# Neuroendocrine Transcriptome in Genetic Hypertension
## Multiple Changes in Diverse Adrenal Physiological Systems

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***Abstract***—The genetic basis of hypertension in the genetically/hereditary hypertensive (BPH/2) mouse strain is incompletely understood, although a recent genome scan uncovered evidence for several susceptibility loci. To probe the neuroendocrine transcriptome in this disease model, 12 488 probe set microarray experiments were performed on mRNA transcripts from adrenal glands of juvenile (prehypertensive) and adult BPH/2 (hypertensive), as well as the genetically/hereditary low-blood pressure (BPL/1), strains at both time points. To determine the impact of strain (BPH/2 versus BPL/1), age (juvenile versus adult), and the interaction of strain and age on gene expression levels, we performed standard 2-factor ANOVA and computed a concordance coefficient to assess the reproducibility of gene expression measurements among replicates. Of genes with significant ($P<0.05$) differential expression, 2647 showed strain differences, 982 showed age differences, and 757 exhibited strain-by-age interaction. Fold-changes in gene expression assayed by microarray were confirmed in a subset by real-time polymerase chain reaction ($R=0.739$, $P=0.0094$). We used a systems biology approach to evaluate alterations in contributing biochemical pathways and we statistically quantified these global pathway disturbances using the Kolmogorov-Smirnov goodness-of-fit test. We found widespread, indeed global, alterations in patterns of gene expression in diverse systems of BPH/2: in sympathochromaffin transcripts suggesting increased sympathetic stimulation; in vasoconstrictor/vasodilator systems; global reductions in carbohydrate intermediary metabolism; and increases in oxidative stress, with changes in oxygen radical forming and disposition enzymes. These analyses highlight widespread derangements in diverse physiological pathways, providing multiple avenues for further investigation into the pathogenesis of genetic hypertension. (***Hypertension*. 2004;43:1301-1311.)**

**Key Words:** hypertension ■ genetics ■ adrenal gland ■ gene expression ■ metabolism ■ lipids ■ oxidative stress



Hypertension displays substantial genetic influence, with heritability estimates for blood pressure ranging from ≈30% to 50%.[1] The multifactorial nature of this condition, however, has made it difficult to elucidate the underlying genetic components. With the advent of gene expression microarray technology, it has become possible to study large numbers of genes in parallel, which is ideal for studying polygenic diseases such as hypertension.[2] High-throughput microarray studies of hypertension are well complemented by the use of genetic models of hypertension. The genetically/hereditary hypertensive "blood pressure high" (BPH/2) mouse was developed by Schlager in a selection program to develop a strain of hypertensive mice inbred to homozygosity.[3] The BPH/2 strain parallels human hypertension, with elevated blood pressure, higher heart rate, and early mortality.[4] Schlager's selection program also included concurrent development of a hypotensive mouse strain, the "blood pressure low" (BPL/1).[3] Although not a normotensive control, the BPL/1 is often used in genetic studies of BPH/2 because extreme phenotypes are expected to improve the likelihood of detecting underlying determinants.

Adrenal gland secretory products, both medullary and cortical, are logical candidates for study in hypertension because they directly influence endocrine, cardiovascular, and sympathetic function. Epinephrine and norepinephrine act through G-protein–coupled adrenergic receptors to affect sympathetic functions, such as the force of contraction of the heart and constriction of blood vessels. Adrenal cortical mineralocorticoid hormones regulate the reabsorption and secretion of sodium and potassium and can therefore also modulate blood pressure. Study of the adrenal neuroendocrine transcriptome, therefore, might be an efficient way to investigate many candidate genes for hypertension simultaneously.

The purpose of this study is thus to use global gene expression patterns in the adrenal gland to explore the genetic basis of hypertension in BPH/2 in an attempt to gain insight into the cause of human essential hypertension.

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## Methods

### Animals

Juvenile (≈5-week-old) and adult (≈12-week-old), hypertensive (BPH/2@J; inbred generation F=55) and hypotensive (BPL/1@J; inbred generation F=55) male mice were obtained from colonies at the Jackson Laboratory (Bar Harbor, Me). A total of 12 mice were obtained: 3 juvenile BPH/2, 3 adult BPH/2, 3 juvenile BPL/1, and 3 adult BPL/1. Systolic blood pressure of the BPH/2 increases early in life. At 7 weeks of age, BPH/2 systolic blood pressure (110 mm Hg) is 40 mm Hg higher than BPL/1 (70 mm Hg).[4] The blood pressure differential increases to 60 mm Hg by 21 weeks of age (BPH/2, 130 mm Hg; BPL/1, 70 mm Hg).[4]

### Microarray Analysis and Real-Time Polymerase Chain Reaction

The methods for adrenal gland mRNA isolation, gene chip hybridization, and independent transcript quantitation by real-time polymerase chain reaction (RT-PCR) are described in an online Methods supplement available at http://www.hypertensionaha.org.

### Data and Statistical Analysis

Analysis of the scaled (globally normalized) microarray data was performed using in-house statistical software. The statistical analysis included an extension of Lin's measure of concordance[5] for assessing the reproducibility of the gene expression values for the 3 replicate readings for each of the 4 experimental conditions (ie, 2 ages and 2 strains), as well as standard 2-factor ANOVA[6] to assess the impact of strain, age, and strain-by-age interactions on gene expression levels. Essentially, the 3 replicate measures taken at a given experimental condition should give the same value if the expression of the gene was measured reliably. The concordance term ranges from −1 to +1, with −1 signifying perfect disagreement among replicates, 0 representing no agreement among replicates, and +1 indicating perfect agreement among replicates (ie, each measurement is exactly equal to the others). Each concordance of the measures was calculated using a modification of Lin's method to account for the 4 different sets of replicates. The in-house software computed 5 statistics for each probe set: concordance, overall, strain, age, and interaction. The overall statistic is an F-statistic generated from the ANOVA calculations that indicates significance of the probe in the overall model. Strain, age, and interaction are ANOVA F-statistics that indicate statistical significance between strains (ie, BPH/2 versus BPL/2), between ages (ie, juvenile versus adult), or a strain-by-age interaction, respectively (online Figure I, http://www.hypertensionaha.org). Each F-statistic (numerator degrees of freedom [df]=1, denominator df=8) corresponds to a $P$ value used to determine statistical significance for probe sets.

