Original Article

Candesartan-Induced Gene Expression in Five Organs of Stroke-Prone Spontaneously Hypertensive Rats

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To test the functional consequences of blocking the local renin-angiotensin system (RAS), we investigated the effects of an angiotensin II type 1 receptor blocker (ARB), candesartan, on the systemic gene expression profile of five important organs (brain, heart, kidney, liver and adipose tissues) in the stroke-prone spontaneously hypertensive rat (SHRSP), an established model of essential hypertension and cardiovascular disorders, and its normotensive control, the Wistar Kyoto (WKY) rat. Rats were treated with candesartan (5 mg/kg/d) for 4 weeks from 12 to 16 weeks of age. DNA microarray technology was used to identify changes in gene expression. Four weeks of treatment with candesartan significantly lowered systolic blood pressure in male rats of both the SHRSP and the WKY strains ($p<0.0005$). Candesartan differentially modulated the gene expression profile in an organ-specific manner in male SHRSP; of the five organs tested, gene expression was most prominently altered in the hearts of SHRSP. In contrast, candesartan treatment exerted minimal or no significant effects on the gene expression profile of the corresponding organs of male WKY rats. The inter-strain differences in gene expression changes induced by candesartan were considered to be associated with both blood pressure–dependent and independent mechanisms. These results help to delineate the mechanisms that underlie the organ or tissue protection conferred by ARB at the levels of cellular biology and genomics in the context of the local RAS. Further studies are warranted to investigate not only individual genes of interest but also genetic "networks" that involve differential organ- or tissue-specific gene expression induced by the blockade of RAS in essential hypertension. (*Hypertens Res* 2008; 31: 1963–1975)

***Key Words*: candesartan, stroke-prone spontaneously hypertensive rat, microarray technology, gene expression, end organ damage**

Introduction

In the renin-angiotensin system (RAS), the focus of research interest has recently shifted from an endocrine role to an autocrine/paracrine role as the key component of tissue/organ- and cell-specific functions; the latter was proposed almost 20 years ago as the concept of the local RAS (*1*). Thus far, the local functions of RAS components, notably angiotensinogen and renin, have been reported in a variety of tissues (*2–4*). These RAS components are regulated at a number of physiological steps that range from the synthesis of renin to the dimerization of angiotensin receptors. Accordingly, tissue concentrations of angiotensin II (Ang II), other RAS compo-

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nents and their active metabolites can be set independently of the circulatory RAS and may either potentiate systemic functions or have entirely separate activities to meet the specific needs of individual tissues (*5*). Accumulating evidence indicates that impairments in the local RAS underlie certain tissue/organ disorders such as stroke and heart failure (*3*, *4*, *6*). This gives rise to the possibility that a class of drugs that act on the local RAS may effectively ameliorate some of these disorders. In fact, a recent study has suggested similar consequences of the actions of such drugs on the local RAS (*7*).

The early, remarkable success of angiotensin converting enzyme (ACE) inhibitors as a remedy for a plethora of diseases, including hypertension, arteriosclerosis, cardiac hypertrophy and diabetic renal disease, has encouraged the further development of drugs designed to inhibit the RAS and associated systems (*8*). Among these drugs, the newest is an Ang II type 1 (AT_1) receptor blocker (ARB), which has been specifically developed to better block the vasoconstricting, growth-promoting effects of Ang II directly at the receptor level. Therapeutic interventions involving ARBs as well as ACE inhibitors are considered to provide benefits at least in part through the interruption of the local RAS. With an enhanced understanding of the molecular biology of the local RAS, new avenues for therapeutic intervention will become possible.

Under these circumstances, we investigated the effects of the ARB candesartan on the systemic gene expression profile of five important organs (brain, heart, kidney, liver and adipose tissue) in the stroke-prone, spontaneously hypertensive rat (SHRSP), an established model of essential hypertension and cardiovascular disorders, and its normotensive control, the Wistar Kyoto (WKY) rat. We used DNA microarray technology to identify large clusters of genes that respond to the administration of candesartan in the context of the local RAS and hemodynamic impacts, such as blood pressure changes, on mRNA expression.

Methods

Animals

This study utilized a Japanese colony of male SHRSP and age-matched male WKY rats. Rats were weaned at 4 weeks after birth and placed on normal rat chow (SP diet, Funabashi Farm, Funabashi, Japan). A selected number of rats were treated with candesartan (5 mg/kg/d), dissolved in 0.15% polyethylene-glycol 300, 0.15% ethanol and 0.055% $NaHCO_3$ for 4 weeks from 12 to 16 weeks of age. Also, to allow for the investigation of blood pressure–dependent mechanisms on systemic mRNA expression—*i.e.*, whether a substantial part of mRNA expression changes are reproducible between different antihypertensive drugs with significant blood pressure decreases—rats were treated with hydralazine (10 mg/kg/d) independently of candesartan. Systolic blood pressure measurements were performed using the tail-cuff method, as previously described (*9*). Briefly, three consecutive blood pressure readings were taken and an average was determined. The rats were killed under pentobarbital anesthesia (50 mg/kg, intraperitoneal infusion), which was immediately followed by the excision and snap freezing of organs at −70°C for subsequent RNA extraction. The intraperitoneal administration of pentobarbital was chosen because it was reported to have minimal effects on the expression of immediate-early genes (*c-fos* and *c-jun*) from 5 to 120 min after the induction of anesthesia in the brain, heart, liver and kidney of rats (*10*).

All animals were treated in compliance with institutional regulations. The study protocol was approved by the animal ethics committee of the Research Institute, International Medical Center of Japan.

Drug Treatment

We first performed a pilot study to determine the dosage of candesartan using three different doses (1, 5, and 10 mg/kg/d) on 12-week-old male SHRSP ($n=2$ each). Based on the results in this pilot study, we set an appropriate dose as 5 mg/kg/d, because no further BP-lowering effects were attained above this level (data not shown). We then proceeded to the main study. Systolic blood pressure was measured by the tail-cuff method every 3 to 4 d for 4 weeks. The vehicle of candesartan used in the present study was 0.15% polyethyleneglycol 300, 0.15% ethanol and 0.055% $NaHCO_3$.

Similarly to the candesartan treatment, we determined an appropriate dose of hydralazine to be 10 mg/kg/d among three doses (1, 5, and 10 mg/kg/d) tested. The vehicle of hydralazine used in the present study was distilled water.

