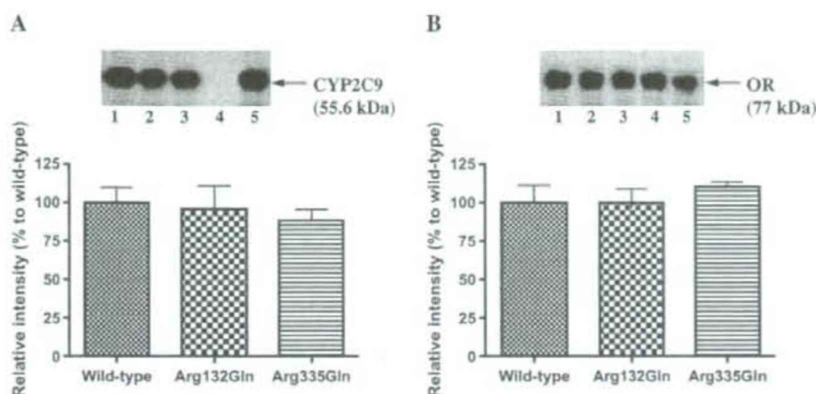

Fig. 2. *Expression of wild-type and two variants of CYP2C9 in insect cell microsomes. Representative Western blots of immunoreactive CYP2C9 (A) and OR (B) proteins (upper) are shown. Lanes 1–3: co-expressed microsomes containing wild-type, Arg132Gln, and Arg335Gln CYP2C9 each with OR; lane 4: microsomes containing solely OR; lane 5: commercially available co-expressed supersomes containing CYP2C9.1 and OR (BD Bioscience, San Jose, USA). Relative intensities of immunoreactive CYP2C9 (A) and OR (B) protein are shown in the lower panels. Each bar represents the mean±SD of three separate experiments.*

Table 2. Kinetic Parameters for Hydroxylation Activities of Wild-Type and Variant CYP2C9 against Diclofenac

Amino acid alteration	K_m (μmol/L)	V_{max} (pmol/min/pmol P450)	Clearance (V_{max}/K_m) (μL/min/pmol P450)
Wild-type	3.4±0.17	79.8±6.6	23.4±0.81
Arg132Gln	1.8±0.05**	7.8±0.4**	4.2±0.31**
Arg335Gln	3.0±0.10*	65.4±2.1*	22.0±0.06*

*$p<0.05$, **$p<0.0001$ *vs.* wild-type. One-way analysis of variance, post-hoc test: Scheffe. Data are represented by means±SD.

tein expression levels were significantly different among the wild-type and two variants ($p=0.77$ for CYP2C9, $p=0.64$ for OR). Catalytic activities of the wild-type and variant (Arg132Gln and Arg335Gln) proteins were assessed using diclofenac as a substrate. Diclofenac 4′-hydroxylation exhibited typical hyperbolic kinetic profiles in both the wild-type and variant proteins (data not shown). The kinetic parameters are summarized in Table 2. The Arg132Gln protein showed a 90% decrease in the V_{max} value and a partial decrease in the K_m value, resulting in fivefold lower intrinsic clearance relative to the wild-type (Table 2). A slight diminution in intrinsic clearance (6%) was observed for the Arg335Gln protein with slightly decreased K_m and V_{max} values (Table 2). The formation of 5-hydroxy diclofenac was observed in neither the wild-type nor variant (Arg132Gln and Arg335Gln) proteins (data not shown), suggesting that these substitutions do not alter the regioselectivity of diclofenac hydroxylation.

CYP2C9 Polymorphisms and the Effectiveness of Losartan in 39 Hypertensive Patients

Among 39 patients taking losartan, 34 patients carried the wild genotype of *CYP2C9*1/*1*, and the other 5 patients carried missense mutations, including *CYP2C9*1/*3* in 2 patients, *CYP2C9*1/*30* in 2 patients, and Arg132Gln mutation in one patient. The changes in systolic and diastolic blood pressure with respect to genotypes at 3 months of losartan treatment are presented in Table 3. Losartan obviously lowered systolic blood pressure in 2 patients with *CYP2C9*3* and in a patient with the Arg132Gln mutation. However, losartan was not effective in 2 patients with *CYP2C9*1/*30*.

