplemented with biotin-11-CTP and biotin-16-UTP (Enzo, Farmingdale, NY). The labeled cRNA was purified using a Qiagen RNeasy spin column (Qiagen). To ensure optimal hybridization to the oligonucleotide array, 15 µg of each cRNA were fragmented at 94°C for 35 minutes in fragmentation buffer (40 mM Tris-acetate, pH 8.1, 100 mM potassium acetate, 30 mM magnesium acetate).

Hybridization of the GeneChip

The fragmented cRNA was then added to a 300-µL hybridization solution (100 mM MES, 1 M NaCl, 20 mM EDTA, 0.01% Tween 20) containing 0.1 mg/mL of herring sperm DNA (Promega, Madison WI), 500 µg/mL acetylated BSA (GIBCO-BRL) and a mixture of control cRNAs to validate the hybridization step. Before hybridization, the fragmented samples were heated to 94°C for 5 minutes, equilibrated at 45°C for 5 minutes, and clarified by centrifugation (16,000 g) at room temperature for 5 minutes. Aliquots of each sample (10 µg of fragmented cRNA in 200 µL of hybridization cocktail) were hybridized to the Affymetrix Rat Genome U34A arrays (Santa Clara, Calif) at 45°C for 16 hours in a rotisserie oven set at 60 rpm. The arrays were then washed with non-stringent wash buffer (6 x SSPE) at 25°C, followed by a stringent wash buffer (100 mM MES [pH 6.7], 0.1 M NaCl, 0.01% Tween 20) at 50°C, stained with streptavidin-phycoerythrin (Molecular Probes, Eugene, OR), washed again with 6 x SSPE, stained with biotinylated anti-streptavidin IgG, followed by a second staining with streptavidin-phycoerythrin, and a third washing with 6 x SSPE. Arrays were read at a resolution of 6 microns with a Hewlett-Packard Gene Array Scanner (Palo Alto, Calif).

Microarray Data Analysis

Expression of each gene on the microarray was quantified by 16 perfect match probe sequences corresponding with different 25-mer regions within the gene. Specific hybridization of each probe was tested by the inclusion of 16 corresponding probes containing a single base mismatch at the middle position of each 25-mer oligonucleotide. The perfect match (PM) and mismatch (MM) probes corresponding to one gene are designated a probe set. Thus, there were 16 distinct specific hybridization events for each gene analyzed.

The Affymetrix RGU34A array provides gene expression data for 8,799 known genes and expressed sequence tag (EST) clusters. Microarray Suite software version 4.0 (Affymetrix) was used to determine average difference (Avg Diff) as a relative indicator of the level of expression of a transcript, which is calculated by taking the difference of the fluorescence intensities between the PM and MM of every probe pair and averaging the differences over the entire probe set.

To reliably compare data from multiple probe arrays, global normalization was applied by the Microarray Suite software. In each comparison between the WKY and SHR samples, the output of the SHR array was multiplied by a factor to make its average intensity equivalent to the average intensity of the WKY array. Then Affymetrix Suite software determined a difference call that identifies the differences between the SHR and WKY samples for a given mRNA. The output of this difference call matrix can be increased, marginally

increased, decreased, marginally decreased, and no change in the SHR samples compared with the WKY samples. We assigned +2 for increased, +1 marginally increased, -1 for marginally decreased, -2 for decreased, and 0 for no change. Because there were 3 WKY samples and 3 SHR samples, this allowed 9 comparisons and the sum of assigned number was ranged from –18 to +18.

To estimate of the quantitative differences in the level of expression of a transcript between two groups, a fold change was calculated for each gene using the GeneSpring software program version 4.1 (Silicon Genetics, Redwood City, Calif). After per-chip normalization where the Avg Diff of each gene was divided by the median Avg Diff of the chip and per-gene normalization, in which the Avg Diff of each gene was divided by median Avg Diff over all samples, normalized Avg Diff values of 3 rats samples in SHR group were averaged and compared with those of WKY group to produce fold change value for each gene. To assure same reverse and in vitro transcription efficiency and similar overall levels of gene expression between the WKY and SHR rats, normalized Avg Diff values for the housekeeping glyceraldehydes 3-phosphate dehydrogenase (GAPDH) gene were calculated and compared in the two groups.

To make statistical group comparison for finding differentially expressed genes between WKY and SHR samples, global error models of GeneSpring software were used. Error models are ways of estimating the precision (standard error) corresponding with a measured expression level.[10] The global error model in GeneSpring combines information from all genes to estimate the curve relating precision to control strength. The control strength is the value by which the raw Avg Diff value is divided to give the normalized Avg Diff value. Because two types of normalization, a per-chip and a per-gene normalization, are selected in this experiment, the control strength will be the product of the two normalization factors. The control strength represents a measure of general expression level for each gene, with respect to the overall gene and chip expression. Two components of precision are fitted, an absolute error component that dominates at low control strength, and a relative error component that dominates at high control strength. In this experiment, replicate measurement data were available, and the error model was fitted using the replicate variability. Robust fitting methods were used to separate the measurement variability from the biological variability. The precision values calculated from the error model was used as the basis of statistical calculations.