Tissue Processing and RNA Isolation

After harvest, whole organs of interest (brain, heart, kidney, liver and adipose tissue) were homogenized separately with a 0.5-inch–diameter generator shaft at a speed of 22,000 rpm for 60 s in a Polytron homogenizer PT1300D (Kinematica AG, Littau, Switzerland). Total RNA was extracted using RNeasy maxi kit (Qiagen, Valencia, USA) according to the manufacturer's protocol. The quality of RNA was checked with an RNA 6000 Nano LabChip on the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA) and then used in DNA microarray experiments. In the microarray analysis, four rats of the SHRSP and WKY strains were used in each treatment group of both candesartan and vehicle. Likewise, as for the treatment groups of hydralazine and vehicle in SHRSP, a microarray analysis was performed for the heart, in which, of the five organs, the most prominent mRNA expression changes were induced by candesartan. Total RNA extracted from each treatment group—that is, total RNA from two animals—was pooled, which led to the preparation of two samples (*i.e.*, two pools derived from four animals) per each treatment group. The cDNA was synthesized from 1 μg of the DNase I–treated total RNA using the Omniscript RT (reverse

Table 1. A List of PCR Primer Sets

Gene symbol	Accession number	Forward	Length	Reverse	Length
Thbs4	X89963	ACGGGATCACCTGTTCTGAC	20	CAGTGCACACCTGCTTGTTT	20
Postn_predicted	AA894092	AATTCAAAGGCCAGACAACAGAGT	24	GATTTGCTTGACAATTAGCTTCTGTTT	27
Ltbp2	NM_021586	CCTCGTGCACTCCTCTTAGG	20	CTACTCCCCAGACCCTCTCC	20
Tnc	AA892824	GGCTCTGAAAATGACTATGTCCG	23	CCTAGCGACAGCAACACCGT	20
Col1a1	Z78279	CGAAGGCAACAGTCGATTCA	20	TGACTGTCTTGCCCCAAGTTC	21
Fos	AW915240	GCTAGCCCTGTGAGCAGTCAG	21	TCTGTAATGCACCAGCTCAGTCA	23
Ren1	AF117820	CTCTGGGCACTCTTGTTGCTC	21	GCACTGATCCTGGTCATGTCTACTC	25
Ppia	NM_017101	CTTCGACATCACGGCTGA	18	CCACCCTGGCACATGAATCC	20

transcriptase) Kit (Qiagen) and subjected to real-time PCR amplification.

Gene Expression Bioarrays

The present study utilized the CodeLink Bioarray Perfect system (GE Healthcare UK Ltd., Buckinghamshire, UK), which uses the UniSet Rat I Expression Bioarray that contains 9,028 gene probes, abbreviated hereafter as the 10K CodeLink array (*11–14*). The 10K CodeLink array accommodates a broad range of genes derived from publicly available, well-annotated mRNA sequences. This array is unique in that it is capable of detecting minimal differences in gene expression, *i.e.*, as low as 1.3-fold with 95% confidence, because of its three-dimensional aqueous gel matrix, on which empirically tested 30-mer oligonucleotides are deposited. This substantially reduces background noise, enhances sensitivity, and allows for the detection and quantification of subtle regulatory relationships among the genes tested.

All reagents were provided in the CodeLink Expression Assay Kit, except where noted. cRNA synthesis was performed according to the manufacturer's instructions using 2 μg of total RNA. The details of the target preparation, array hybridization, post-hybridization processing, and scanning were the same as described elsewhere (*15*).

Microarray Data Analysis and Statistics

The CodeLink software package normalized the overall raw signal intensities on each array to their median value of all the rat probes. Normalized data were uploaded to GeneSpring GX (Agilent Technologies, Santa Clara, USA) for analysis. The threshold of detection was calculated using the normalized signal intensities for 300 negative controls on the array. Probes with signal intensity below this threshold were considered to be absent and were eliminated from the list. Since the low-intensity signals were most susceptible to noise, we filtered the data for spot quality by excluding all genes with a signal intensity smaller than 0.75 (compared with the median signal of 1.0) for each array. We assessed the differential expression of genes that passed these intensity filters by averaging the normalized signal intensities of two pools per group and calculating the ratio of the averaged values in the drug-treated group to those in the vehicle-treated group. Statistical significance was determined by Student's *t*-test (two-tailed, unpaired). Then, only genes that had *p*-values of <0.05 were included in the analysis and further filtered for a level of 1.5-fold expression change. Gene ontology reports (Biological Process, Cellular Component, and Molecular Function, based on the Gene Ontology Consortium, http://www.geneontology.org/GO.doc.html) (*16*) were sought through the GO Ontology Browser in GeneSpring GX. We also utilized GeneSpring GX's "Post Hoc Test" program, which enables users to identify genes as common (or unique) from multiple datasets of differentially expressed genes, to produce a list of genes that were up- or down-regulated in the current experimental setting. For readers' reference, we partially applied gross, functional categorization (*17*) to the ontology information on individual genes showing significant expression changes in the Results section.

Real-Time PCR Amplification

To validate the expression ratios of a subset of genes, we performed quantitative real-time PCR. The PCR primer sequences for target genes were originally designed (Table 1). The expression levels of seven rat genes were compared within four samples (*i.e.*, two microarray pools) per group (drug *vs*. vehicle treatment group) using the 10K CodeLink arrays and within five samples per group using real-time PCR with standard curve methods. Real-time PCR amplifications were performed in a 384-well plate in the ABI 7900 HT Sequence Detection System (PE Applied Biosystems, Foster City, USA) in a total volume of 10 μL, which included 2 μL of template cDNA plus SYBR green I master mixture and a final concentration of 400 nmol/L each of forward and reverse primers. Each sample was analyzed in duplicate, and PCR amplification was performed as follows: 2 min at 50°C and 10 min at 95°C, followed by a total of 40 temperature cycles (15 s at 95°C and 1 min at 60°C). The numbers of copies of the PCR template in a starting sample were calculated by using the SEQUENCE DETECTOR system version 2.2.2,

incorporated into the ABI 7900 HT Sequence.

Results

Physiological Phenotypes

In SHRSP, 4 weeks of treatment with candesartan (5 mg/kg/d) significantly lowered systolic blood pressure, which was 158.5±3.5 mmHg in the candesartan-treated group and 242.6±9.5 mmHg in the vehicle-treated group (mean±SEM, $p<0.0005$, $n=5$ each). At the same dose, candesartan significantly lowered systolic blood pressure in WKY rats: 103.5±4.3 mmHg in the candesartan-treated group and 134.7±2.8 mmHg in the vehicle-treated group ($p<0.0005$, $n=5$ each). Also, in SHRSP, candesartan significantly alleviated cardiac hypertrophy, as indicated by heart weight per body weight: 3.18±0.03 mg/g in the candesartan-treated group and 3.86±0.03 mg/g in the control group fed on normal rat chow, SP diet (mean±SEM, $p<0.001$, $n=4$ *vs*. $n=35$).