Discussion

In the present study, the large-scale direct resequencing effort of the *CYP2C9* allowed us to detect 31 genetic variations in 724 Japanese individuals. We also obtained accurate frequencies of the known variations, *CYP2C9*3*, **13*, **14*, **27* and **30*, that are specific to Asians, except for **3*. As for the novel alleles, Arg132Gln and Arg335Gln, their effects on both protein expression levels and enzymatic activity were assessed using a baculovirus expression system.

The most frequently identified missense mutation in the present study was *CYP2C9*3* (Ile359Leu), with a frequency

Table 3. Patient Characteristics and Blood Pressure Response to Losartan with Respect to Genotypes: Essential Hypertensive Patients Taking Losartan

	CYP2C9 genotype					
	**1/*1*	**1/*3*		**1/*30*		Arg132Gln
Case number	34	2		2		1
Sex (male/female)	21/13	0/2		2/0		1/0
Age (years)	65.10±7.04	70	67	77	71	70
BMI (kg/m^2)	25.10±3.07	21.47	24.20	24.33	25.59	20.7
SBP						
At baseline (mmHg)	151.10±14.75	130[a]	156	155	172	157
At 3 month (mmHg)	142.80±16.23	119	141	151	173	128
Change (mmHg)	−8.70±14.35	−11	−15	−4	1	−29
DBP						
At baseline (mmHg)	88.80±9.26	71[a]	104	81	98	82
At 3 month (mmHg)	84.90±9.98	75	96	83	95	70
Change (mmHg)	−4.20±6.91	4	−8	2	−3	−12

Values are mean±SD. BMI, body mass index; SBP, DBP, systolic and diastolic blood pressures. [a]Office blood pressure in this patient with *CYP2C9*1/*3* was 130/71 mmHg. Losartan was prescribed because this patient had higher home SBP (over 150 mmHg).

of 0.033, which was in good agreement with the previously published results in Japanese populations (*11, 36, 37*). The frequency of *CYP2C9*13* (Leu90Pro), 0.0014 in the present study, was comparable to that recently reported in a Japanese population (*11*) but much lower than those in previous studies of other Asian populations (*6, 9*). *CYP2C9*13* was first identified in a Chinese individual who showed poor metabolizer phenotype for both lornoxicam and tolbutamide (*6*). Functional analysis of the *CYP2C9*13* protein showed decreased enzymatic activity for tolbutamide and diclofenac (*10*). Another recently published allele, *CYP2C9*14* (Arg125His), was detected in an individual in the present study. This allele was first identified in an Indian patient, and the variant protein exhibited 80–90% lower catalytic activity toward tolbutamide (*7, 8*). *CYP2C9*27* (Arg150Leu) and **30* (Ala477Thr), both detected recently in a Japanese population (*11*), were also identified in 3 and 2 individuals in the present study, respectively. The *in vitro* study revealed that the *CYP2C9*30* protein had a twofold higher K_m value and a threefold lower V_{max} value than the wild-type towards diclofenac, whereas the catalytic activity of the *CYP2C9*27* protein was similar to the wild-type (*11*).

The novel Arg132Gln variant exhibited a 90% decrease in the V_{max} value toward diclofenac 4′-hydroxylation (Table 2). Arg132 is located in a loop region between the C and D helices (Fig. 1) and is highly conserved in the CYP2C family (http://drnelson.utmem.edu/humP450.aln.html). Arg133, the corresponding residue of CYP2B4, is suggested to play a prominent role in binding its redox partners, cytochrome b5 and P450 reductase (*38*). Accordingly, the loss of catalytic activity of the Arg132Gln variant might reflect the altered affinity of variant protein to these redox partners due to electrostatic changes as proposed for **2* (Arg144Cys), **14* (Arg125His), and **26* (Thr130Arg) (*8, 11, 39*).

The Arg335Gln variant showed a similar holo-CYP2C9 content to wild-type in insect cell microsomes. Furthermore, the intrinsic clearance of the Arg335Gln variant was only slightly lower than that of the wild-type. In contrast to Arg335Gln, a substitution in the same position, Arg335Trp (**11*), was reported to exhibit a threefold increase in K_m and more than a twofold decrease in the intrinsic clearance for tolbutamide when expressed in a bacterial cDNA expression system (*40*). In addition, catalytically active *CYP2C9*11* holo protein was expressed at a very low level due to its decreased stability in insect cells (*41*). To confirm whether or not the protein stability of the Arg335Gln variant might be influenced by the *in vitro* expression system used, the wild-type and variant proteins were expressed in a mammalian expression system using COS-1 cells. The protein expression level of Arg335Gln variant in COS-1 microsomes was decreased by only 30% compared with that of the wild-type (data not shown), indicating that the protein stability of the Arg335Gln product was not substantially different between mammalian expression systems and baculovirus/insect cell systems. Thus, the substituted residues (Trp *vs.* Gln) at this position might quite differently influence the stability of protein as well as catalytic activities.