Results of the statistical analysis were clustered by empirical criteria (ie, ANOVA F-statistic), gene ontology (GO) (www.geneontology.org), and known gene function categories. Empirical clustering was performed by sorting the probe sets into strain, age, and interaction clusters based on ANOVA F-statistics. Probe sets not significant (at $P<0.05$) were excluded. Empirical clusters were not mutually exclusive, because probe sets could display significance in any combination of strain, age, or interaction.

The 3 empirical clusters (age, strain, interaction) were grouped into GO Biological Process categories using DAVID,[7] a tool created at the National Institutes of Health (NIH) to explore microarray data. Parameters used in DAVID are as follows: classification type=biological process; level=terminal node; minimum number of hits threshold=10 (for strain and interaction clusters); or minimum number of hits threshold=5 (for age cluster). Functional clustering (eg, catecholamine biosynthetic and elimination pathways) was performed by hand annotation of the data set based on particular biochemical pathways.

Other pathways not as well understood were studied in a high throughput manner with GenMAPP 1.0 software.[8] The GenMAPP derived pathways were also hand-annotated for completeness because the software is not specifically designed to handle Affymetrix probe annotation. Genes within functional clusters were examined for strain, age, and age-by-strain interaction differences. Two different ages were investigated, primarily to explore time-dependent adaptations or responses to the hypertensive state by identifying age-by-strain interaction differences, as opposed to early potentially pathogenic processes.

Complete well-defined biochemical pathways constructed by functional clustering were statistically analyzed for global perturbations resulting from strain differences using the Kolmogorov-Smirnov (KS) goodness-of-fit test.[9] The KS test was used to test the hypothesis that a given data set (ie, strain F-statistics for genes in a particular pathway) was drawn from a specific distribution (ie, 2-factor ANOVA F-distribution with numerator df=1, denominator df=8). The test was performed by computing the maximum deviation between the theoretical cumulative distribution function (CDF) and the empirical cumulative distribution function (ECDF) for the data set of each pathway. The maximum deviation between the CDF and ECDF is the test statistic and is known as "D". Each D value was translated to a $P$ value used to assign statistical significance to perturbations of entire pathways.

**TABLE 1. Empirical Cluster Groups**

| F-Statistic | $P$ | Age | Strain | Interaction |
|---|---|---|---|---|
| >5.32 | <0.05 | 982 | 2647 | 757 |
| >11.3 | <0.01 | 325 | 1226 | 191 |
| >14.7 | <0.005 | 204 | 869 | 122 |
| >25.4 | <0.001 | 74 | 410 | 46 |

Statistical breakdown of probes sorted by level of stringency ($P$ value), as well as by age, strain, and interaction. Columns 1 and 2 contain threshold levels of ANOVA F-statistics (numerator df=1, denominator df=8) and their corresponding $P$ values, respectively. Each integer corresponds to the number of probes meeting or exceeding the specified statistical criterion listed in that row. The Affymetrix MG-U74Av2 mouse chip encodes 12 488 genes (including quality-control points).

## Results and Discussion

### Statistical Analysis: Concordance and ANOVA

Concordance results (agreement among replicates) are documented in an expanded Results/Discussion section available online at http://www.hypertensionaha.org.

Significant ($P<0.05$) strain differences ranged from 0.07-fold to 21.54-fold ([BPH fluorescence]/[BPL fluorescence]). As expected in studying a complex multifactorial trait such as hypertension, most of the genes with strain significance show subtle fold changes between 0.5-fold and 2.0-fold; such modest fold changes reinforce the importance of a systems biology approach to analyzing the data (see later).

### RT-PCR: Verification of Chip Quantification of Transcripts

For the results, see the supplementary Results/Discussion online at http://www.hypertensionaha.org.

### Empirical (Statistical) Clustering

By 2-way ANOVA, 21% (2647/12488) of the probe sets exhibited strain significance, 8% (982/12 488) exhibited age significance, and 6% (757/12488) exhibited interaction significance at the $P<0.05$ level (Table 1). Differential gene expression contributing to the pathogenesis of the blood pressure trait might be expected to show a strain effect (including early prehypertensive changes), whereas responses (or adaptations) to the blood pressure elevation with increas-

ing age might be expected to display interaction effects, reflecting the time-dependent differences in trait exposure between the 2 strains. Increasing statistical stringency greatly reduced the size of the clusters (Table 1), but for initial screening of candidate loci we elected to use the lowest stringency ($P<0.05$) to minimize false-negative results.

## GO Clustering

GO clustering was performed to gain a wider perspective on disruptions in biological processes within the BPH/2 that may lead to or result from its hypertensive state (Figure 1). Two important observations arise from the strain differences in GO clusters: (1) the biological processes altered in the BPH/2 are large in number and diverse in process, perhaps not surprising in a polygenic disease such as hypertension; (2) approximately half of the genes do not yet have an agreed on biological process classification. This lack of classification arises because the functions of many genes in the mouse genome are still unknown, and perhaps half (≈6000) of the probe sets on the GeneChip represent expressed sequence tags. Thus, percentage assignments of differentially expressed transcripts to particular GO clusters might vary in the future as more expressed sequence tags are assigned known functions. Nonetheless, the spectrum of clusters encompassing differentially expressed transcripts is quite broad.

Strain differential expression is likely to provide clues to the pathogenesis of the trait, whereas interactions might point toward adaptations to (or consequences of) the trait with advancing age. In each case (strain, age, and interaction comparisons; Figure 1), differential gene expression patterns were documented for a variety of GO-clustered physiological processes (eg, transcriptional regulation, transport, electron transport, metabolism, protein phosphorylation, proteolysis, development, signal transduction, G protein-coupled receptor (GPCR) signaling, cell adhesion, and cell cycle/growth/proliferation).

Strain-by-age interaction differences included transcripts in several categories. Thus, the response (or adaptation) to genetic hypertension seems to involve activation (or deactivation) of a wide spectrum of gene expression programs.

## Functional Clustering (by Known Actions/Pathways of the Gene Product): Global Alterations in Pathways

Because of the often-subtle changes in gene expression we observed at multiple loci, we searched the data set for functional clusters of genes: loci involved in catecholamine metabolism and processing, sympathetic nerve function, the renin-angiotensin-aldosterone system (RAAS), vasoconstriction and vasodilation, intermediary metabolism, and the oxidative stress response (Table 2; online supplementary Tables III and IV, http://www.hypertensionaha.org). These specific functional categories were chosen because of preexisting understanding of hypertension pathophysiology in those areas.