Using Microarray Suite and GeneSpring software, all of the following thresholds had to be surpassed for mRNAs to be declared as differentially expressed between WKY and SHR kidney samples: (1) there had to be statistically significant ($P < .05$) differences in the mean Avg Diff value between two groups in parametric test using global error model; and (2) the sum of the assigned number for difference call in 9 comparison must be less than –11 or more than +11. These selection criteria may prevent detection of some differentially expressed genes, resulting in potential false negatives. Thus, some underestimation of the actual number of differentially expressed genes likely exists.

Real Time Reverse Transcription (RT) PCR

To confirm the differential expression of mRNAs detected with the microarray analysis, real time RT-PCR was performed for selected genes. For each sam-

Table 1. Primers and Probe Sequences for Real Time RT PCR

Name	Sequence	Position, nucleotide	Size, bp
Stearoyl-CoA desaturase 2			
Forward primer	5'-CCCTATGACTACTCTGCCAGTGAGTAC-3'	1257-1283	27
Reverse primer	5'-CCCAGTTCTCTTAATCCTGGCTAA-3'	1400-1377	24
TaqMan probe	5'-FAM-ACCACGTTCTTCATCGACTGCATGGC-TAMRA-3'	1302-1327	26
Cu/Zn superoxide dismutase			
Forward primer	5'-TGTACCACTGCAGGACCTCATT-3'	224-245	22
Reverse primer	5'-GCCACACCGTCCTTTCCA-3'	339-322	18
TaqMan probe	5'-FAM-CCAACATGCCTCTCTTCATCCGCTG-TAMRA-3'	276-300	25
Glutamylcysteine gamma synthetase light chain			
Forward primer	5'-CATCCTCCAGTTCCTGCACAT-3'	113-133	21
Reverse primer	5'-CATGATCAAAGGACACCAACATG-3'	216-194	23
TaqMan probe	5'-FAM-ACCACGCAGTCAAGGACCGGCA-TAMRA-3'	136-157	22
Glutathione S-transferase			
Forward primer	5'-CTTGCTTATGACATTCTTGACCAGTAC-3'	511-537	27
Reverse primer	5'-TTCATGTAGGCAGAGATCTTCTTCA-3'	632-608	25
TaqMan probe	5'-FAM-CCTGGACGCCTTCCCAAACCTGA-TAMRA-3'	558-580	23
Heme oxygenase 3			
Forward primer	5'-CAGAGAGCCCGTGGTAGCA-3'	896-914	19
Reverse primer	5'-AGTTCACCAGGCAGCTTTCTTG-3'	990-969	22
TaqMan probe	5'-FAM-CCACGAGCGAACTTGAGCCTCAGG-TAMRA-3'	917-940	24
Protein kinase B kinases			
Forward primer	5'-GCCTCTCAAAGCTCATCCATTCT-3'	1069-1091	23
Reverse primer	5'-TGCCATAACAGTCTTCATCATCTTC-3'	1190-1166	25
TaqMan probe	5'-FAM-TCCATCACGTGGGAGAATCTGCACC-TAMRA-3'	1097-1121	25
Phosphoinositide 3-kinase p85 alpha			
Forward primer	5'-TCTGTAGTGGTAGATGGCGAAGTC-3'	1172-1295	24
Reverse primer	5'-CCAGCTCTTTCAGGGAGCTGTA-3'	1277-1256	22
TaqMan probe	5'-FAM-TCATCAACAAGACTGCCACCGGCTATG-TAMRA-3'	1206-1232	27

ple, 50 ng of total RNA were reverse transcribed in a 5-µL reaction containing 1X TaqMan RT buffer, 5.5 mM $MgCl_2$, 500 µM of each 2'-deoxynucleotide 5'-triphosphate, 2.5 µM random hexamers, 0.4 U/µL RNase inhibitor, and 1.25 U/µL Multiscribe reverse transcriptase (PE Applied Biosystems, Forster City, Calif). Control reactions for each RNA sample containing no RT enzyme were also set up to assess any genomic DNA contamination. RT was performed in a DNA Thermal Cycler 480 (PE Applied Biosystems) at 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min.

For real-time RT PCR, all primers and TaqMan probes were designed using Primer Express software version 1.0 (PE Applied Biosystems) based on the sequence data of Genebank. PCR reactions were performed, recorded, and analyzed by using the ABI 7700 Prism Sequence Detection system (PE Applied Biosystems). Primer and TaqMan probe sequences for the selected genes are presented in Table 1. The housekeeping GAPDH gene was used as internal control. The TaqMan probe and primers for rat GAPDH were supplied by PE Applied Biosystems in a control reagent kit. We have found that no difference in GAPDH mRNA expression between WKY and SHR samples in these experiments (data not shown). PCR reactions were carried out in 50 µL volumes consisting of 1 X TaqMan buffer A (including passive reference), 5.5 mM MgCl2, 200 µM deoxyATP, 200 µM deoxyCTP, 200 µM deoxyUTP, 400 µM deoxyUTP, 100 nM TaqMan probe, 200 nM forward primer, 200 nM reverse primer, 0.01

U/μL AmpErase UNG, and 0.025 U/μL AmpliTaq Gold DNA polymerase (PE Applied Biosystems). A 5-μL cDNA sample and no RT control preparations were amplified using the following conditions: 50°C for 2 minutes and 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Target and GAPDH genes are amplified in a separate tube, and all experiments were performed in triplicate.