Thirty-nine patients were taking losartan, which is known to exhibit considerable inter-individual variation in its antihypertensive effects. Losartan is primarily oxidized by CYP2C9 to an active carboxylic acid metabolite, E-3174 (*14–16*). CYP3A4 also plays a limited role in the metabolic activation of losartan *in vitro*; however, its significance *in vivo* has not been demonstrated (*3, 15, 16*). We evaluated the impact of *CYP2C9* variations on the antihypertensive effect of losartan based on the patients' average resting blood pressure measured before and three months after losartan treatment.

Two Japanese hypertensive patients carrying the *CYP2C9*3* heterozygous allele showed lowered systolic blood pressure by losartan (Table 3). This is in line with the previous report that no significant differences in the pharmacokinetics of losartan and E-3174 were observed between *CYP2C9*1/*3* and **1/*1* (*42*). Contrary to our result, a Danish prospective study of optimal monotherapy with losartan in type 1 diabetic patients with nephropathy showed that the reduction in systolic 24 h blood pressure was significantly greater in wild-type patients ($n=48$) than in *CYP2C9*3* carriers ($n=12$) (*43*). Furthermore, similar changes in diastolic and systolic 12 h blood pressures were also observed between *CYP2C9*1/*1* ($n=4$) and **1/*3* ($n=3$) Japanese patients (*20*). The role of heterozygous *CYP2C9*3* in the blood pressure–lowering response to losartan in hypertensive patients should be further studied in a large cohort of patients.

Inconsistent with our *in vitro* study, systolic blood pressure in a patient with Arg132Gln was obviously lowered by losartan (Table 3). For this variation, the substrate-dependent differences between diclofenac and losartan oxidation are unlikely because Arg132 might interact with redox partners but not with substrates as described above. However, the change in enzymatic activity toward losartan should be further analyzed.

However, losartan was not effective in 2 patients carrying the heterozygous *CYP2C9*30* (Ala477Thr) allele. A serious impact on the pharmacodynamics of losartan was not demonstrated statistically because of the small sample size of individuals with **30*. Ala477 is located in the substrate recognition site-6 region in the β2 sheet, which shows very strong hydrophobic interactions with the substrates (*44*), suggesting the importance of this residue in metabolic activity of CYP2C9 toward various substrates. Therefore, insufficient conversion of losartan to E-3174 by this defective mutation might be responsible for the therapeutic failure of these patients. Pharmacokinetic analysis of *CYP2C9*30* towards losartan would be necessary to further elucidate its clinical relevance.

In conclusion, multiple rare functional variations of *CYP2C9* were detected in a Japanese population. Approximately 7% of the Japanese individuals analyzed (53 of 724) carried one of the functionally deleterious alleles (*CYP2C9*3*, **13*, **14*, **30*, and Arg132Gln). In addition to *CYP2C9*3*, *CYP2C9*30* might also be used for determining inter-individual responses to losartan treatment in Japanese hypertensive patients.

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A Polymorphism Regulates CYP4A11 Transcriptional Activity and Is Associated With Hypertension in a Japanese Population