In SHRSP, 4 weeks of treatment with hydralazine (10 mg/kg/d) significantly lowered systolic blood pressure: 184.8±3.5 mmHg in the hydralazine-treated group and 253.6±7.8 mmHg in the vehicle-treated group (mean±SEM, $p<0.0001$, $n=5$ each).

Effects of Candesartan on Gene Expression in the Brain, Heart, Kidney, Liver and Adipose Tissues of SHRSP and WKY Rats

Using the 10K CodeLink array, the expression profiles of approximately 10,000 rat genes were analyzed in the brain, heart, kidney, liver and adipose tissues of SHRSP and WKY rats after 4 weeks of treatment with candesartan. The expression profiles were additionally analyzed in the hearts of SHRSP after 4 weeks of treatment with hydralazine to examine the potential impacts of blood pressure–dependent mechanisms on differential gene expression.

The present study was originally designed to extensively identify "signature" genes altered by candesartan treatment in individual organs of interest, irrespective of hypertension status, *i.e.*, gene alteration commonly found in both SHRSP and WKY rats. However, we found only one gene in a single organ—namely, *Ren1* in the kidney—to be differentially expressed in both SHRSP and WKY rats (Fig. 1). This finding is considered to corroborate the effectiveness of candesartan treatment in the present study.

The results of microarray analysis for candesartan treatment are shown separately for each rat strain (Tables 2 and 3).

Microarray Data in SHRSP (Table 2)

In the brain of SHRSP, only two genes, *Ank2* and *D123*, were differentially expressed (down-regulated) between candesartan- and vehicle-treated animals. No genes were found to be up-regulated.

In the hearts of SHRSP, 69 genes were differentially expressed in the same experimental setting. Of these, 33 genes were up-regulated, and the functions of 20 genes (of the 33) are known to be involved in cellular metabolism, cell organization/biogenesis, regulation of cellular physiological process, transport, cell motility, and cell growth. The rest (36 genes) were down-regulated, of which 31 are known to be involved in cellular metabolism, transport, regulation of cellular physiological process, cell death, cell proliferation, cell motility, cell cycle, cell organization/biogenesis, cell homeostasis, and cell division.

In the kidneys of SHRSP, candesartan treatment up-regulated four genes, half of which (two genes) are known to play a role in the regulation of blood pressure. *Ren1* is a rate-limiting enzyme of the RAS. *Atp1a1* mediates Na^+ and K^+ transport to maintain water and electrolyte balance. Likewise, only three genes, *Fos*, *Cd24* and *Arc*, were down-regulated. *Fos* is an immediate early gene encoding a nuclear protein involved in signal transduction, whereas *Cd24* may be involved in neuronal migration during development. *Arc* is known to be involved in neuronal plasticity, long-term potentiation, and memory consolidation.

In the livers of SHRSP, candesartan treatment up-regulated two genes, *Cckar* and *Slco1c1*. *Cckar* is a G-protein–coupled receptor activated by cholecystokinin and may be involved in food intake and insulin regulation. *Slco1c1* mediates the high-affinity transport of sulfated bile acids into hepatocytes.

In the adipose tissue of SHRSP, candesartan treatment up-regulated 13 genes, 12 of which are known to be involved in transport, cell organization/biogenesis, cellular metabolism, cell motility, and extracellular matrix organization/biogenesis. Likewise, 19 genes were down-regulated, eight of which are known to be involved in cellular metabolism, transport, regulation of cellular physiological process, cell death, cell proliferation, cell motility, cell cycle, cell organization/biogenesis, cell homeostasis, and cell division.

Microarray Data in WKY Rats (Table 3)

In the brains of WKY rats, only one gene was differentially expressed (up-regulated) between candesartan- and vehicle-treated animals. However, this gene is not well-characterized.

In the kidneys of WKY rats, two genes were differentially expressed. Of these two, *Ren1* was up-regulated in a similar fashion as in the kidneys of SHRSP, and *Calb1*, which binds calcium, was down-regulated.

In the livers of WKY rats, three genes were differentially expressed, of which *Zfp354a* was up-regulated and is known to be a C2H2 class zinc finger–containing DNA–binding protein. Two genes were down-regulated, of which *Tnfrsf12a* is involved in cell proliferation, adhesion and angiogenesis.

In the heart and adipose tissue of WKY rats, no genes were significantly changed at the level of expression.