Probes for detection of target were labeled with a fluorescent dye FAM (6-carboxy-fuorescein) and a quencher dye TAMAR (6-carboxy-tetra-methylrhodamine). The probe for detection of the standard GAPDH was labeled with a fluorescent dye VIC (6-carboxyrhodamine 6G) and quencher dye TAMAR. After the target amplification, the probe annealed to the amplicon and was displaced and cleaved between the reporter and quencher dyes by the nucleolytic activity of the polymerase. The amount of product resulting in detectable fluorescence at any given cycle within the exponential phase of PCR is proportional to the initial number of template copies. The number of PCR cycles (the threshold cycle, CT) needed to detect the amplicon is, therefore, a direct measure of template concentration.

The primer pairs for the targets were selected using the PE Biosystems Guidelines for Amplifying Custom Target Sequences for Quantitation. Following these guidelines allows the amplification of all targets to be performed using the same reaction conditions and thermal cycling parameters. The mean CT value of the WKY group was used as a "calibrator" to which SHR samples were compared. To use comparative CT method for relative quantitation, ΔCT for WKY and SHR sample are calculated; where ΔCT is the difference in threshold cycles for target and GAPDH. Then the expression of the SHR target gene relative to the calibrator WKY target gene was evaluated using the expression 2-ΔΔCT ; where ΔΔCT is the difference in ΔCT for SHR and WKY samples.

RESULTS

General Data

As expected, the SHR group exhibited marked elevation of arterial pressure when compared with the WKY controls (203 ± 5.77 mm Hg versus 133 ± 5.77 mm Hg; $P < .001$). No significant difference was found in serum creatinine (0.45 ± 0.03 mg/dL versus 0.48 ± 0.05 mg/dL) or creatinine clearance (4.55 ± 0.64 mL/min/kg vs. 4.70 ± 0.84 mL/min/kg) between the two groups.

Microarray Data and Real Time RT PCR Results

Of the 8,799 gene and EST probe sets analyzed by the Affymetrix RGU34A microarray, 74 genes and ESTs were observed to have different expression levels between the WKY and SHR kidneys, according to our selection criteria. Of these genes and ESTs with changed expression, 43 were up-regulated (Table 2) and 31 were down-regulated (Table 3) in SHR. Because whole kidney was the source of RNA for this study, a variety of cell types may contribute to these differentials in gene expression, including endothelial cells, mesangial cells, interstitial cells, glomerulotubular, and vascular cells. Functional classification of differentially expressed genes is shown in Table 4. Table 5 shows normalized Avg Diff values for GAPDH control probe sets. There was no difference in mean signal intensity of GAPDH transcripts between the WKY and SHR groups pointing to comparable transcription efficiency and overall expression of this housekeeping gene in the two groups.

Significant differences were seen in expression of certain genes involved in lipid and carbohydrate metabolisms, signal transduction, and intracellular traf-

Table 2. Genes Upregulated in SHR as Compared with WKY Rat

Genebank accession No.	Fold change	Gene description	Normalized signal intensity	
			WKY	SHR
S62516	>20	SA gene	0.03	2.03
M15114	9.85	DNA polymerase alpha mRNA, 3' end	0.19	1.84
Z75029	9.61	Heat shock protein (HSP) 70.2 mRNA for HSP 70	0.26	2.45
L16764	9.01	HSP 70 mRNA, complete cds	0.32	2.87
X65083	7.48	Cytosolic epoxide hydrolase	0.26	1.96
L19998	7.13	Minoxidil sulfotransferase mRNA, complete cds	0.28	1.99
AI136977	7.12	EST	0.27	1.92
U75397	6.77	Krox-24 mRNA, 3' untranslated region, partial sequence	0.34	2.27
L07281	6.23	Carboxypeptidase E (CPE) gene, exon 9	0.32	2.00
M35601	2.40-5.70	Alpha-fibrinogen mRNA, 3' end	0.35-0.58	1.40-2.02
AA818604	5.12	HSP 70-1	0.50	2.54
AA875269	5.02	Stearoyl-CoA desaturase	0.35	1.76
AI639294	4.88	EST	0.39	1.89
AF023087	4.88	Nerve growth factor induced factor A mRNA, partial 3'UTR	0.42	2.06
M18416	4.39	Nerve growth factor-induced (NGFI-A) gene, complete cds	0.47	2.08
AA998683	3.02	Heat shock 27 kDa protein	0.73	2.21
AA946439	2.96	H4 gene for somatic histone H4	0.52	1.55
S63521	2.89	Glucose-regulated protein GRP 78	0.46	1.32
AI014169	2.66	EST	0.52	1.39
D13871	2.65	Glut 5 protein, complete cds	0.54	1.43
AF013144	2.65	MAP-kinase phosphatase (cpg21) mRNA, complete cds	0.54	1.44
AI236601	2.64	EST	0.58	1.54
L37333	2.61	Glucose-6-phosphatase mRNA, complete cds	0.66	1.71
H33426	2.56	EST	0.55	1.41
M11794	2.46	Metallothionein-2 and metallothionein-1 genes, complete cds	0.64	1.57
U30186	2.45	Growth arrest, DNA-damage-inducible protein GADD153 mRNA, complete cds	0.62	1.53
AI102562	2.42	Metallothionein-1	0.60	1.44
AI176546	2.40	EST	0.67	1.60
L14004	2.15	Polymeric immunoglobulin receptor mRNA sequence	0.66	1.43
AA892264	2.12	EST	0.63	1.33
U53922	2.09	DnaJ-like protein (RDJ1) mRNA, complete cds	0.65	1.35
D12769	2.02	Basic transcription element binding protein, complete cds	0.66	1.34
AA900476	2.01	MRG1:melanocyte-specific gene 1-related gene	0.75	1.51
U75393	2.01	Succinyl-CoA synthetase alpha subunit mRNA	0.73	1.46
X13044	1.80-2.0	CD74 antigen (Rat mRNA for MHC class II-associated invariant chain gamma)	0.71-0.74	1.33-1.42
AA800318	1.98	EST	0.62	1.23
M25804	1.96	Rev-ErbA-alpha protein mRNA, complete cds.	0.74	1.45
AI176456	1.94	EST (Highly similar to metallothionein-2)	0.72	1.40
Y00404	1.89	Cu/Zn superoxide dismutase	0.70	1.33
AA955477	1.87	EST (similar to human MAPK activated protein kinase-3 mRNA)	0.73	1.37
M15562	1.78	MHC class II RT1.u-D-alpha chain	0.77	1.37
AI176658	1.76	Heat shock 27 kD protein 1	0.79	1.39
U02506	1.75	Polymeric immunoglobulin receptor mRNA	0.75	1.32