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Abstract—CYP4A11 oxidizes arachidonic acid to 20-hydroxyeicosatetraenoic acid, a metabolite with renovascular and tubular function in humans. A previous study demonstrated a significant association between the CYP4A11 gene polymorphism and hypertension; however, the precise mechanism of the association has not been clarified. To assess the involvement of *CYP4A11* in the pathogenesis of hypertension, we sought to identify a functional polymorphism of *CYP4A11* and examined its impact on predisposition to hypertension in the Tanno-Sobetsu Study. The −845A/G polymorphism was identified in the promoter region of *CYP4A11* by direct sequencing. Luciferase expression driven by the promoter of *CYP4A11* containing the wild-type −845GG genotype was 30% lower than expression with the variant −845AA genotype. Gel mobility shift assays with nuclear protein extracts showed specific binding to probes containing the variant −845GG. To assess the effect of *CYP4A11* polymorphisms on hypertension, we also carried out a case-control study using 4 single nucleotide polymorphisms (−845A/G, −366C/T, 7119C/T, and 8590T/C) in the Tanno-Sobetsu Study. The odds ratio for hypertension in participants with the AG+GG genotype of −845A/G was 1.42 ($P=0.008$), and the odds ratio for hypertension of the TT genotype of 7119C/T was 1.37 ($P=0.037$) after adjusting for confounding factors. The haplotype-based case-control analysis using 4 single nucleotide polymorphisms revealed a significant haplotype (G-C-T-T) that was significantly associated with hypertension, with an odds ratio of 1.44 ($P=0.006$) after adjusting for confounding factors. We have identified a functional variant (−845A/G) of *CYP4A11* that is significantly associated with hypertension and that appears to be a novel candidate for a predisposing factor for hypertension. (***Hypertension*. 2008;52:1142-1148.**)

Key Words: genetics ■ hypertension ■ single nucleotide polymorphism (SNP) ■ CYP4A11 gene ■ renal circulation ■ transcription factor

The metabolism of arachidonic acid by cytochrome P450 (CYP) enzymes leads to the formation of various biologically active eicosanoids, such as hydroxyeicosatetraenoic acids, epoxyeicosatrienoic acids, and dihydroxyeicosatrienoic acids.[1–3] 20-Hydroxyeicosatetraenoic acid (20-HETE) is a strong vasoconstrictor and acts as a second messenger for vasoactive peptides (angiotensin II and endothelin). Recent reports have revealed that 20-HETE plays a dual role in the regulation of blood pressure (BP) by inducing renal vasoconstriction (prohypertensive function), as well as inhibiting sodium reabsorption in tubules (antihypertensive function).[4]

20-HETE is normally produced in renal and cerebral arterioles,[5,6] the glomerulus, and the renal tubules[7,8] and has been implicated in the regulation of contractile state, ion flux, and mitogenesis. 20-HETE is generated from arachidonic acid by CYP4A, also known as omega/omega-1 hydroxylase.[9,10] It has been reported that administration of antisense oligonucleotides to CYP4A1 reduces BP in spontaneously hypertensive rats,[11] and the expression of CYP4A is involved in early changes in eicosanoid formation and renal function in the young spontaneously hypertensive rats.[12] The *Cyp4a14* gene-disrupted mice showed increases in plasma androgens, kidney *Cyp4a12* expression, and the formation of prohypertensive 20-HETE, which resulted in hypertension.[13] A recent report demonstrated that *CYP4A11*, originally isolated from human kidney and liver cDNA libraries,[14,15] is the human homologue of mouse *CYP4a14*, expressed in human renal tubule and not in afferent arterioles. The report also described CYP4A11 regulation of 20-HETE.[16] Despite the potential involvement of CYP4A11 in regulating BP through 20-HETE, the exact function of the CYP4A11 gene is still unknown.

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Genetic approaches may provide a powerful tool for clarifying the pathogenesis of essential hypertension;[17] however, a recent report of a genome-wide association study[18] failed to establish a consensus in identifying a "hypertensive gene." In contrast, several recent reports have suggested a critical role for the *CYP4A11* polymorphism in predisposition to hypertension.[19–21]

Our investigation of a candidate gene for hypertension included 3 major objectives. We sought to detect a functional polymorphism of the CYP4A11 gene in Japanese people and to determine the molecular outcomes associated with the detected polymorphism in relation to hypertension. To achieve our third objective of clarifying the genetic involvement of CYP4A11 in the pathogenesis of hypertension, we carried out a case-control study within the Tanno-Sobetsu Study.

Methods

Polymorphism Identification and Genotyping

We generated primers specific for the promoter and exon regions of the CYP4A11 gene and used the primers for PCR amplifications. Four amplicons in the promoter region and 12 in the exon region were purified using ExoSAP-IT (GE Healthcare UK Ltd) and subjected to sequence analysis. Sequencing was performed using the 1.1 Big-Dye deoxy terminator cycle sequencing kit (Applied Biosystems, Inc), and analysis of sequencing reactions was carried out on the ABI Prism 3100 Genetic Analyzer (Applied Biosystems). The sequences were aligned with wild-type sequences obtained from the National Center for Biotechnology Information Web site (CYP4A11; AY369778) and examined for the presence of mutations.