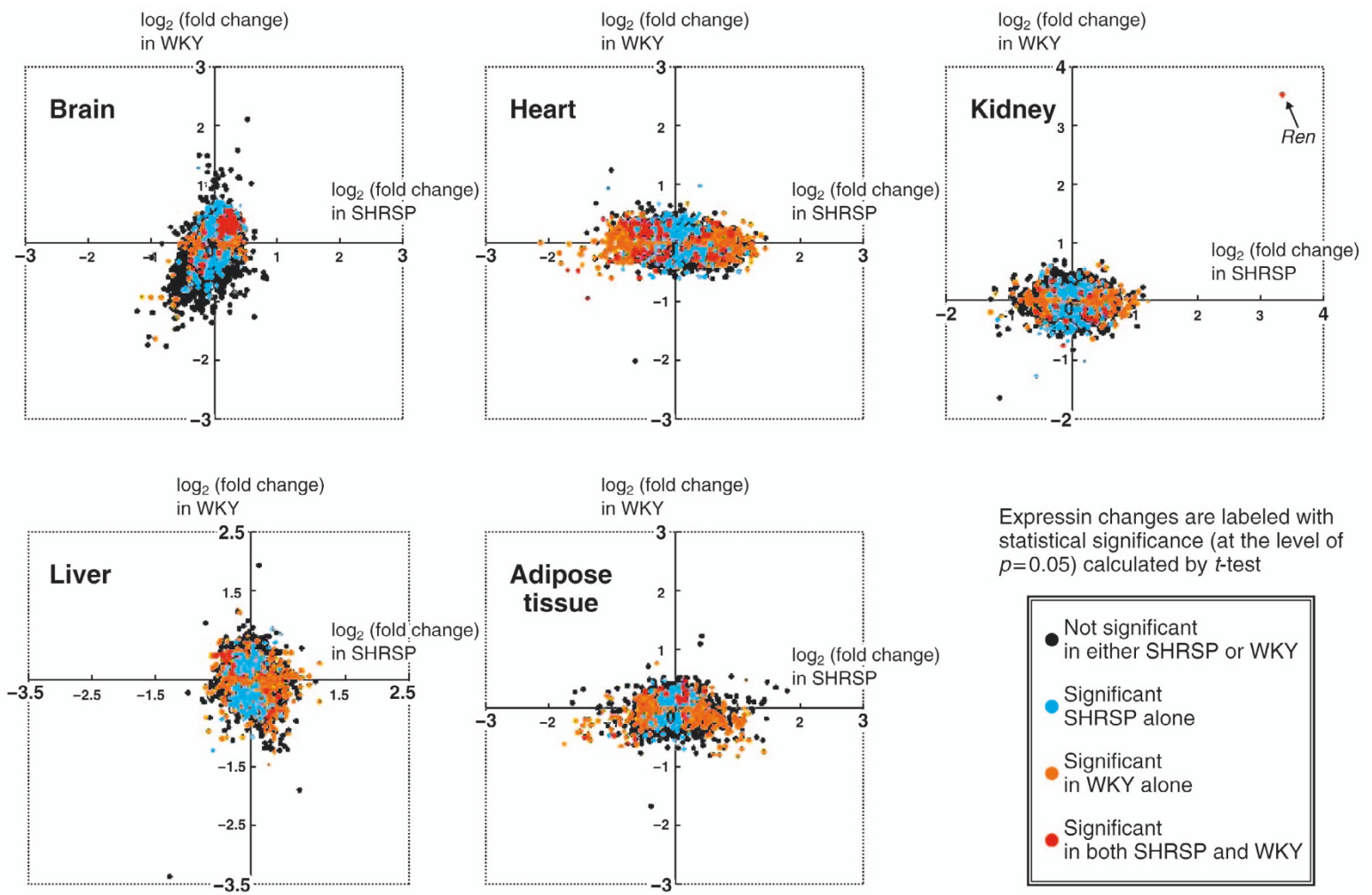

Fig. 1. *Scatter plots of gene expression changes induced by candesartan treatment in the five tested organs. The $\log_2$ expression ratio of [treatment/vehicle] is plotted against the results for SHRSP (x-axis) and WKY rats (y-axis) in each organ, where individual expression changes are labeled with statistical filters to show differentially regulated genes. That is, based on the statistical significance (at the level of $p < 0.05$) calculated by t-test, microarray data points are largely categorized into four classes: not significant in either SHRSP or WKY rats (black), significant in SHRSP alone (blue), significant in WKY rats alone (orange), and significant in both SHRSP and WKY rats (red). A plot corresponding to the renin (Ren) gene is marked in the kidney.*

Table 2. A List of Genes Showing Significant Changes in mRNA Expression in SHRSP

Tissue (up- or down-regulated)	Accession nunber	Gene symbol	Ratio (fold change)	*p*-value
Brain				
Down-regulated	BI286775	Ank2	0.448	0.04
	NM_053877	D123	0.494	0.032
Heart				
Up-regulated	NM_012754	Esr2	2.693	0.01
	BI275314	hom. to (Q984G4) Mlr8016 protein	2.574	0.04
	NM_133601	Cblb	2.453	0.044
	BM384301	LOC689625	2.431	0.034
	AA892791	Ercc1 pred.	2.391	0.013
	CA509498	Wdr1	2.322	0.003
	AI007919	Strap	2.319	0.021
	M17412	LOC360721	2.318	0.003
	BE110378	secretagogin, EF-hand calcium binding protein	2.316	0.002
	NM_134349	Mgst1	2.310	0.007
	AW527553	RGD1305235	2.296	0.019
	NM_031134	Thra	2.249	0.041
	AW141128	hom. to histone acetyltransferase MORF	2.246	0.039
	AW251633	Ascc3l1	2.235	0.045
	NM_133570	Grp	2.226	0.023
	NM_031587	Pxmp2	2.175	0.018
	NM_024139	Chp	2.168	0.009
	NM_017284	Psmb2	2.158	0.039
	AA799661	Fyco1 pred.	2.157	0.036
	NM_080481	Atp5i	2.125	0.003
	NM_053598	Nudt4	2.112	0.003
	AA818952	Pde4dip	2.104	0.047
	AI010272	LOC689898	2.096	0.015
	BF390029	Tbca	2.080	0.002
	AI009128	Ptrf pred.	2.073	0.02
	AA800029	Phr1 pred.	2.072	0.005
	AW435110	LOC290704	2.061	0.025
	BF558524	sim. to adenylosuccinate synthetase, muscle isozyme	2.057	0.028
	AI411425	Esd	2.045	0.019
	AW916127	Acox3	2.039	0.008
	AW251339	sim. to sex-determination protein hom. Fem1a	2.022	0.047
	AA848357	Perp pred.	2.022	0.022
	BE100918	Rufy1	2.003	0.004
Down-regulated	X89963	Thbs4	0.228	0.022
	AA894092	Postn pred.	0.281	0.02
	NM_021586	Ltbp2	0.286	0.022
	AA892824	Tnc	0.306	0.005
	NM_012826	Azgp1	0.313	0.02
	BE111688	Akap7	0.344	0.008
	NM_031590	Wisp2	0.353	0.0003
	NM_019334	Pitx2	0.360	0.042
	NM_013197	Alas2	0.362	0.012
	BE096084	Scn3b	0.363	0.008
	NM_030868	Nov	0.375	0.033
	NM_019212	Acta1	0.382	0.045
	CB580520	Nes	0.409	0.006
	NM_019273	Kcnmb1	0.413	0.017
	NM_017058	Vdr	0.417	0.005
	NM_019296	Cdc2a	0.429	0.007
	NM_012997	P2rx1	0.436	0.031
	AA818342	2,556 bp at 5′ side: sim. to COL8A1	0.446	0.038
	Z78279	Col1a1	0.450	0.022
	NM_031517	Met	0.452	0.01
	NM_024369	Fstl1	0.464	0.023
	AI764027	Nes	0.469	0.037
	BF558723	Col15a1	0.475	0.001
	NM_012733	Rbp1	0.476	0.017
	AI407095	Qprt	0.477	0.018
	BI275000	Mfap5 pred.	0.477	0.008
	NM_031747	Cnn1	0.482	0.005
	AI599031	Loxl1	0.483	0.022
	NM_016994	C3	0.486	0.006
	BF551173	RGD1311830 pred.	0.488	0.014
	NM_147211	Cr16	0.492	0.02
	CF110715	Tpm4	0.493	0.023
	AW918237	Col8a2 pred.	0.494	0.04
	NM_053630	Kcnh4	0.496	0.024
	NM_033096	Ppm1b	0.499	0.033
	J04035	Eln	0.500	0.012
Kidney				
Up-regulated	J02941	Ren1	10.052	0.007
	BF556836	RGD1311454	2.291	0.016
	CB713772	Atp1a1	2.061	0.028
	BF406412	RGD1311045 pred.	2.031	0.036
Down-regulated	AW915240	Fos	0.398	0.018
	NM_012752	Cd24	0.425	0.029
	NM_019361	Arc	0.452	0.007
Liver				
Up-regulated	NM_012688	Cckar	2.145	0.031