ficking, antioxidant system, drug metabolism, stress response, anti-inflammatory system, regulation of gene transcription, and DNA synthesis, as well as several other functions. In an attempt to validate the results of the microarray experiments, we performed real-time RT PCR on 7 genes dealing with lipid metabolism, insulin signal transduction, and the antioxidant defense, which are of interest to our group. The real-time RT PCR results are shown in Table 6. Of the 7

Table 3. Genes Downregulated in SHR as Compared with WKY Rat

Genebank accession No.	Fold change	Gene description	Normalized signal intensity	
			WKY	SH
U50412	>20	Phosphoinositide 3-kinase p85 alpha	2.53	0.03
AA946368	10.86	CD 36 antigen	2.97	0.27
AF072411	6.14-6.67	Fatty acid translocase/CD 36 mRNA, complete cds	2.09-2.49	0.34-0.37
M25157	6.07	Cu/Zn superoxide dismutase mRNA, complete cds	2.00	0.33
M33746	5.56	UDP glucuronosyltransferase-5 mRNA, complete cds	2.14	0.39
M13100	5.35	Long interspersed repetitive DNA sequence LINE3 (L1Rn), heme oxygenase-3	2.39	0.45
M96601	4.81	Taurine transporter mRNA, complete cds	1.89	0.39
Y15748	4.57	Protein kinase B kinase (3-phosphoinositide dependent protein kinase-1)	2.01	0.44
AI178971	4.26	Hemoglobin, alpha 1	1.75	0.41
D78018	4.08	Nuclear factor IA, complete cds	1.50	0.37
X07686	4.07	L1Rn B6 repetitive DNA element	1.97	0.49
AA859837	3.43	Guanine deaminase	2.10	0.61
U83119	3.30	L1 retrotransposon ORF2 mRNA, consensus sequence, partial cds	1.91	0.58
M10094	3.28	MHC class I truncated cell surface antigen mRNA	1.55	0.47
X04229	3.27	Glutathione S-transferase mu type 2	1.65	0.51
AA891943	3.24	EST	1.55	0.48
L23128	3.12	MHC class I mRNA, complete cds	1.65	0.53
AA875500	3.10	EST	1.67	0.54
AA800566	3.01	EST	1.63	0.54
M13101	3.01	Long interspersed repetitive DNA sequence LINE4 (L1Rn)	1.72	0.57
L00117	2.88	Elastase I gene, exon 8	1.52	0.53
X13905	2.70	cDNA for ras-related rab1B protein	1.47	0.54
X53581	2.61	Long interspersed repetitive DNA containing 7 ORF's	1.64	0.63
D10852	2.59	N-acetylglucosaminyltransferase III, complete cds	1.48	0.57
J05181	2.57	Gamma-glutamylcysteine synthetase mRNA, complete cds	1.47	0.57
M88601	2.35	Meprin beta-subunit mRNA, complete cds	1.50	0.64
AA892446	2.33	EST	1.52	0.66
AF081204	2.24	Small intestine sodium dependent multivitamin transporter mRNA, complete cds	1.51	0.67
AA892388	2.11	CD 59 antigen	1.39	0.66
X61295	1.90	L1 retroposon, a portion of its ORF2 sequence	1.5	0.79
U28504	1.89	Sodium-dependent inorganic phosphate cotransporter-1 (RNaPi-1a; RNaPi-1b)	1.46	0.77

genes studied, five showed concordance with the microarray results. However, significant down-regulation of protein kinase B kinase mRNA level shown by microarray technique could not be confirmed by real-time RT PCR. Two probe sets representing Cu/Zn superoxide dismutase (SOD) showed discordant results in microarray analysis (Y00404, increased in SHR; M25157, decreased in SHR). However, real-time RT PCR analysis showed no difference between the WKY and SHR groups. It is of note that review of the published literature provided confirmation of our microarray data for several other genes. These included up-regulation of epoxide hydrolase,[11] *HSP70*,[12] and SA genes,[13] and down-regulation of CD36 gene mRNA.[7]