Luciferase Assay

DNA fragments of the 5′-flanking region of the CYP4A11 gene (nucleotide positions −891 to −260), with and without the −845GG genotype, were amplified from genomic DNA by PCR. The primers used for PCR amplification contained *Bgl*II and *Spe*I sites (upstream; 5′-GA*AGATCT* (*Bgl*II) GGTCAGGACTCCTAAACAGAG, downstream; 5′-GA*CTAGT* (*Spe*I) GGCAACAGTGGGAGAAA), and the PCR products were digested and fused into the luciferase reporter vector phRL-null (Promega). Normal human renal proximal tubule epithelial cells (RPTECs) were used for the luciferase assays. Transient transfections were performed with NRK-52E (Amaxa), according to the manufacturer's instructions. The promoter/luciferase reporter plasmid (5 mg) and an internal luciferase control were transfected into RPTECs, and cells were incubated for 48 hours. Cell extracts were assessed for luciferase activity using the Dual-Glo Luciferase Assay System (Promega), according to the manufacturer's protocol. The measurements were performed in a luminometer (ARVO MX, PerkinElmer) in triplicate. *Firefly* luciferase activity, as a measure of *CYP4A11* promoter activity, was normalized against the *Renilla* luciferase internal control and expressed in relative activity units. The pGL3-control vector (Promega) was used as a negative control.

Electrophoretic Mobility Shift Assay

Nuclear extracts from RPTECs were prepared using the Nuclear/Cytosol Fractionation kit (BioVision, Inc) according to the manufacturer's instructions and diluted to a final protein concentration of 1 mg/mL. The double-stranded oligonucleotides used for binding were as follows: 4A11-wt (−855 to −825), 5′-GTGTAATTACATACTATTGTAGGGTGAAAGA, and 4A11-mt (−855 to −825), 5′-GTGTAATTACGTACTATTGTAGGGTGAAAGA. A total of 10 mg of nuclear extract was incubated with 1 mg of poly [d(I-C)] plus the digoxigenin-labeled oligonucleotide (0.8 ng; Dig Gel Shift kit, 2nd Generation, Roche, Inc) in the presence or absence of unlabeled oligonucleotides for 15 minutes at 15°C to 25°C. The binding reaction was carried out in a solution containing 20 mmol/L of HEPES (pH 7.6), 1 mmol/L of EDTA, 10 mmol/L of $(NH_4)_2SO_4$, 1 mmol/L of dithiothreitol, 0.2% Tween 20, and 30 mmol/L of KCl. The reaction mixtures (final volume: 20 mL) were directly loaded onto an 8% 90 mmol/L Tris/64.6 mmol/L boric acid/2.5 mmol/L EDTA (pH 8.3) polyacrylamide gel (Invitrogen, Inc), which had been run for 5 minutes before sample loading. After electrophoresis (80 V for 60 minutes at 4°C), the gels were transferred to nylon membranes in 90 mmol/L Tris/64.6 mmol/L boric acid/2.5 mmol/L EDTA (pH 8.3) buffer (300 mA for 60 minutes at 4°C) using the NuPAGE system (Invitrogen, Inc) and cross-linked at 120 mJ/cm^2 for 3 minutes, followed by chemiluminescent detection using the CSPD working solution (Roche, Inc).

Table 1. Patient Baseline Characteristics

Parameters	Hypertensive (n=495)	Normotensive (n=494)	*P*
Age, y	65.6±8.0	65.7±8.0	ns
Sex, male, %	46.06	46.96	ns
BMI	24.8±3.2	23.3±2.9	<0.0001*
Systolic BP, mm Hg	157.5±16.0	123.2±11.9	0.0001*
Diastolic BP, mm Hg	85.2±11.0	71.1±9.1	<0.0001*
Triglyceride, mg/dL	113.6±62.5	97.4±50.3	<0.0001*
Fasting BS, mg/dL	101.8±30.3	97.7±22.5	0.017*

BMI indicates body mass index; BS, blood sugar; ns, not significant. Data are means±SDs unless otherwise specified.

*The *P* value of genotypes and alleles were calculated using Fisher's exact test.