For functional clusters that could be mapped onto complete, well-defined biochemical pathways, we used a series of hypothesis-driven KS tests to show that global perturbations in these pathways are highly unlikely to occur by chance alone. In other words, the entire pathways were significantly perturbed from normal functioning. Hypothesis-independent "guilt-by-association" methods (eg, k-means, quantitative threshold clustering, self-organizing maps) are commonly used to empirically cluster statistically significant genes based solely on mathematical properties of the expression data, in hopes that biological meaning could be found in the clusters, an approach that has shown potential usefulness in phylogenetic analyses.[10] Essentially, our strategy involved functionally clustering genes, initially ignoring any statistical criterion, into biochemical pathways as a first step, and then analyzing the statistical distribution of the pathway as a sum of its genetic components.

Because a 2-factor ANOVA ($\alpha=0.05$) performed on 12 488 probe sets may lead to false-positive results, finding that a pathway is defective by simultaneously analyzing all of its components may be more convincing than simply considering differential expression of just a few individual genes in that pathway. The joint probability that the distribution of genes within an entire pathway deviates from that expected by chance alone is likely to be substantially smaller than the probability that one or a few genes within a pathway is differentially expressed.

Results of the KS analysis suggest global disturbances ($P<0.05$ for the entire pathway) in the following pathways: cholesterol biosynthesis, electron transport chain, fatty acid degradation, fatty acid synthesis, glycolysis/gluconeogenesis, mitochondrial long-chain fatty acid $\beta$-oxidation, pentose phosphate, and the tricarboxylic acid (TCA) Krebs cycle (Table 3, Figure 2). The implications of alterations in each individual pathway are explored. Although the KS test takes into account the data for all genes within a particular pathway, the data for genes not statistically significant are excluded from online supplementary Table IV (http://www.hypertensionaha.org). It is important to note that some of the pathways analyzed with the KS test have data missing for certain genes because the MG-U74 Av2 chip did not contain probe sets for those genes. The missing data should not influence the conclusions drawn from the results of the KS test because a small number of missing data points do not have the power to shift the overall statistical distribution of the pathway.

## Catecholamines and Sympathetic Activity

Genes coding for catecholamine biosynthetic enzymes were overexpressed in BPH/2, whereas genes coding for catecholamine degradation enzymes were underexpressed (Table 2; online supplementary Table III). Tyrosine hydroxylase (the rate-limiting enzyme in catecholamine biosynthesis) and phenylethanolamine N-methyltransferase (the enzyme that converts norepinephrine to epinephrine in the last step of catecholamine biosynthesis) were overexpressed 1.95-fold and 1.78-fold, respectively, in the BPH/2. GTP cyclohydrolase I was also overexpressed 2.07-fold in BPH/2; the enzyme is rate limiting in the formation of tetrahydrobiopterin, an essential cofactor of tyrosine hydroxylase. Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein $\theta$ polypeptide (Ywhaq), an activator of tyrosine hydroxylase, was overexpressed 1.77-fold in BPH/2.