DISCUSSION

The present study identified some 43 upregulated and 31 downregulated genes in the adult SHR. For the purpose of the present discussion, the differentially expressed genes with known functions have been grouped into several

Table 4. Functional Classification of Differentially Expressed Genes

Category	Gene product	Change	Function
Lipid metabolism	Fatty acid translocase/CD 36	Decreased	Long chain fatty acid translocase, clear fatty acid from circulation
	Taurine/beta-alanine transporter	Decreased	Catalyzes the transport of taurine into the cells. Taurin improve insulin sensitivity and hyperlipidemia
	SA gene (acyl-CoA synthetase)	Increased	Activate medium chain nonesterified fatty acid to its CoA derivative
	Soluble epoxide hydrolase	Increased	Hydrolyze vasodilating and natriuretic epoxyeicosatrienoic acids
	Stearoyl-CoA desaturase 2	Increased	Synthesis of monounsaturated fatty acid
	Succinyl-CoA synthetase alpha	Increased	Citric acid cycle enzyme, roles in the mitochondrial metabolism leading to insulin secretion
Carbohydrate metabolism	Glucose transporter 5	Increased	Fructose transporter, increase triglyceride concentration
	Glucose-6-phosphatase	Increased	Key enzyme in homeostatic regulation of blood glucose levels, increase endogenous glucose production
	Succinyl-CoA synthetase alpha	Increased	Citric acid cycle enzyme, roles in the mitochondrial metabolism leading to insulin secretion.
Signal transduction	Phosphoinositide 3-kinase p85 alpha	Decreased	Central part in the mediation of insulin-stimulated glucose disposal
	Protein kinase B kinase	Decreased	Enable phosphatidylinositol 3,4,5-trisphosphate to activate protein kinase B
	MAP-kinase phosphatase	Increased	Dephosphorylation and inactivation of MAP kinases
	MAP-kinase activated protein kinase-3	Increased	Activated by stress and cytokines, substrate of CSBP p38 MAP kinase, able to phosphorylate HSP27
Protein vesicle trafficking	Rab 1b	Decreased	Involved in vesicular traffic, control vesicle traffic from the trafficking endoplasmic reticulum to the Golgi apparatus
	Mannoside acetyl glucosaminyl transferase 3	Decreased	Synthesis of glycoprotein oligosaccharides, catalyzes the addition of n-acetylglucosamine to the beta-linked mannose
Antioxidant system	Gamma glutamylcysteine synthetase light chain	Decreased	Rate-limiting enzyme for de novo glutathione biosynthesis
	Glutathione S-transferase	Decreased	Conjugation of reduced glutathione to a wide number of exogenous and endogenous hydrophobic electrophiles
	Heme oxygenase-3	Decreased	Cleaves the heme ring to form biliverdin and carbon monoxide
Drug metabolism and detoxification	UDP-glucuronosyltransferases	Decreased	Phase II conjugation pathway, conjugation and elimination of potentially toxic xenobiotics and endogenous compounds
	Glutathione S-transferase	Decreased	Phase II conjugation pathway, conjugation of glutathione to exogenous and endogenous hydrophobic electrophiles
	Minoxidil sulfotransferase	Increased	Phase II conjugation pathway, catalyses o-sulfation of phenols, n-o-sulfation of minoxidil and tyrosine esters
	Soluble epoxide hydrolase	Increased	Phase I metabolism, role in xenobiotic metabolism by degrading potential toxic epoxides
Cytoprotection	CD 59	Decreased	Restrain complement-mediated injury
	Taurine/beta-alanine transporter	Decreased	Catalyzes the transport of taurine into the cells, attenuate the deleterious effects of radiation and oxidative damage
	Heat shock protein (HSP) 70	Increased	Protect cell against oxidative stress, ischemia and other harmful stimuli
	Metallothionein	Increased	Bind various heavy metals, transcriptionally regulated by heavy metals and glucocorticoids
HSP/molecular chaperone	HSP 70	Increased	Cytoprotective and molecular chaperone in protein biogenesis
	DnaJ-like protein (HSP 40)		Co-chaperone of hsc 70, seems to play a role in protein import into mitochondria
	Succinyl-CoA synthetase alpha	Increased	HSP 70 substrate and HSP 70 interacting proteins
	HSP 27 kDa	Increased	Involved in stress resistance and actin organization
	Glucose-regulated protein GRP 78	Increased	Minor HSP, induced under condition of glucose deprivation
Transcription regulators	Nuclear Factor IA	Decreased	Binds the sequence 5'- TTGGCN5GCCAA-3' in promoters, activate or repress the initiation of gene transcriptions
	Basic transcription element binding protein	Increased	Activate or repress the transcription of genes with GC-box sequences in their promoter
	Krox-24	Increased	Transcription factor, an immediate-early serum response gene
	Nerve growth factor-induced protein	Increased	Transcriptional regulator, binds to the egr-site, activate the (EGR1) transcription of target genes for mitogenesis and differentiation
	MRG1	Increased	Transcription factor, Cbp/p300-interacting transactivator
	Rev-ErbA-alpha protein	Increased	Orphan nuclear receptors that function as dominant transcriptional silencers, regulate mammalian muscle and adipocyte differentiation
DNA synthesis, replication	DNA polymerase alpha subunit	Increased	Nuclear DNA replication
	H4 gene for somatic histone H4	Increased	Play a central role in nucleosome formation