Population Study: The Tanno-Sobetsu Study

A total of 1501 participants were recruited from the Tanno-Sobetsu Study designed by the Second Department of Internal Medicine, Sapporo Medical University. The study started in 1977 with a cohort base in the northern part of Japan, Hokkaido, and the detailed epidemiological findings have been reported previously.[22] BP was measured twice in each participant while seated, after 5 minutes of rest. Hypertension was defined as systolic BP ≥140 mm Hg, diastolic BP ≥90 mm Hg, or the current use of antihypertensive agents. Normotensive (n=748; mean age: 57.9 years; 38.4% men) and hypertensive (n=753; mean age: 67.3; 39.7% men) participants were divided based on this definition. To match the 2 groups by age and sex, the participants were reduced by a random score method. The random scores were assigned to the remaining cases by multiplying age and sex by a uniform 0-to-1 random variable. A random selection with an age or sex bias was achieved by removing cases, starting with the lowest scores. This process was performed incrementally until the mean age for the 2 groups had been sufficiently matched. By the matching method, the mean age and the ratio of men in the normotensive (n=494) and hypertensive (n=495) groups were, respectively, 65.7 years and 46.96% men in the normotensive group and 65.6 years and 46.06% men in the hypertensive group (Table 1).

All of the participants gave written informed consent to the genetic analysis and all of the other procedures associated with the study. The ethics committee of Osaka University approved the study protocol.

Genotyping

The single nucleotide polymorphism (SNP) assays for −845A/G, −366C/T, 7119C/T, and 8590T/C (described in detail in the Results section) were performed using the TaqMan PCR method. The primers and probes are shown in Table 2. Probes were labeled with the fluorescence FAM (mutant type) or VIC (wild-type). Genotyping was performed twice with 5 ng of DNA per reaction in a total reaction volume of 10 mL on 386-well plates. The thermocycler

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Table 2. The Primer and Probe Sequences for Genotyping of the CYP4A11 Gene

SNP	Primer and Probe	Sequence
	Fw-primer	CTTGGTCAGGACTCCTAAACAGAGT
−845A/G	RV-primer	AGCCTGTTTACCAATTCTCTTTCAC
rs9332978	Probe for a major genotype	VIC-CTGATTGTGTAATTACATACT
	Probe for a minor genotype	FAM-CTGATTGTGTAATTACGTACTA
	Fw-primer	CAAATTCCTTGAGGTCAGCATTCA
−366C/T	RV-primer	CAGGCAGTATGCTAGGTGTGT
No rs#	Probe for a major genotype	VIC-CTGCGAGGAAAAC
	Probe for a minor genotype	FAM-CTGCAAGGAAAAC
	Fw-primer	TGCTGAGGTGGACACGTTCA
7119C/T (His323His)	RV-primer	CCATCACCCAGGAGGCTG
rs28451040	Probe for a major genotype	VIC-TTGAGGGCCACGACA
	Probe for a minor genotype	FAM-TTGAGGGCCATGACA
	Fw-primer	GCTTCCACCTCTGGGAGTCC
8590T/C (Phe434Ser)	RV-primer	GACTTCCCTCATTCCTCTATTCGA
rs1126742	Probe for a major genotype	VIC-TTGACCCTTTCCGTTTT
	Probe for a minor genotype	FAM-TTTGACCCTTCCCGTTT

Fw indicates forward; RV, reverse.

parameters were as follows: 50°C for 2 minutes, 95°C for 10 minutes, 45 cycles of 95°C for 15 seconds, and 60°C for 60 seconds. Allelic discrimination was measured automatically on the ABI Prism HT7900 (Applied Biosystems) using the Sequence Detection System 3.1 software.

Statistical Analysis

A power analysis for this case-control study (a 1-to-1 relationship between hypertensive and normotensive subjects) showed that a sample size of 495 individuals in each group would be required to replicate these results with a power of 80% and an α of 0.05 when the estimated odds ratio (OR) for the case is set at 1.42; this analysis was made using the online calculator available at the Genetic Power Calculator Web site (http://pngu.mgh.harvard.edu/~purcell/gpc).