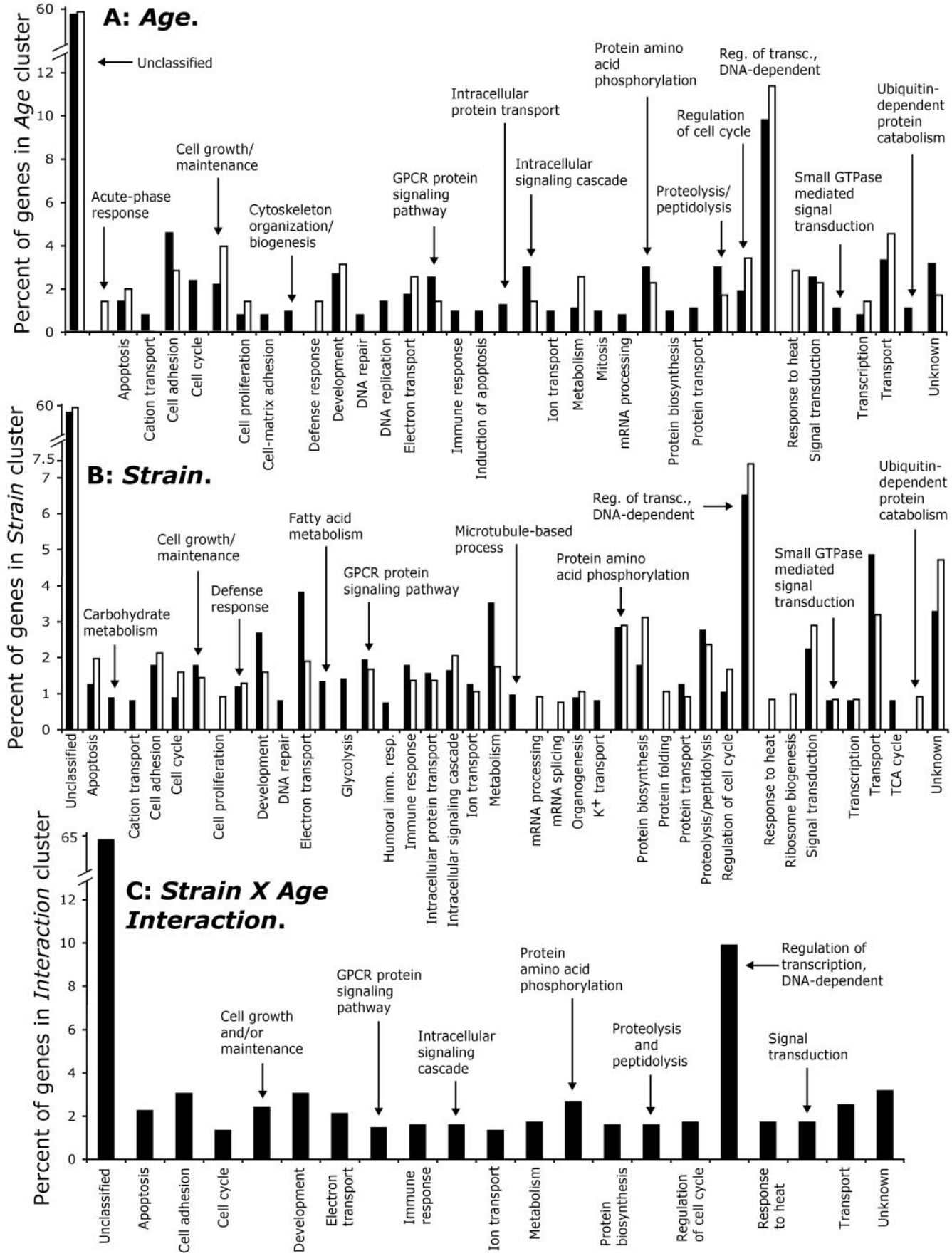

**TABLE 2. List of Genes Differentially Expressed (by Strain or Age-by-Strain Interaction) in Functional Clusters**

| **Functional Cluster**, Gene Name | Fold Change (BPH/BPL) | Gene Name | Fold Change (BPH/BPL) |
|---|---|---|---|
| **Catecholamine biosynthesis** | | | |
| GTP cyclohydrolase 1 | 2.0692 | Tyrosine hydroxylase | 1.9454 |
| Phenylethanolamine N-methyltransferase | 1.7844 | Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, theta polypeptide | 1.7749 |
| **Catecholamine degradation** | | | |
| Alcohol dehydrogenase 1 (class I) | 1.4057 | Monoamine oxidase A | 0.3022 |
| Alcohol dehydrogenase 4 (class II), pi polypeptide | 0.3523 | Sulfotransferase family 4A, member 1 | 0.4204 |
| Catechol-O-methyltransferase | 0.6929 | | |
| **Catecholamine uptake** | | | |
| Dopamine transporter | N/A | Norepinephrine transporter | 0.5026 |
| Solute carrier family 22 (organic cation transporter), member 5 | N/A | Solute carrier family 22 (organic cation transporter), member 2 | 0.7460 |
| Serotonin transporter | 1.4959 | | |
| **Oxidative stress** | | | |
| ATP-binding cassette, subfamily B (Mdr/Tap), member 1A | 1.8149 | Cytochrome P450, 2C40 | 0.5724 |
| ATP-binding cassette, subfamily C (Cftr/Mrp), member 5 | 1.1713 | Cytochrome P450, 3A16 | 0.3699 |
| ATP-binding cassette, subfamily G (white), member 2 | 1.2929 | Glutathione peroxidase 2 | 0.7327 |
| Cytochrome B-245, alpha polypeptide (NADPH oxidase, p22 phox subunit) | 1.3121 | Glutathione peroxidase 4 | 0.7814 |
| Cytochrome P450, family 2, subfamily c, polypeptide 39 | 2.1757 | Glutathione peroxidase 5 | 0.7411 |
| Cytochrome P450, family 2, subfamily e, polypeptide 1 | 2.7820 | Glutathione S-transferase, mu 1 | 0.7564 |
| Cytochrome P450, subfamily IV B, polypeptide 1 | 1.3498 | Glutathione S-transferase, mu 3 | 0.8053 |
| Glutamate-cysteine ligase, catalytic subunit | 1.1601 | Glutathione S-transferase, mu 6 | 0.4083 |
| Glutathione S-transferase, omega 1 | 1.2453 | Glutathione S-transferase, theta 1 | 0.7944 |
| Glutathione S-transferase, alpha 3 | 1.3659 | Microsomal glutathione S-transferase 1 | 0.2677 |
| Microsomal glutathione S-transferase 3 | 1.3811 | Paraoxonase 1 | 0.6371 |
| Paraoxonase 2 | 1.2079 | Peroxiredoxin 1 | 0.7883 |
| Peroxiredoxin 2 | 1.4106 | Superoxide dismutase 2, mitochondrial | 0.6085 |
| Peroxiredoxin 3 | 1.3057 | Uncoupling protein 1, mitochondrial | 0.3356 |
| Superoxide dismutase 1, soluble | 1.1226 | Uncoupling protein 3, mitochondrial | 0.3095 |
| Transporter 2, ATP-binding cassette, sub-family B (Mdr/Tap) | 1.3612 | | |
| Xanthine dehydrogenase | 1.4331 | | |
| Cytochrome P450, family24, subfamily a, polypeptide 1 | 0.1795 | | |
| **Renin-angiotensin-aldosterone system** | | | |
| Angiotensin I-converting enzyme (peptidyl-dipeptidase A) 2 | 1.9165 | Angiotensin II, type I receptor-associated protein | 0.7527 |
| Angiotensin II receptor, type 2 | 1.3849 | Hydroxysteroid 11-beta dehydrogenase 2 | 0.7687 |
| Angiotensinogen | 1.6969 | Renin | 0.3162 |
| **Sympathetic neuroeffector junction** | | | |
| Chromogranin A | 1.4293 | Adrenergic receptor, beta 1 | 0.6398 |
| Neuropeptide Y | 1.9997 | Adrenergic receptor, beta 3 | 0.5510 |
| Tyrosine hydroxylase | 1.9454 | Norepinephrine transporter | 0.5026 |
| **Vasoconstrictor systems** | | | |
| Arginine vasopressin receptor 1A | 2.1947 | Arginine vasopressin | 0.6003 |
| Endothelin 3 | 2.0351 | Urotensin II receptor | 0.7160 |
| Neuropeptide Y | 1.9997 | | |
| **Vasodilator systems** | | | |
| Preproenkephalin 1 | 1.3366 | Nitric oxide synthase 2, inducible, macrophage | 0.6498 |
| Kallikrein 5 | 0.5732 | Nitric oxide synthase 3, endothelial cell | 0.6440 |
| Kallikrein 6 | N/A | Pancreatic polypeptide | 0.8030 |
| Kininogen | 0.4734 | | |

Criteria for entry: member of a functional cluster and significant ($P<0.05$; $F>5.32$) $P$ value for comparison by strain or interaction.
Fold change (BPH/BPL): chip fold change; mean BPH/2 fluorescence divided by mean BPL/1 fluorescence.
N/A: Fold change not applicable because gene displayed only interaction significance.
Functional clusters indicated in **bold** type.



Catechol-O-methyltransferase, which catalyzes the transfer of a methyl group from S-adenosylmethionine in one of the major degradative pathways of catecholamines, and monoamine oxidase A, an enzyme of the mitochondrial outer membrane that catalyzes the oxidative deamination of catecholamines, were underexpressed 0.69-fold and 0.30-fold, respectively, in BPH/2.

**Figure 1.** Biological process GO clusters. A, Age. Percent of genes in each GO cluster, for genes significant ($P<0.05$) by age (n=982), separated by overexpression in juvenile (n=631; solid bars) or adult (n=351; open bars) mice. B, Strain. Percent of genes in each GO cluster, for genes significant by strain (n=2647), separated by overexpression in BPH/2 (n=1314; open bars) or BPL/1 (n=1333 genes; solid bars). (C) Age-by-strain interaction. Percent of genes in each GO cluster, for genes significant by interaction (n=757; all solid bars). By the DAVID tool for display of GO clusters, the minimum number of hits (based on F-criteria) threshold=10 (for strain and interaction clusters) or minimum number of hits (based on F-criteria) threshold=5 (for age cluster).