Table 2. Continued

Tissue (up- or down-regulated)	Accession nunber	Gene symbol	Ratio (fold change)	*p*-value	Tissue (up- or down-regulated)	Accession nunber	Gene symbol	Ratio (fold change)	*p*-value
	NM_053441	Slco1c1	2.141	0.004		AI406896	RGD1310358 pred.	0.364	0.019
Adipose tissue									
Up-regulated	BG377148	1,743 bp at 3′ side: sim. to protein fosB	2.670	0.022		BE111629	Eraf pred.	0.371	0.015
						BF416240	RGD1311874 pred.	0.372	0.035
	NM_012778	Aqp1	2.284	0.018		AI547453	Csmd1	0.387	0.02
	NM_019367	Ppt2	2.265	0.01		BI296106	Rapsn pred.	0.388	0.015
	NM_031066	Fez1	2.195	0.044		NM_138861	Prlpk	0.393	0.001
	NM_031324	Prep	2.159	0.027		BG374248	Rgr pred.	0.396	0.008
	NM_053309	Homer2	2.153	0.031		NM_012633	Prph1	0.396	0.012
	CF111976	Eif2ak2	2.149	0.027		AA943126	RGD1565474 pred.	0.407	0.012
	NM_024396	Abca2	2.092	0.025					
	NM_053543	Ncdn	2.084	0.026		X69523	Rbp3	0.432	0.002
	NM_022397	Hnrpf	2.083	0.009		NM_013158	Dbh	0.457	0.025
	NM_031350	Pex3	2.074	0.007		NM_053931	Gp1bb	0.473	0.012
	NM_012499	Apc	2.068	0.04		BF420752	RGD1565470 pred.	0.497	0.008
	NM_013138	Itpr3	2.053	0.018					
Down-regulated	BE113155	Iqgap3 pred.	0.296	0.007		AA998971	RGD1307749 pred.	0.500	0.048
	AI574903	Hist3h2ba pred.	0.338	0.002					
	NM_017102	Slc2a3	0.344	0.002		NM_199382	Bpgm	0.500	0.037
	BI297863	RGD1309350 pred.	0.356	0.001					

SHRSP, stroke-prone spontaneously hypertensive rats; hom., homologue; sim., similar; pred., predicted.

Table 3. A List of Genes Showing Significant Changes in mRNA Expression in WKY

Tissue (up- or down-regulated)	Accession nunber	Gene symbol	Ratio (fold change)	*p*-value
Brain				
Up-regulated	AI102591	Snrpd1 pred.	2.296	0.0280
Kidney				
Up-regulated	J02941	Ren1	11.506	0.0040
Down-regulated	AI233253	Calb1	0.414	0.0350
	NM_031984	Calb1	0.490	0.0450
Liver				
Up-regulated	NM_052798	Zfp354a	2.217	0.0450
Down-regulated	H35647	Tnfrsf12a	0.370	0.0200
	AW915240	Fos	0.438	0.0110

WKY, Wistar Kyoto rats.

Inter-Strain Differences in Gene Expression Changes Induced by Candesartan

When gene expression changes were compared between SHRSP and WKY rats, the most marked inter-strain difference was observed in the heart among the five tested organs (Figs. 1 and 2). The overall baseline signal intensity of the microarray (*i.e.*, at the vehicle-treated condition) tended to be higher in the hearts of SHRSP than in those of WKY rats, as indicated by the down-skewed scatter plots in Fig. 2B (upper scatter plots). To find the cause of the inter-strain difference in gene expression changes, we investigated the relationships between the comparative degree of gene expression in vehicle-treatment groups (which was represented by the $\log_2$-transformed ratio of baseline signal intensity in SHRSP to that in WKY rats) and the extent of expansion or reduction of inter-strain differences in gene expression (which was represented by inter-strain differences in $\log_2$-transformed fold