Continued

Table 4. Functional Classification of Differentially Expressed Genes (Continued)

Category	Gene product	Change	Function
Others	Long interspersed elements (LINE)	Decreased	Retrotransposone, repetitive, mobile genetic element, site of recombination
	MHC class I	Decreased	Involved in the presentation of foreign antigens to the immune system
	MHC class II	Increased	Involved in the presentation of foreign antigens to the immune system
	Meprin 1 beta	Decreased	Hydrolysis of protein and peptide substrates, degrade extracellular matrix
	Elastase I	Decreased	Hydrolysis of proteins including elastin
	Guanine deaminase	Decreased	Catalyzes the hydrolytic deamination of guanine, producing xanthine and ammonia
	HBA1 (hemoglobin, alpha 1)	Decreased	Encode hemoglobin alpha 1
	Sodium dependent multivitamin transporter	Decreased	Transports pantothenate, biotin and lipoate in the presence of sodium
	Alpha-fibrinogen	Increased	Yield monomers that polymerize into fibrin and acting as a cofactor in platelet aggregation
	Carboxypeptidase E	Increased	Removes residual c-terminal Arg or Lys remaining after initial endoprotease cleavage during prohormone processing
	GADD153, DNA-damage inducible transcript 3	Increased	Member of the error-free postreplication repair pathway
	Polymeric immunoglobulin receptor	Increased	Role in host defense
	Sodium-dependent inorganic phosphate cotransporter-1	Decreased	Maintain intrcellular phosphate, may mediate sodium-sodium and sodium-lithium exchange activities

functional categories. However, we wish to acknowledge the risk of extrapolation from mRNA abundance to pathophysiologic function which ultimately depends on the synthesis, catabolism and functional status (such as phosphorylation) of the corresponding proteins. Accordingly, significant alterations in gene expression may result in negligible biologic impact if not accompanied by parallel changes in the abundance of the functionally active protein. Likewise, minimal changes in gene expression may be associated with profound biologic impact in the face of discordant protein abundance and activity. Finally, minimal alteration in protein abundance and activity may be sufficient to exert major impact under steady state chronic conditions. Thus, definitive conclusions as to the functional significance of our findings await measurements of the abundance and activities of the corresponding proteins.

The SHR group showed dysregulations of several genes involved in lipid metabolism. Our results confirmed the previous studies, which showed up-regulation of SA and epoxide hydrolase genes and down-regulation of CD 36 gene in SHR compared to WKY rat.[7,11,13] Human SA gene product has acyl-CoA synthetase activity for medium-chain fatty acids and presumably leads to increased accumulation of triglycerides.[14] CD 36 facilitates a major fraction of long chain fatty acid uptake by the heart, oxidative skeletal muscle, and adipose tissue.[7] The observed dysregulations of SA and CD 36 genes can contribute to visceral obesity, defective fatty acid metabolism, and insulin resistance.[7,14] Epoxide hydrolase metabolizes membrane phospholipid-derived epoxyeicosatrienoic acids to dihydroxyeicosatetraenoic acid. Epoxyeicosatrienoic acids are known to have vasodilating and natriuretic properties.[15] Up-regulation of epoxide hydrolase can, therefore, contribute to hypertension by accelerating inactivation of epoxyeicosatrienoic acids which can, in turn, promote vasoconstriction and volume expansion.[11]

This study revealed up-regulation of stearoyl-CoA desaturase-2 gene and down-regulation of taurine/beta-alanine transporter gene in SHR compared with WKY rat. Stearoyl-CoA desaturase-2 is responsible for the synthesis of monounsaturated fatty acids and, as such, its up-regulation can alter the ratio of oleic acid to stearic acid.[16] Abnormal alteration of this ratio has been shown to

Table 5. Normalized Average Difference Values for GAPDH Control Gene

Rats		Portion of GAPDH gene		
		3'	5'	middle
WKY	1	0.907	0.910	0.860
	2	0.968	1.048	0.973
	3	1.039	1.198	1.096
	Mean ± SD	0.971± 0.066	1.052 ± 0.144	0.976 ± 0.118
SHR	1	1.093	0.998	1.034
	2	0.742	0.792	0.868
	3	1.032	1.002	1.027
	Mean ± SD	0.956 ± 0.048	0.931 ± 0.120	0.976 ± 0.094

play a role in several physiologic and pathologic states, including diabetes, obesity, hypertension, neurologic diseases, immune disorders, cancer, and aging.[17] Increased production of oleate by stearoyl-CoA desaturase may lead to increased triglyceride synthesis, resulting in obesity and insulin resistance.[18] It is also reported that adipose tissue stearoyl-CoA desaturase mRNA expression is elevated,[19] and its enzyme activity is not suppressible by high fat diet in the obese diabetic Zucker rats.[18] In contrast, the enzyme activity is markedly suppressed by high fat diet in the lean littermate.[18] In view of these considerations, up-regulation of stearoyl-CoA desaturase-2 gene could contribute to dyslipidemia and insulin resistance in SHR. Taurine/beta-alanine transporter catalyzes the cellular uptake of taurine from the circulating blood. Taurine has been shown to lower serum cholesterol and triglycerides in a rat model of spontaneous type II diabetes presumably via increased cholesterol conversion to bile acids and decreased cholesterol production.[20] Taurine also attenuates hypertension and improves insulin sensitivity in the fructose-fed rat, an animal model of insulin resistance.[21] Therefore, down-regulation of taurine/beta-alanine transporter gene could be another mechanism of dyslipidemia and insulin resistance in SHR.