**TABLE 3. Perturbations of Gene Expression in Entire Functional Pathways: Kolmogorov-Smirnov Goodness-of-Fit Test Results**

| Biochemical Pathway | KS Test Statistic D | *P* | Pathway Significantly Perturbed? |
|---|---|---|---|
| Cholesterol biosynthesis | 0.3554 | <0.05 | Y |
| Electron transport chain | 0.5381 | <0.01 | Y |
| Fatty acid degradation | 0.5084 | <0.01 | Y |
| Fatty acid synthesis | 0.6376 | <0.01 | Y |
| Glycolysis/gluconeogenesis | 0.4498 | <0.01 | Y |
| Mitochondrial long chain fatty acid $\beta$-oxidation | 0.6877 | <0.01 | Y |
| Pentose phosphate pathway | 0.8246 | <0.01 | Y |
| TCA cycle | 0.7028 | <0.01 | Y |

The Kolmogorov-Smirnov (KS) test was used to determine if the distribution of ANOVA F-statistics within each pathway significantly deviates from that which is expected in an F-distribution for degrees of freedom 1,8.

$P<0.05$ indicates that the pathway listed is significantly perturbed from its normal functioning. TCA indicates tricarboxylic acid; Y, yes.

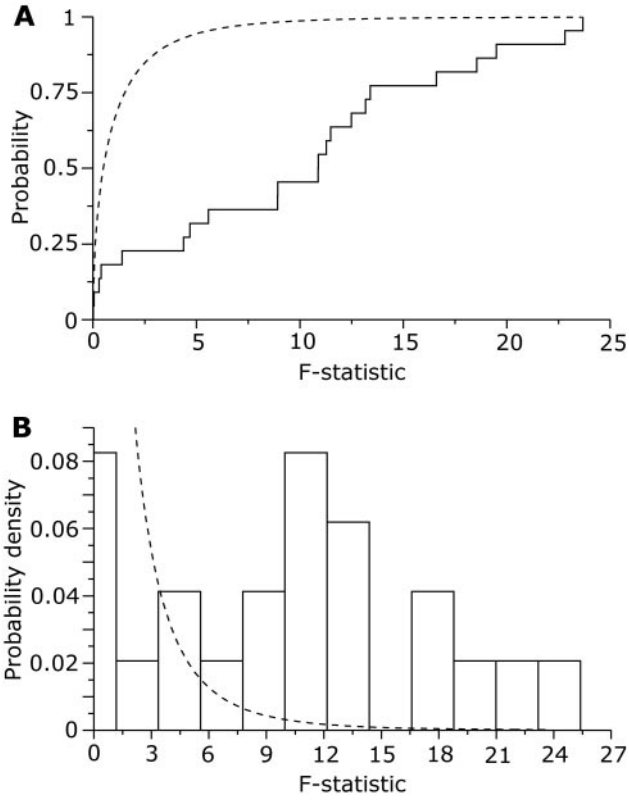


**Figure 2.** Differences in gene expression across entire biological pathways. Representative KS results for the 22 transcripts giving rise to the enzymes of the TCA cycle. A and B, The x-axis represents strain F-statistic and the y-axis represents probability. A, Plots are shown of the theoretical (expected) CDF (dashed line) for the F-distribution (df=1,8) and the ECDF (observed) (solid line) for strain F-statistics of genes of the TCA cycle. The KS test calculates the maximum vertical deviation between the CDF and ECDF to determine whether the data set distribution significantly deviates from the theoretical F-distribution. B, Plots are shown of the probability density function for the theoretical (expected) F-distribution (df=1.8; dashed line) and the relative histogram distribution of observed data of the TCA cycle (bars). It is clear that the distribution of observed data within the TCA cycle significantly deviates from that of the expected F-distribution (df=1,8): KS test D statistic=0.7028, $P<0.01$.

This specific combination of differentially expressed genes (increased production with decreased destruction) would tend to increase steady-state catecholamine concentrations in the animal. Chromogranin A is the major soluble protein in neurotransmitter and catecholamine secretory vesicles,[11,12] with roles in vesiculogenesis and regulation of catecholamine secretion.[13] The gene coding for chromogranin A is upregulated 1.43-fold in BPH/2, consistent with an overabundance of catecholamines in this model.

The $\beta$1 adrenergic receptor (Adrb1) was underexpressed 0.64-fold, whereas the $\beta$3 receptor (Adrb3) was underexpressed 0.55-fold in BPH/2. Adrb3 regulates lipolysis in adipocytes. Underexpression of Adrb3 may contribute to global reduction in intermediary metabolic function (see Intermediary Metabolism).

Norepinephrine is inactivated in the synaptic cleft by neuronal reuptake through the norepinephrine transporter (Net; Slc6a2).[14] Net was underexpressed 0.50-fold in BPH/2. Organic cation transporter (Oct) also eliminates catecholamines, primarily at extraneuronal sites.[15] Oct2 was underexpressed 0.75-fold, whereas Oct2n displayed interaction significance, with decreased expression in BPH/2 as the animal ages.

Thus, the overall pattern of catecholamine biosynthetic/removal differential gene expression displayed in BPH/2 suggests the possibility of an increased abundance of norepinephrine in the synaptic cleft through a combined mechanism of overproduction of catecholamines coupled with diminished enzymatic degradation and synaptic reuptake.

## Renin-Angiotensin-Aldosterone System

Differential expression of RAAS components shows an interplay between potentially prohypertensive events and possible compensatory measures (Table 2; online supplementary Table III and supplementary Results). Upregulation (1.70-fold) of the angiotensinogen (Agt) gene in BPH/2 is likely to increase blood pressure, because angiotensinogen is the precursor of angiotensin I. Linkage analysis of a BPH/2–BPL/1 intercross found that an Agt-containing region on chromosome 8 cosegregated with blood pressure.[16] The renin (Ren) gene is downregulated 0.32-fold in BPH/2. The ≈0.77-fold diminution of hydroxysteroid 11-$\beta$ dehydrogenase 2 (Hsd11b2) expression suggests that BPH/2 might experience an excess of effective mineralocorticoid activity.

Other RAAS genes differentially expressed are angiotensin II type-2 receptor (elevated 1.38-fold), angiotensin receptor-associated protein (diminished 0.75-fold), and angiotensin I-converting enzyme 2 (elevated 1.92-fold).