All of the continuous variables were expressed as means±SD. Differences in continuous variables between hypertensive and normotensive participants were analyzed using 1-way ANOVA. Differences in categorical variables were analyzed using the Fisher's exact test. The Hardy-Weinberg equilibrium was assessed by χ^2 analysis. The difference in CYP4A11 genotype or allele distribution between hypertensive and normotensive participants was analyzed using χ^2 analysis. Based on the genotype data of the genetic variations, we performed linkage disequilibrium analysis and haplotype-based case-control analysis using the expectation maximization algorithm[23] and the software Haploview 4.1 (Daly Laboratory, Broad Institute). Pairwise linkage disequilibrium analysis was performed for −845A/G, −366C/T, 7119C/T, and 8590T/C. We used |D'| values of >0.5 to assign SNP locations to 1 haplotype block. SNPs with an r^2 value of <0.5 were selected as tagged, which means they were available for the haplotype. In the haplotype-based case-control analysis, haplotypes with a frequency of <0.01 were excluded. The frequency distribution of the haplotypes was calculated by χ^2 analysis.

In addition, logistic regression analysis was performed to assess the contribution of the major risk factors. ORs were calculated as an index of the association of the CYP4A11 genotypes with the prevalence of hypertension. Gene expression and luciferase activity were compared by 2-tailed unpaired t tests. Statistical significance was established at $P<0.05$. Statistical analyses were performed using JMP software for Macintosh 5.1.1 (SAS Institute, Inc).

Results

Polymorphism Identification

We performed sequence analysis of the CYP4A11 gene and promoter region in 32 healthy human samples. Distribution of the SNPs detected in the promoter and exon regions of the CYP4A11 gene are shown in Figure 1. Four polymorphisms in the promoter and exon regions of CYP4A11 were detected, −845A/G, −366C/T, 7119C/T, and 8590T/C, with an allelic frequency of >1%. We chose the promoter polymorphism (−845A/G) for the following experiments and functional analysis, because the frequency of the variant allele of −845A/G was much higher than that of −366C/T (27.6% and 7.0%, respectively); thus, this polymorphism was thought to have the potential to alter the transcription efficiency of the CYP4A11 gene.

Functional Analysis of the −845A/G Polymorphism

To determine the functional consequence of the −845A/G polymorphism in the promoter region, we constructed luciferase reporter vectors driven by the wild-type −845AA promoter (phRL-4A11wt) and mutant −845GG promoter (phRL-4A11mt) and performed luciferase assays. The results demonstrated that promoter activity of the variant −845GG promoter was significantly lower than that of the wild-type −845AA promoter (Figure 2).

Next we used a gel mobility shift assay to assess DNA binding activity on the promoter region containing the polymorphism. Human RPTEC nuclear extracts were incubated with the digoxigenin-labeled wild-type AA genotype or variant GG genotype probes, in the absence or presence of excess of unlabeled probe (Figure 3). Notably, we observed DNA binding activity (shifted band, ≈35 kDa) with the oligonucleotide containing the variant −845GG genotype but not with the wild-type AA genotype.

Figure 1. SNPs in the CYP4A11 gene.

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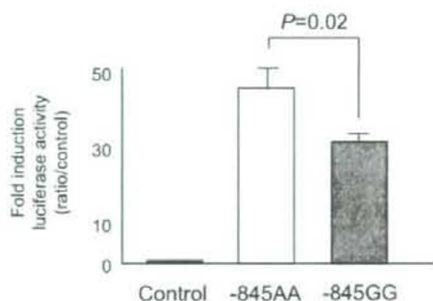

Figure 2. Comparison of the transcriptional activity of CYP4A11 promoter genotypes.

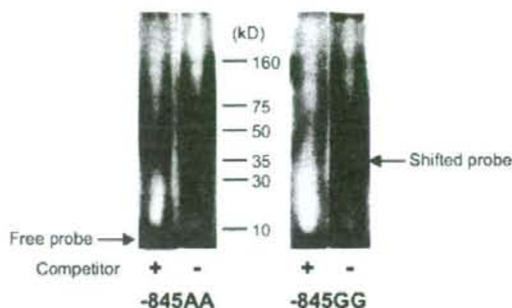

Figure 3. Results of the gel mobility shift assay for the CYP4A11 promoter.

Population Study

Table 2 shows the baseline characteristics of the age- and sex-matched normotensive and hypertensive groups. Those with hypertension had higher values for systolic BP, diastolic BP, triglyceride, and blood sugar levels. We performed genotyping of the 4 SNPs (−845A/G, −=366C/T, 7119C/T, and 8590T/C) in the Tanno-Sobetsu Study population, and the resulting genotype frequencies were not significantly different from the predicted Hardy-Weinberg expectation (data not shown).