This study also found dysregulations of several genes involved in carbohydrate metabolism in SHR, including up-regulation of glucose transporter 5, glucose-6-phosphatase (G6Pase), and succinyl-CoA synthetase alpha genes. Glucose transporter 5 is a fructose transporter.[22] Because increased fructose consumption has been shown to cause hypertriglyceridemia and hyperinsulinemia,[23] up-regulation of glucose transporter 5 could potentially contribute to insulin resistance and hypertension in SHR. G6Pase, which promotes endogenous glucose production, is normally inhibited by insulin.[24] Therefore, up-regulation of G6Pase gene may, in part, contribute to insulin resistance in SHR. Alternatively, elevation of G6Pase despite the presence of hyperinsulinemia may represent another facet of insulin resistance in this model. Similar finding was reported in type 2 diabetes in which G6Pase expression is insensitive to insulin.[25] Succinyl CoA synthetase generates GTP in the citric acid cycle which could play a central role in the beta cell mitochondrial metabolism leading to insulin secretion.[26]

Down-regulations of phosphoinositide 3-kinase, rab 1 b, and mannoside acetyl glucosaminyl transferase-3 genes in SHR were among other findings of this study. Activated phosphoinositide 3-kinase and its 3-phosphorylated inositol lipid products mediate most insulin-induced cellular responses, such as translocation of glucose transporter-4 (GLUT4) vesicles, glycogen synthesis,

Table 6. mRNA Expression Ratio Between SHR and WKY Rat

Gene	Ratio (SHR/WKY)
Heme oxygenase-3	0.25
Glutathione S-transferase	0.34
Phosphoinositide 3-kinase p85 alpha	0.41
Gamma glutamylcysteine synthetase light chain	0.53
Stearoyl-CoA desaturase 2	1.45
Cu/Zn superoxide dismutase	0.98
Protein kinase B kinases	1

and insulin-induced protein synthesis.[27] Therefore, down-regulation of phosphoinositide 3-kinase could be a major underlying mechanism for insulin resistance in SHR. Rab 1 b controls vesicle traffic from the endoplasmic reticulum to the Golgi apparatus.[28] Mannoside acetyl glucosaminyl transferase-3 catalyzes the addition of N-acetylglucosamine to the mannose of N-linked oligosaccharides in the process of protein glycosylation.[29] GLUT4, the insulin-responsive glucose transporter, contains a single N-glycosylation site, and glycosylation of GLUT4 appears to play an important role in regulation of its function.[30] Therefore down-regulations of rab 1 b and mannoside acetyl glucosaminyl transferase 3 could result in major disturbance of GLUT4 vesicle trafficking pathway.

Several recent studies have shown that certain forms of genetic or acquired hypertension are associated with oxidative stress.[31-34] The present study showed down-regulation of gamma glutamylcysteine synthetase, glutathione S-transferase, and heme oxygenase (HO) 3 genes. Gamma glutamylcysteine synthetase is the rate-limiting enzyme for glutathione synthesis.[35] Glutathione S-transferase adds glutathione to electrophiles with a variety of chemical structures and protects cells from oxidative stress.[36] Thus, down-regulations of gamma glutamylcysteine synthetase and glutathione S-transferase can lead to glutathione deficiency and defective protection against oxidative injury. These events can, in turn, contribute to oxidative stress and hypertension in SHR. The latter proposition is supported by the result of our previous study in which induction of oxidative stress by glutathione depletion caused severe hypertension in genetically normal rats.[37] HO, which exists in constitutive (HO-2 and HO-3) and inducible (HO-1) isoforms, degrades pro-oxidant heme to produce carbon monoxide and biliverdin, which is a potent antioxidant.[38] Endogenous carbon monoxide is a potent vasodilator and possesses anti-inflammatory and anti-apoptotic properties.[39] Therefore, down-regulation of constitutive HO 3 isoform as shown in this study could potentially contribute to oxidative stress, inflammation, and hypertension in SHR.