## Kallikrein-Kinin System

BPH/2 displayed 0.57-fold underexpression of kallikrein 5 (Klk5) and 0.47-fold underexpression of kininogen (Kng); these changes are potentially prohyperensive events because Klk5 cleaves Kng to form the vasodilator bradykinin. Altered

expression of kallikrein 6 also has the potential to increase blood pressure, because its expression decreases in the BPH/2 as the animal grows older.

## Other Vasodilators/Vasoconstrictors

A discussion of other vasodilators/vasoconstrictors is available online at http://www.hypertensionaha.org.

## Intermediary Metabolism

Differential gene expression in pathways of intermediary metabolism (Table 3; Figures 2, 3, 4; online supplementary Table IV) of the hypertensive mouse provides compelling evidence for global alterations of metabolic function in the BPH/2. Multiple components of the electron transport chain, fatty acid degradation and synthesis, glycolysis, gluconeogenesis, pentose phosphate, and TCA cycle pathways are almost globally depressed in BPH/2, whereas the cholesterol biosynthetic pathway is enhanced. For gene-specific examples and discussion, see the online supplementary Results/Discussion and Table IV, as well as Figure 3 and 4. The fact that the reduction in metabolic function is so global in an oligogenic model of hypertension[17] suggests that the changes are secondary effects resulting from a primary genetic cause.

Gene expression data in the cholesterol biosynthesis pathway suggest that BPH/2 may produce increased cholesterol, a condition that is commonly observed in human hypertensives. HMG CoA reductase (Hmgcr), the rate-limiting enzyme in this pathway, is overexpressed 2.06-fold in BPH/2. Cholesterol is the precursor of glucocorticoid and mineralocorticoid steroid hormones, but only 1 steroid biosynthetic gene (3BH6) is overexpressed in BPH/2. High levels of cholesterol, with consequent cardiovascular risk, might contribute to the early mortality of BPH/2.

Hypertensive humans often have insulin resistance and dysglycemia (ie, the inability to properly regulate blood glucose levels). Impaired glucose transport and consequent cellular glucose starvation may constitute an impetus for release of glucagon, cortisol (corticosterone in the mouse), and catecholamines. Paradoxically, these actions further raise blood glucose. Our data provide evidence for insulin resistance (Glut4, Glut8 underexpression; see online Results/Discussion and online Table IV), dysglycemia (global depression of intermediary metabolism; see online Results/Discussion and online Table IV), and enhanced catecholamine biosynthesis (see Catecholamines and Sympathetic Activity). In addition, abnormally high levels of glucose in the blood can form so-called advanced glycation end products[18] of proteins. These glycated proteins can induce inflammation, impair vascular dilation,[19–21] and generate ≈50-fold more reactive oxygen species (ROS) than nonglycated proteins.[18,22] ROS have been implicated in the pathogenesis of many diseases, including hypertension.[23–28]

## Oxidative Stress

Aberrant increases in ROS, which result in a condition commonly referred to as "oxidative stress," can lead to damage of lipids, carbohydrates, proteins, and DNA, and ultimately to apoptotic cell death.[29,30] Oxygen radicals can influence blood pressure by inactivating the vasodilator nitric oxide to peroxynitrite ($ONOO^-$). Oxidative stress has been implicated in human[23–25] and rodent[26–28] hypertension, although the hypertensive rat has been studied much more extensively than the hypertensive mouse.

## Oxidative Stress: ROS Sources

Under normal conditions, a potent source of ROS within the cell is electron transfer processes within the mitochondria,[31] but ROS are also byproducts of cytochrome P450s,[32,33] xanthine oxidase/dehydrogenase,[34,35] and NADPH oxidase.[36] Our gene expression data show that many subunits of complexes within the electron transport chain are underexpressed in the BPH/2 (online Table IV; see Intermediary Metabolism). Another source of ROS is cytochrome P450s. Our data show differential expression of 6 of these genes (online Table III). Three cytochrome P450s are overexpressed in the BPH/2 (Cyp2c39, 2.18-fold; Cyp2e1, 2.78-fold; Cyp4b1, 1.35-fold), whereas 3 are underexpressed (Cyp24a1, 0.18-fold; Cyp2c40, 0.57-fold; Cyp3a16, 0.37-fold). Xdh plays a role in the formation of the powerful antioxidant urate;[37] however, xanthine oxidase can generate superoxide and hydrogen peroxide from molecular oxygen.[37] Xdh is overexpressed 1.43-fold in the BPH/2. NADPH oxidase is a key enzyme in superoxide production in the vasculature,[38–40] and its subunit p22 phox plays a critical role in NADPH oxidase-mediated superoxide production.[36] The cytochrome b-245 $\alpha$ polypeptide gene, which encodes p22 phox, is upregulated 1.31-fold in BPH/2.

## Oxidative Stress: First Line of Defense

Glutathione (Glu-Cys-Gly) and glutathione-dependent enzymes function in a 2-tiered "defense" against oxidative stress.[41] The enzyme glutamate-cysteine ligase (or $\gamma$-glutamylcysteine synthetase) catalyzes the rate-limiting step in glutathione synthesis.[42,43] Upregulation of the glutamate-cysteine ligase catalytic subunit 1.16-fold in BPH/2 suggests an increase in synthesis of glutathione.

Also involved in the first line of oxidative stress defense is superoxide dismutase (Sod), an enzyme that attacks the superoxide anion and catalyzes its dismutation to hydrogen peroxide. Superoxide dismutase 1 (Sod1), a soluble form of the enzyme found in the cytoplasm, is overexpressed 1.12-fold in BPH/2. The mitochondrial form of the enzyme, superoxide dismutase 2 (Sod2), is actually underexpressed 0.61-fold in the BPH/2. Glutathione peroxidases (Gpx) are first-line-of-defense enzymes that remove hydrogen peroxide. Several Gpx isoenzymes exist that act in different parts of the cell. The BPH/2 underexpressed Gpx2 0.73-fold and Gpx4 0.78-fold (these are located in the cytosol), along with Gpx5 0.74-fold (located in the extracellular space).