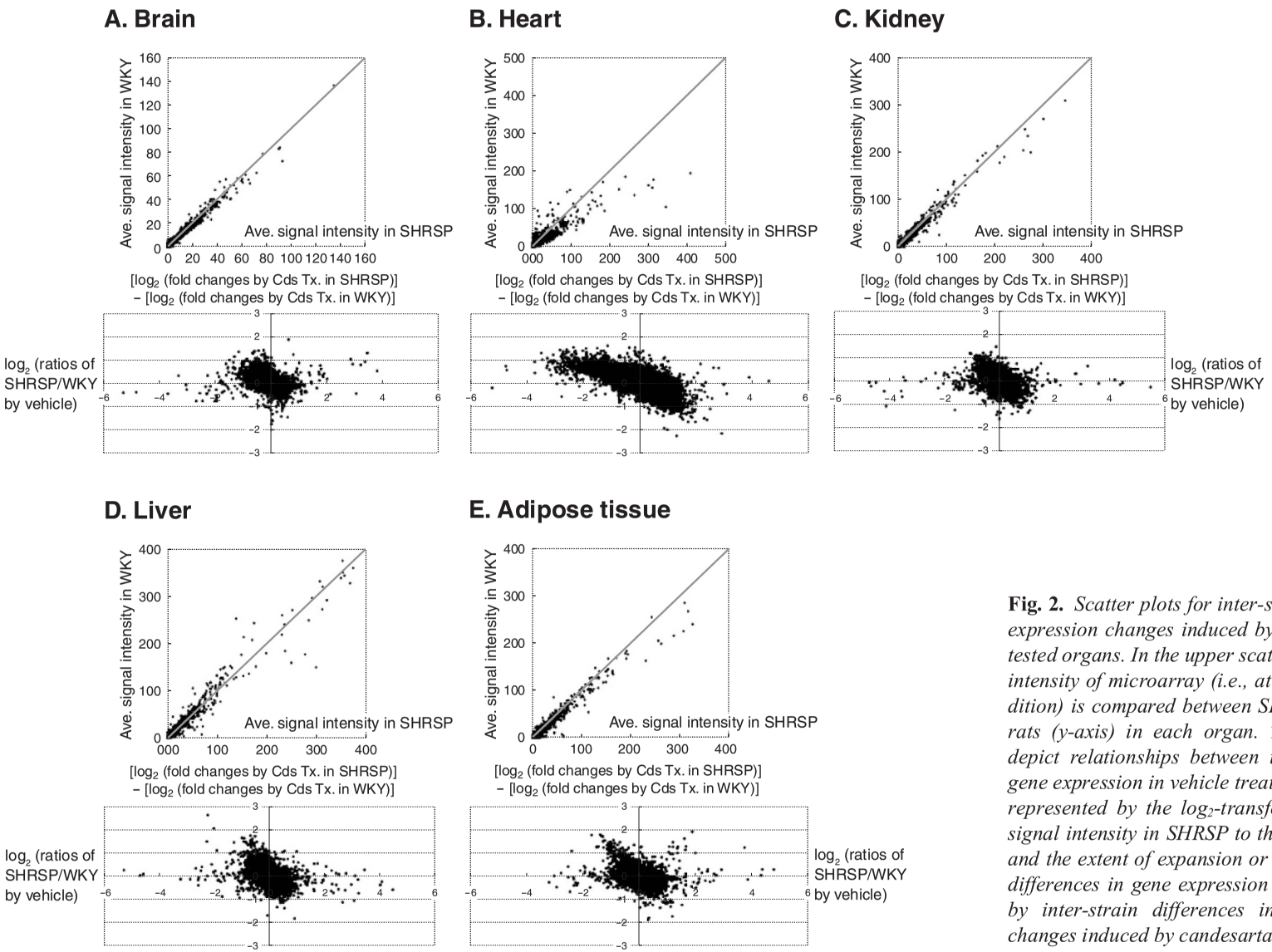

Fig. 2. *Scatter plots for inter-strain differences in gene expression changes induced by candesartan in the five tested organs. In the upper scatter plots, baseline signal intensity of microarray (i.e., at the vehicle-treated condition) is compared between SHRSP (x-axis) and WKY rats (y-axis) in each organ. The lower scatter plots depict relationships between the comparative size of gene expression in vehicle treatment groups (which was represented by the log_2-transformed ratio of baseline signal intensity in SHRSP to that in WKY rats) (x-axis) and the extent of expansion or reduction of inter-strain differences in gene expression (which was represented by inter-strain differences in log_2-transformed fold changes induced by candesartan treatment) (y-axis).*

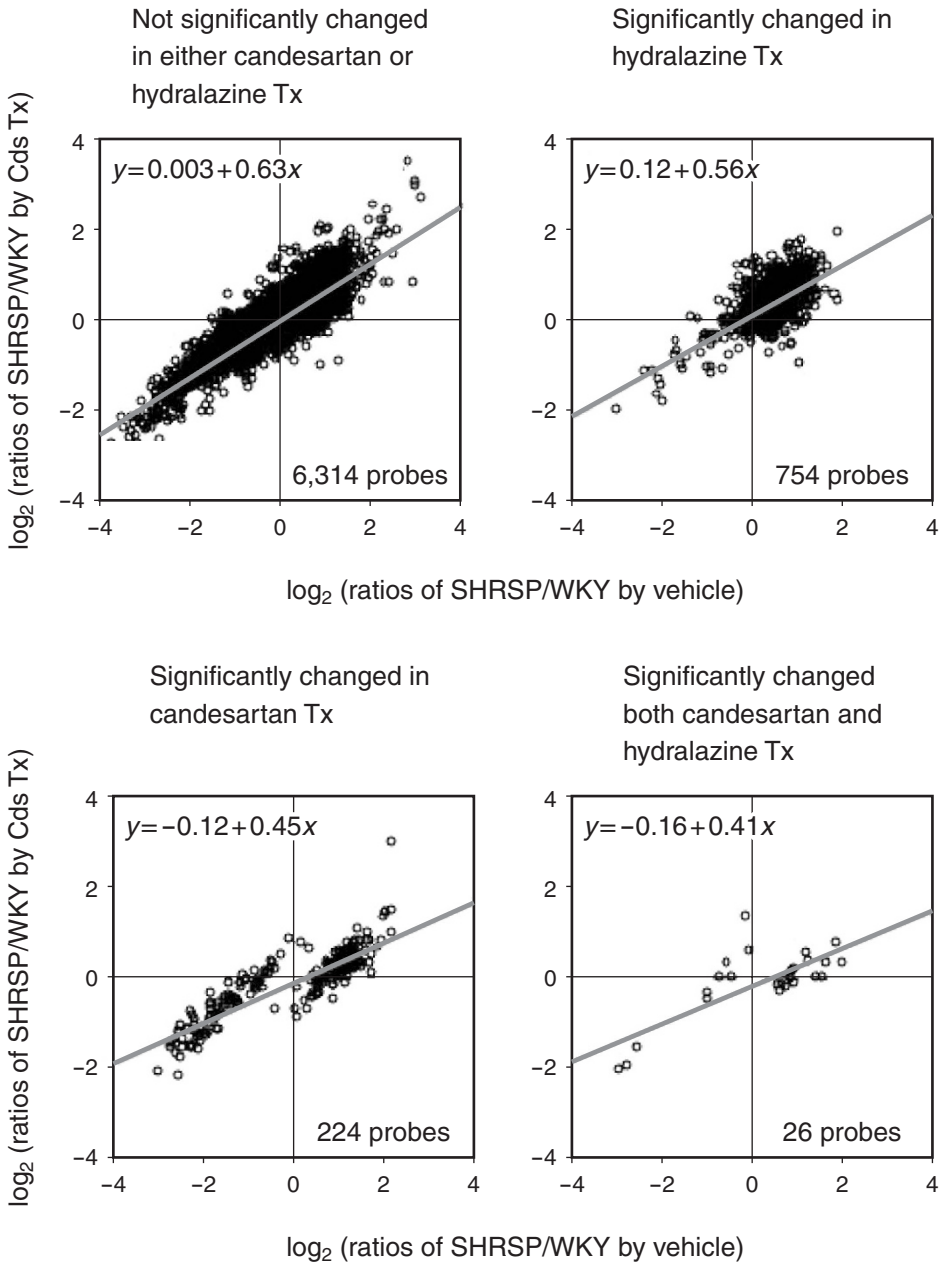

Fig. 3. *Scatter plots depicting log_2-transformed ratios of SHRSP and WKY rats for candesartan treatment (y-axis) against those for vehicle treatment (x-axis) according to the patterns of individual gene expression changes by candesartan and hydralazine. Genes (probes) tested in microarray analysis are categorized into four plots (from upper left to lower right in the figure) as follows: genes without significant changes by either of drugs (n = 6,314), genes with significant changes by hydralazine (n = 754), genes with significant changes by candesartan (n = 224), and genes with significant changes by both drugs (n = 26). The regression lines are shown in each plot together with the corresponding equations; that is, y = a + bx, where b represents the slopes of the regression lines and becomes smaller as inter-strain differences in gene expression could be reduced by candesartan treatment.*

changes induced by candesartan treatment) (Fig. 2A–E, lower scatter plots). There appeared to be an inverse correlation between these two parameters, suggesting that candesartan treatment reduced the "preexisting" inter-strain difference in gene expression. That is, the larger the inter-strain difference was for a given gene expression in the vehicle-treated group, the more prominent was the gene expression change induced by candesartan treatment in the hearts of SHRSP. Physiolog-