Expressions of several genes involved in the phase II drug metabolism,[40] such as 17-beta-hydroxysteroid UDP-glucuronosyltransferase and minoxidil sulfotransferase genes as well as glutathione S-transferase gene were altered in SHR. If true in humans with essential hypertension, who are frequently treated with various blood pressure lowering and other drugs, these changes would be of considerable pharmacological interest. In addition, dysregulation of these enzymes can potentially contribute to hypertension in SHR by altering conjugation and ultimate elimination of vasoactive substances, such as, steroids and catecholamines which are usually metabolized by glucuronidation and sulfation reactions.[40,41]

The SHR group exhibited a marked increase in heat shock protein 70 (HSP 70) mRNA in the kidney. This observation is consistent with earlier reports by other investigators.[12] In fact, as reviewed by Hamet,[42] hypertensive animals and humans exhibit increased thermosensitivity and a locus of thermosensitivity segregates with blood pressure in rodents. In this regard, HSP gene family has been considered as a potential candidate for development of hypertension.[42] Conversely, up-regulations of HSP-related genes and metallothionein genes may represent a protective response to biologic stresses associated with hypertension and oxidative stress in SHR. This supposition is supported by the reported rise in HSP70, abundance in genetically normotensive animal rendered acutely hypertensive by administration of either phenylephrine, angiotension-II, endotheline, vasopressin, or catecholamines.[43] The protective effect of HSP's probably is mediated by their capacity to function as molecular chaperones to prevent inappropriate protein aggregation and to mediate transport of immature proteins to the target organelles for final packaging, degradation, or repair.[44] Metallothioneins are induced by a variety of inducers that include toxic heavy metals, reactive oxygen species, and different types of stress. They contain numerous cysteine residues that bind heavy metals and serve as antioxidants in mammals[45]

Although up-regulations of HSPs and metallothioneins serve a protective function, down-regulations of taurine/beta-alanine transporter gene and the complement regulatory membrane protein CD59 gene could have deleterious effects on oxidative stress and hypertension in SHR. In addition to its role in lipid metabolism,[20] taurine functions as an osmolyte in a variety of tissues and is known to attenuate the deleterious effects of radiation and oxidative damage in a number of tissues and cell types.[46] Thus, down-regulation of taurine/beta-alanine transporter gene may contribute to tissue injury associated with hypertension in SHR. CD 59 has an important role in restraining complement-mediated injury via limiting membrane attack complex (MAC) formation.[47] Insertion of the MAC into endothelial cell membranes causes the release of growth factors that stimulate tissue growth and proliferation.[47] MAC can also act on vascular smooth muscle cell to promote the release of monocyte chemotactic protein-1, thus leading to chemoattraction of monocytes.[48] Immunocompetent cells infiltrate the kidney in several models of experimental hypertension.[9] In fact, lymphocytes and macrophages have been shown to infiltrate the kidney of SHR and produce inflammation and oxidative stress, which could play a role in the genesis and maintenance of hypertension in this model.[49] Because the complement regulatory membrane protein, CD59, restricts MAC formation, down-regulation of CD59 gene can potentially facilitate infiltration of immunocompetent cells in the kidney and, thereby, aggravate hypertension, tissue injury and vascular complication in SHR.

Many genes involved in transcriptional regulation and DNA synthesis are significantly altered in SHR. Functional significance of each of the altered genes and its relevance to the pathogenesis of hypertension and the associated disorders in SHR requires further study.

Several types of long interspersed elements (LINEs) are underexpressed in SHR compared with the WKY rat. LINEs are repetitive mobile DNA elements that occasionally transpose into new site within the genome via RNA intermediates such as retrotransposone.[50] Therefore, they can be a site of recombination (unequal crossing over)

between repetitive sequences, leading to concomitant duplication and deletion of other genes located in the vicinity of the LINEs.[51] For example, it is suggested that aforementioned CD36 gene deficiency in SHR is caused by a genomic deletion event resulting in creation of a dysfunctional chimaeric gene.[7,52] Underexpression of LINEs in SHR shown in this study suggests that SHR might have originated from WKY rat by the mechanism of gene deletion between LINEs. This recombination also can explain partially the difference in CD 36 gene mutation observed between two SHR strains: the original SHR/Izm strain with normal CD36 gene and NIH-derived strain SHR/NCrj with mutant CD36 gene. This is possibly caused by a spontaneous de novo mutation.[53] In the present study, we used NIH-derived strain. Therefore, some of the observed discrepancies in gene expression between SHR and WKY in this experiment might be the result of the de novo mutations during breeding at the NIH.

Ideally, all genes described as differentially expressed by the microarray analysis should be confirmed by additional techniques, such as RT-PCR or Northern blot analysis. Thus, the modifications described in mRNA expression of genes, which were not confirmed by real-time RT-PCR, should be viewed with caution. Because this experiment was performed at 22 weeks of age, at which time blood pressure is markedly elevated in SHR, some of the dysregulated genes shown in this study could be a consequence rather than a cause of the hypertension. Further study using young prehypertensive SHR is required to address this issue.

The majority of published studies exploring the mechanism and treatment of hereditary hypertension in experimental animals have employed male gender. Thus, the available data on this important topic in female gender are extremely limited. Moreover, we are aware of at least one group conducting similar studies using male SHR. We, therefore, elected to study the female SHR to generate badly needed data on genetic hypertension in female gender while avoiding duplication of the ongoing study by the other groups and providing the opportunity to discern possible gender differences. We realize that hormonal variations inherent in fertile female animals employed here could have also influenced the results. Further studies are planned using ovariectomized rats with and without estrogen replacement in this model to discern the effects of menopause and hormone replacement.

In conclusion, this study provided a potential basis for insulin resistance, defective insulin signal transduction pathway, abnormal lipid and glucose metabolisms, impaired antioxidant and anti-inflammatory defense systems and oxidative stress in adult SHR.

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