## Oxidative Stress: Second Line of Defense

ROS and the toxic compounds they produce that escape the first line of defense are subjected to a multitude of enzymes that comprise the second line of defense.[41] For example, the glutathione S-transferase (Gst) family of enzymes conjugate glutathione to ROS and ROS byproducts to deactivate the toxic compounds and prepare them to be purged from the cell. Two Gst enzymes, Gsto1 and Gsta3, are upregulated 1.25-

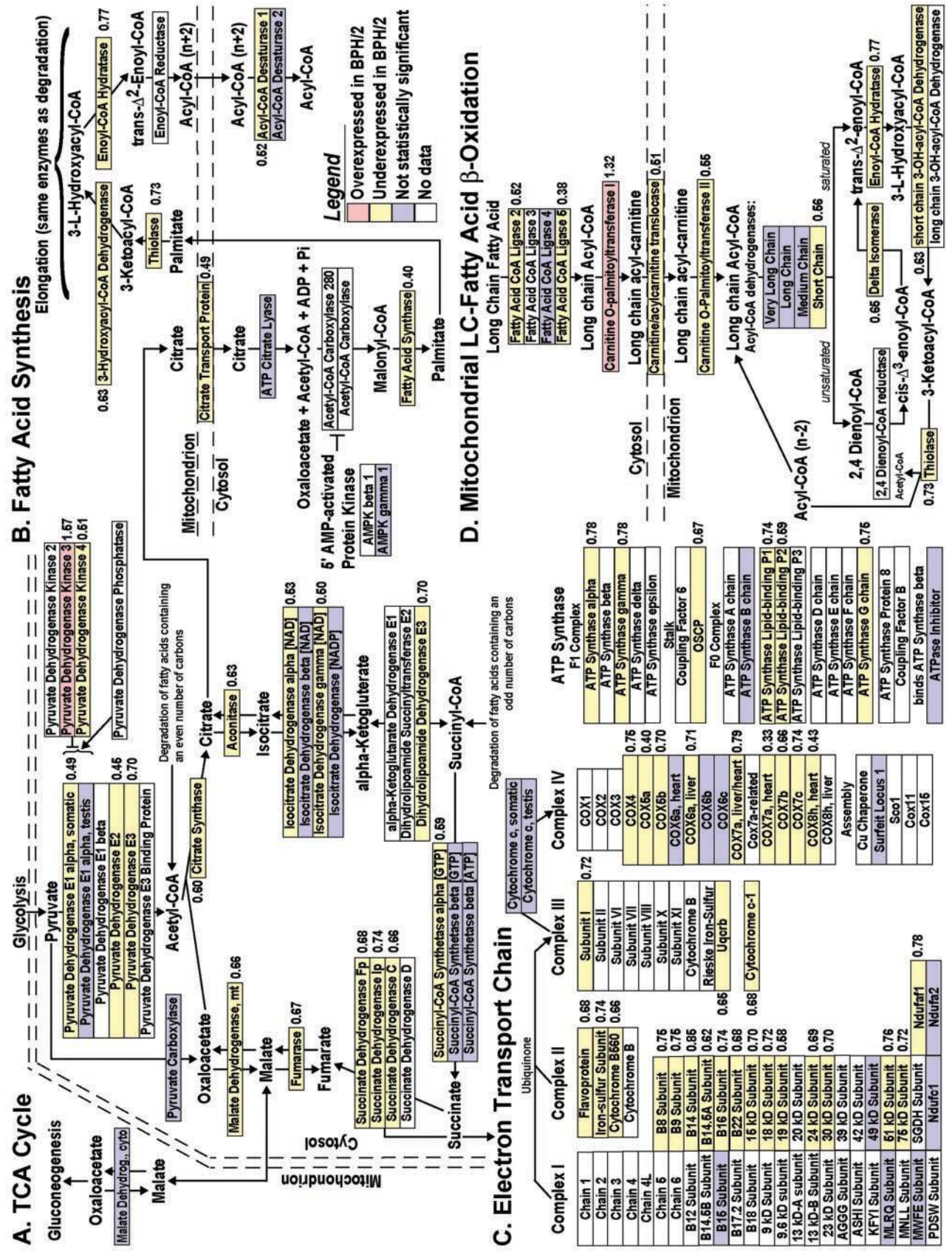


**Figure 3.** Intermediary metabolism pathways in BPH/2. A, TCA cycle. B, Fatty acid synthesis. C, Electron transport chain. D, Mitochondrial long chain fatty acid β-oxidation. A legend is shown (B). Red indicates gene overexpressed in BPH/2. Yellow indicates gene underexpressed in BPH/2. Blue indicates gene not statistically significant. White indicates no data for gene, ie, no probe on chip. Value listed next to each gene is the fold change (BPH/BPL).