Table 4. Comparison of Differential Expression Ratios of 7 Genes between 10K CodeLink Array and Quantitative RT-PCR

Tissue origin (strain/organ)	Accession nunber	Gene symbol	10K CodeLink array				Quantitative RT-PCR			
			Ratio (fold change)	Actual signal intensity		Unpaired *t*-test *p*-value	Ratio (fold change)	Number of transcripts per control gene (*Ppia*)*		Unpaired *t*-test *p*-value
				Candesartan	Vehicle			Candesartan	Vehicle	
SHRSP/Heart	X89963	Thbs4	0.23	4.205	18.447	0.022	0.24	0.366±0.066	1.528±0.092	0.014
SHRSP/Heart	AA894092	Postn_predi	0.28	2.352	8.357	0.020	0.28	0.0007±0.0002	0.0026±0.0005	0.015
SHRSP/Heart	NM_021586	Ltbp2	0.29	1.024	3.575	0.022	0.33	0.562±0.062	1.687±0.083	0.003
SHRSP/Heart	AA892824	Tnc	0.31	0.883	2.889	0.005	0.39	0.076±0.013	0.195±0.037	0.006
SHRSP/Heart	Z78279	Col1a1	0.32	0.750	2.371	0.012	0.59	0.102±0.011	0.172±0.005	0.002
SHRSP/Kidney	AW915240	Fos	0.40	3.246	8.146	0.018	0.65	0.0012±0.0001	0.0020±0.0001	0.109
SHRSP/Kidney	AF117820	Ren 1	10.1	15.09	1.50	0.007	15.1	0.0816±0.0037	0.0054±0.0004	4.6×10^{-5}
WKY/Kidney	AF117820	Ren 1	11.5	14.55	1.26	0.004	14.9	0.0755±0.0070	0.0051±0.0003	8.4×10^{-4}

SHRSP, stroke-prone spontaneously hypertensive rats; WKY, Wistar Kyoto rats. *Values are means±SEM.

ically, candesartan could have caused the marked inter-strain difference in the reduction of blood pressure and also could have significantly alleviated the cardiac hypertrophy in SHRSP. Thus, it is plausible that the observed inter-strain difference in gene expression profile that was ameliorated by candesartan treatment can be ascribed mainly to blood pressure–dependent mechanisms. Similarly to the heart, the inter-strain difference was noticeable in the kidney and adipose tissue but not necessarily in the brain and liver (Fig. 1).

Comparison of Gene Expression Changes in the SHRSP Heart between Candesartan and Hydralazine Treatments

Although not completely equivalent to candesartan, hydralazine (10 mg/kg/d) was found to significantly lower systolic blood pressure in SHRSP. For hydralazine treatment, we performed microarray analysis in the heart, in which the most marked inter-strain difference was observed for candesartan treatment, to examine whether this inter-strain difference could be substantially ascribed to blood pressure–dependent mechanisms.

A total of 7,318 genes (probes) passed our microarray data filtering for spot quality. Among them, only 26 genes (0.35%) showed significant ($p<0.05$ by unpaired *t*-test, and <0.67 or ≥1.5 fold) expression changes induced by both drugs in common, while 224 (3.1%) and 754 (10.3%) genes showed significant changes in expression by candesartan and hydralazine, respectively, at the relevant statistical thresholds (Fig. 3). In line with Fig. 2, we further evaluated the extent of expansion or reduction of inter-strain differences in gene expression by plotting the $\log_2$ ratios of SHRSP and WKY rats for candesartan treatment (in the *y*-axis) against those for vehicle treatment (in the *x*-axis) according to the patterns of individual gene expression changes from the two drugs. Here, in each plot, the regression lines would make a smaller angle with the *x*-axis, as inter-strain differences in gene expression could have been reduced by candesartan treatment. The slopes of the regression lines appeared to near the *x*-axis (from 0.63 to 0.41) in the following order: genes without significant changes by either of drugs, genes with significant changes by hydralazine, genes with significant changes by candesartan, and genes with significant changes by both drugs (Fig. 3). Gene expression changes in the hearts of SHRSP were found to be more prominent for hydralazine than for candesartan treatment as a whole, and there was a relatively weak but significant correlation between the two drug treatments (correlation coefficient $=0.083$, $p<0.0001$, $r^2=0.007$). Thus, both hemodynamic and drug-specific factors cooperatively contributed to the overall tendency of reduction in inter-strain differences in gene expression induced by candesartan.

Validation of DNA Microarray Data by Real-Time PCR Amplification

We validated the accuracy of the differential expression ratios from the 10K CodeLink array by comparing them with the data obtained using quantitative RT-PCR (TaqMan® assay; PE Applied Biosystems, Foster City, USA) for a subset of seven genes (Table 4). While only two pools (two samples per microarray pool, *i.e.*, $n=4$ in total) were compared between candesartan and vehicle treatment groups in the present study, the 10K CodeLink turned out to generate expression ratios that were in good agreement with those of TaqMan®.

Discussion

The present study, for the first time, provides comprehensive profiles of gene expression induced by candesartan treatment in five organs of the hypertensive rat strain SHRSP, where the local RAS is supposed to have important cardiovascular function. Candesartan differentially modulated gene expression profiles in the brain, heart, kidney, liver and adipose tissues of SHRSP in an organ-specific manner. Of the five organs tested, gene expression was most prominently altered in the hearts of SHRSP. In contrast, candesartan treatment exerted

minimal or no significant effects on gene expression profiles in the corresponding organs of normotensive WKY rats. These inter-strain differences in gene expression changes induced by candesartan seemed to be associated with both blood pressure–dependent and independent mechanisms.

The present study is the first to provide a global profile of cardiac gene expression as modulated by ARB in a genetic model of hypertension. The RAS components are locally expressed in the heart and capable of generating Ang II. It is known that the cardiac RAS is involved in the induction of hypertrophy, fibrosis and remodeling and is therefore of significant clinical relevance to the study of hypertension and heart failure (*4–6*, *18*). Our observation that candesartan induces a prominent alteration in cardiac gene expression strengthens the general understanding that locally generated angiotensin peptides have multiple and novel diverse actions, including cell growth, cell proliferation and cell death (*5*). Along this line, notably, some experimental and clinical studies have demonstrated that AT_1 receptor antagonists can attenuate most of the deleterious effects of Ang II in the heart (*19*). The cardiac genes altered in SHRSP in the present study are involved in cellular events including metabolism, regulation of physiological process, ion transport, organization and biogenesis, motility, death, proliferation, cycle and growth, and homeostasis, which partly overlap with the genes previously reported (*20–22*) but also include novel genes, *e.g.*, thrombospondin 4 (*Thbs4*), latent transforming growth factor β binding protein 2 (*Ltbp2*) and tenascin C (*Tnc*) (Table 4). These observations provide a basis on which we can dissect the complex cardioprotective mechanisms that underlie the local cardiac RAS and its interaction with other systems.