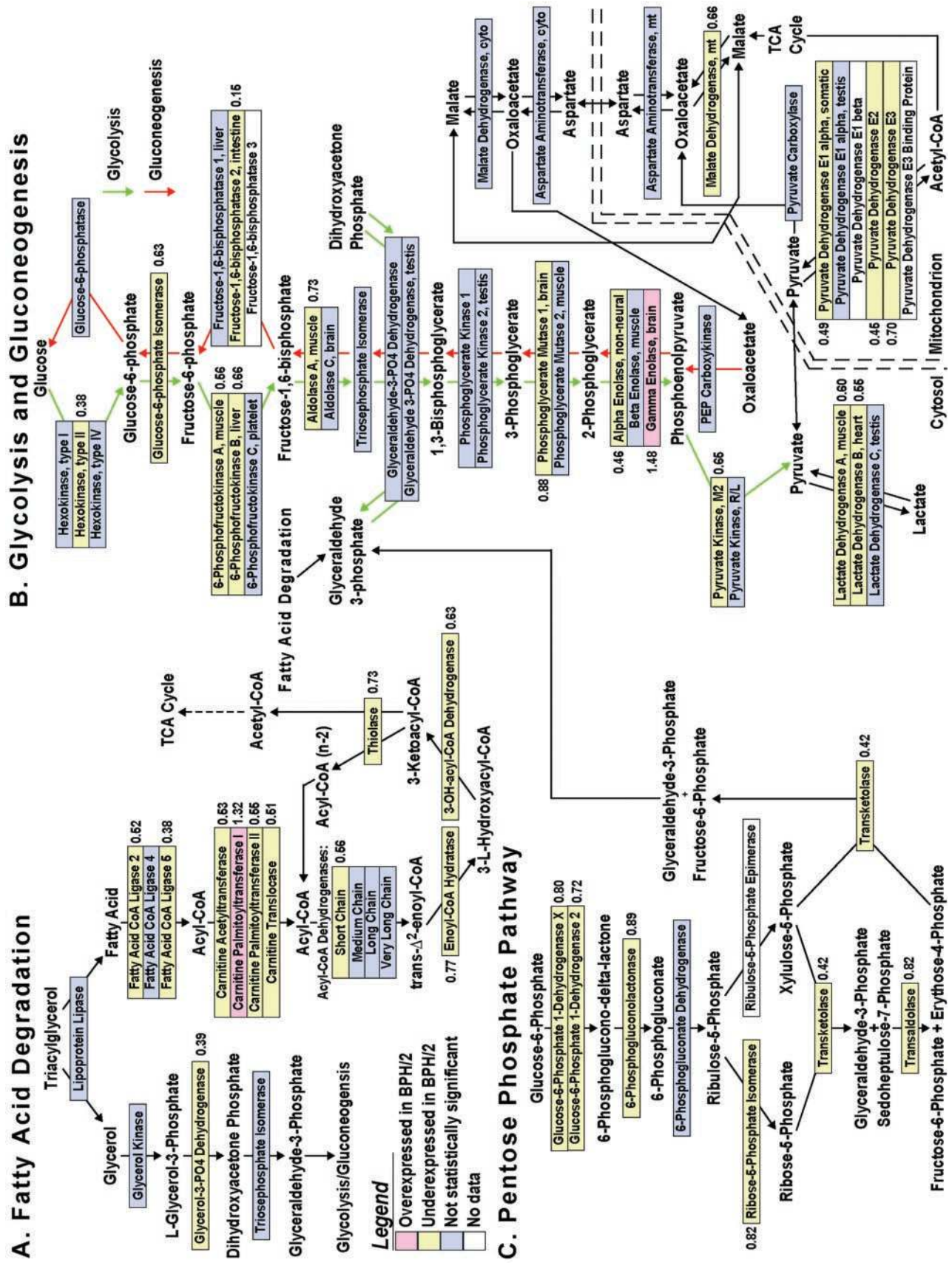


**Figure 4.** Intermediary metabolism pathways in BPH/2. A, Fatty acid degradation. B, Glycolysis and gluconeogenesis. C, Pentose phosphate pathway. A legend is given (A). Red indicates gene overexpressed in BPH/2. Yellow indicates gene underexpressed in BPH/2. Blue indicates gene not statistically significant. White indicates no data for gene, ie, no probe on chip. Value listed next to each gene is the fold change (BPH/BPL).

fold and 1.37-fold, respectively, in the BPH/2, whereas 4 are downregulated (Gstm1, 0.76-fold; Gstm3, 0.81-fold; Gstm6, 0.41-fold; Gstt1, 0.79-fold).

ROS detoxified in the first and second lines of defense are eliminated from the cell through glutathione S-conjugate transporters, also known as multidrug resistance proteins or ATP-binding cassettes (Abc). Four of these transporters, namely Abcb1a, Abcc5, Abcg2, and Tap2, are overexpressed 1.81-fold, 1.17-fold, 1.29-fold, and 1.36-fold, respectively, in the BPH/2, further supporting the notion that BPH/2 experience increased oxidative stress.

## Perspectives

We present the first global analysis of patterns of gene expression in the genetically hypertensive mouse (BPH/2). Our data suggest that the hypertensive mouse may have transcriptional changes leading to increased sympathetic activity, a condition similar to the apparent mineralocorticoid excess syndrome (marked by low renin and aldosterone), RAAS that exhibits prohypertensive and vasodilatory changes, differential expression in vasoconstrictor systems and apparent responsive changes in vasodilator systems, increased cholesterol production, a global depression in intermediary metabolism, and an enhanced oxidative stress response.

The spontaneously hypertensive rat (SHR) has become the paradigm for animal research in genetic hypertension, modeling human essential hypertension. Only a few microarray analyses of the SHR have been published, evaluating adipose tissue,[44] kidney,[45,46] and vascular smooth muscle cells.[47] The adipose tissue study, performed by Aitman et al,[44] was a landmark experiment because microarray data were used to pinpoint the Cd36 gene (fatty acid translocase) as a genetic underpinning of insulin resistance, defective fatty acid metabolism, and hypertriglyceridemia in the SHR. Other studies in the SHR did not present biochemical pathway data and typically reported genes ranked only by fold change or fluorescent intensity. Furthermore, such rat studies usually did not present data on the same genes that we found differentially expressed. A study of chromosome 2 congenic kidneys[45] did, however, find a significant reduction of glutathione S-transferase $\mu 2$ (Gstm2), a gene involved in the oxidative stress response, and mapped this gene through comparative genome analysis to corresponding regions on rat chromosome 2, mouse chromosome 3, and human chromosome 1. RT-PCR investigation of the adrenal gland of the SHR found a statistically significant 2.5-fold increase of tyrosine hydroxylase expression in the SHR as compared with its normotensive control, the Wistar-Kyoto rat.[48] This finding parallels the 1.95-fold overexpression of tyrosine hydroxylase that we observed in the BPH/2.

A biometrical genetic analysis of BPH/2–BPL/1 intercrosses and backcrosses suggested that ≈4 to 5 major loci (as yet not specifically identified) are responsible for a major fraction of the difference in blood pressure between the parental strains.[17] Our previous genome-wide scans[49] of BPH/2–BPL/1 intercrosses and backcrosses subsequently identified only 2 chromosomal regions with "significant" (logarithm of odds [LOD] >3.3) linkage to blood pressure, whereas 5 chromosomal regions displayed "suggestive" (LOD >2.3) linkage. It seems likely, then, that many (if not most) of the ≈2647 genes differentially expressed in BPH/2 are not the underlying disease loci, but instead are responding to such primary loci, either evoking secondary hypertensive effects or acting in a compensatory manner to curb the effects of the primary prohypertensive insults. Our results identified as many as 2647 genes with expression significantly different by strain, 982 genes significant by age, and 757 genes significant by interaction. The ≈4 to 5 major genes[17] likely responsible for blood pressure increase in BPH/2 might exhibit differential gene expression, depending on whether the underlying mutations confer qualitative changes in the gene product or quantitatively alter gene expression. Mutations conferring quantitative changes in gene expression are especially likely to lie in our empirical or functional clusters. Our studies compared gene expression in a hypertensive mouse (BPH/2) to a hypotensive control strain (BPL/1). It is conceivable that comparison of BPH/2 to a truly normotensive (normal blood pressure) strain might have yielded different patterns of differential gene expression. Finally, our experiments cannot address whether differential gene expression might have changed during the course of selection of high and low blood pressure in the BPH/2 and BPL/1 strains over many generations of brother/sister mating.[3,4] Nonetheless, our observations provide a broad picture of biochemical functions in the hypertensive mouse that might participate in genetic susceptibility to blood pressure elevation.

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