The local RAS is also assumed to play a role in the brain and has been implicated in the pathogenesis and outcome of stroke (*3*, *7*). Ang II exerts most of its well-defined physiologic actions through the stimulation of an AT_1 receptor subtype. This receptor subtype not only may contribute to stroke-related pathologic mechanisms (*e.g.*, hypertension and atherothrombosis) but also may be involved in post-ischemic damage to the brain (*23*). Animal studies to date have shown that only the effective blockade of brain AT_1-receptors exerts neuroprotective effects in ischemic neuronal tissues and consequently improves the neurological outcome of focal brain ischemia (*23*). In this context, it is reported that candesartan is the most effective ARB to cross the blood-brain barrier and the current dosage seems to be sufficient to block AT_1-receptors in the brain according to the dosage (0.1–3 mg/kg/d) used in the previous studies (*23–25*). The SHRSP animal model tested in this study is known to exhibit a propensity for stroke and seems to be appropriate for physiological investigation of neuroprotective effects of candesartan treatment. Surprisingly, we could find much less modest changes in gene expression in the brains of SHRSP than in the hearts of SHRSP. In fact, only two genes involved in the cell cycle and signal transduction process turned out to be significantly modulated (down-regulated) by candesartan treatment in the current experimental setting. Zhou *et al*. (*24*) also performed microarray analysis on brain microvessels after 4 weeks of candesartan administration (0.3 mg/kg/d) *via* a subcutaneously implanted osmotic mini-pump. This study reported that a larger number of probes (genes) were significantly altered at the level of mRNA expression by candesartan treatment in WKY rats than in the spontaneously hypertensive rat (SHR), while a total of 16 probes including stress-related genes such as stathmin were down-regulated to the same extent in both strains. Accordingly, the authors argued that candesartan could target many genes in brain microvessels through blood pressure–independent mechanisms. This appears to be in accordance with our observations of the brain; that is, that fold changes of gene expression tended to be larger in WKY rats than in SHRSP (Fig. 1), although differentially expressed genes were equivalent in number at the arbitrarily defined cutoff levels in Table 2 ($p<0.05$ by unpaired *t*-test, and <0.5 or ≥ 2.0 fold).

All components of the RAS are found in the kidney, with each being compartmentalized in the organ's tubular and interstitial networks. Above all, it should be noted that the *Ren1* gene was most prominently (>10 fold) up-regulated by candesartan treatment in the kidneys of both rat strains, which indicated a successful blockade of the local RAS in the current experimental setting. As considerable amounts of Ang II are formed locally, it is debated that intra-renally formed Ang II is more important than circulating Ang II in controlling renal function. However, we could find much subtler gene expression changes in the kidneys than in the hearts of SHRSP. Likewise, we found a limited number of genes to be significantly altered in the livers of SHRSP.

Of particular note in this study is the fact that, next to the heart, the adipose tissue had the second most significant alterations in gene expression induced by candesartan. The SHRSP is generally known to be a model of insulin resistance in humans and is characterized by reduced insulin-mediated glucose disposal and defective fatty acid metabolism in the isolated adipocytes (*26*). Accumulating evidence has been provided for the existence of the local RAS in human adipose tissues, which may function independently of circulating RAS and has been implicated in the pathogenesis of metabolic syndrome (*27*). Importantly, enhanced Ang II signaling and the subsequent metabolic disturbances in the adipose tissue are considered potential therapeutic targets for the prevention of cardiovascular complications in metabolic syndrome (*28*). Along this line, the present data may help clarify the mechanisms linking the adipose tissue RAS to the development of insulin resistance in hypertension and metabolic syndrome.

Recently, it has been shown that hypertension-related end-organ damage largely depends on the level of blood pressure (*29*), but some people have argued that it is also substantially influenced by the activation of the local RAS (*30*). Generally, it is difficult to separate blood pressure–dependent mechanisms from blood pressure–independent mechanisms when

we evaluate pharmacological impacts of a particular antihypertensive drug on end-organ damage. It would be valuable to compare the results for a hypertensive rat strain (SHRSP in this study) with those for a normotensive rat strain (WKY rats in this study) (Fig. 1); the extent of blood pressure reduction by antihypertensive treatment significantly differed between these strains, implying that differential gene expression unique to SHRSP is substantially explained by blood pressure–dependent mechanisms. To verify this speculation, we compared treatment with candesartan and hydralazine with respect to gene expression changes in the heart of SHRSP and found that both hemodynamic (*i.e.*, blood pressure–dependent) and drug-specific (*i.e.*, blood pressure–independent) factors could cooperatively contribute to the overall tendency of reduction of inter-strain differences in gene expression induced by candesartan. This also seems to be applicable for the results of gene expression in the kidneys of SHRSP. The lack of apparent inter-strain differences in the brains of SHRSP, on the other hand, needs to be carefully interpreted, which may simply indicate the relatively mild gene expression changes in the brain because of the drug's organ specificity observable in the current experimental setting. Of particular note is the fact that differential gene expression was prominent in the adipose tissue of SHRSP after candesartan treatment. The underlying mechanism remains to be elucidated, but one possible explanation is that the "enhanced" RAS in the adipose tissue may be calmed down by the pharmacological property of candesartan, whether it is the primary cause of hypertension or the phenomenon secondary to increased blood pressure (*31*). When the reference of pharmacological property was set to WKY rats, candesartan treatment appeared to reduce the "pre-existing" inter-strain difference in gene expression in most of the organs tested (Fig. 2). In this context, it must be noted that candesartan treatment induces only a modest degree of gene expression changes at the current dose in the normotensive WKY rats (Table 3). Thus, candesartan treatment is considered to ameliorate—in an organ-specific manner and by acting principally on the local RAS—gene expression changes through both blood pressure–dependent and –independent mechanisms at a variety of pathophysiological stages *in vivo*.

While a number of genes altered by candesartan in SHRSP have been characterized, detailed functions of many genes remain unknown. The list of genes thus detected in the present study can provide insights into the beneficial effects of ARB, such as candesartan, on the cardiovascular system. Moreover, differential organ- or tissue-specific gene expression changes caused by candesartan may help delineate the mechanisms that underlie the organ or tissue protection conferred by ARB at the levels of cellular biology and genetics, and these findings will contribute to pharmacogenetics. Further studies are warranted to investigate not only the individual genes of interest but also the genetic "networks" that involve differential organ- or tissue-specific gene expression induced by the blockade of RAS in essential hypertension.

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