To determine whether an association exists between the variants and hypertension, we performed Fisher's exact tests for all 4 of the SNPs. Table 3 shows the results of the association assessments between each polymorphism and prevalence of hypertension. We identified a significant correlation between the −845A/G and 7119C/T polymorphisms and hypertension. The OR for hypertension of the AG+GG genotype of −845A/G was 1.42 after adjustment for confounding factors (serum triglyceride level, fasting blood sugar level, and body mass index; 95% CI: 1.10 to 1.85; P=0.008). The OR for hypertension of the TT genotype of 7119C/T was 1.37 after adjusting for triglyceride level, fasting blood sugar level, and body mass index (95% CI: 1.02 to 1.85; P=0.037; Table 3). The −366C/T and 8590T/C variants showed no significant association with hypertension.

Table 4 shows the linkage disequilibrium patterns for the CYP4A11 gene with $|D'|$ and r^2 values. All 4 of the SNPs were located in 1 haplotype block, because all $|D'|$ were >0.5. In the haplotype-based case-control study, we constructed haplotypes using these 4 SNPs, because all of the r^2 values were <0.5. In the haplotype-based case-control study, 4 haplotypes were established in the total group (Table 5). The frequency of the G-C-T-T form was significantly higher in the hypertensive group than in the normotensive group (P=0.0093), and the A-C-C-T haplotype frequency was significantly lower in the hypertensive group than in the normotensive group (P=0.0133). However, only the G-C-T-T haplotype was significantly associated with hypertension after adjusting for triglyceride level, fasting blood sugar level, and body mass index (OR: 1.44; 95% CI: 1.11 to 1.87; P=0.006).

Discussion

Recent studies have attempted to clarify human CYP4A gene (for human, *CYP4A11*) function as a 20-HETE synthase by pursuing the potential association between *CYP4A11* and hypertension. *CYP4A11* is known to be highly polymorphic, expanding the possibilities for studying the mechanism of this gene, especially as a risk factor for cardiovascular diseases.[24] A product of *CYP4A*, 20-HETE, has been demonstrated to function in both prohypertensive and antihypertensive mechanisms. In the renal arterioles, 20-HETE may act as a strong vasoconstrictor, whereas in renal tubules, 20-HETE may attenuate sodium transport and function as a natriuretic, antihypertensive substance. Ito et al[16] reported recently that *CYP4A11* is highly expressed in human renal tubules and regulates 20-HETE, which is associated with the function of renal tubules and salt-sensitive hypertension.

We directly sequenced human DNA samples and identified 4 SNPs and 1 microsatellite polymorphism. In addition to the previously reported SNPs, this study identified some other specific polymorphisms in the Japanese population. Gainer et al[19] reported that the 8590T/C polymorphism of *CYP4A11* was functional, affecting the catalytic activity of the 20-HETE synthase. In that report, 8590C was associated with the prevalence of hypertension in 2 independent cohort studies. Gainer et al[19] were likely the first to demonstrate a positive association between 8590T/C and hypertension in whites as a loss-of-function variant; the −845A/G polymorphism in the promoter region was also identified, although they did not report investigating it further.

In the current study, we selected −845A/G as a representative of the SNPs, because this polymorphism is in the promoter region and occurred at a higher allele frequency, and we focused on its functional analysis. The first finding of this study is that the −845GG genotype dramatically decreased promoter activity compared with the −845AA genotype as assessed by luciferase experiments. The second finding was that the −845GG genotype mutation of this region stimulated DNA binding in a gel mobility shift assay by an unidentified protein and potential transcription factor. When this result is coupled with the results from the luciferase assay, the implication is that the −845GG binding factor could function as a transcription repressor; thus, the −845G allele may decrease transcriptional activation of *CYP4A11*.

Accordingly, we used the TFMATRIX Web site (http://www.genome.jp/dbget) to determine whether the sequence including −845A/G could change the suspected transcription

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Table 3. Genotype and Allele Distributions for Normotensive and Hypertensive Participants

			Unadjusted				Adjusted*	
SNP	Genotype		Hypertensives (n=495)	Normotensives (n=494)	OR (95% CI)	*P*	OR (95% CI)	*P*
−845A/G		AA	256	298				
rs9332978		AG	205	170		0.023†		
		GG	34	26				
	Dominant model	AA	256	298				
		AG+GG	239	196	1.42 (1.10 to 1.83)	0.006†	1.42 (1.10 to 1.85)	0.008†
	Recessive model	GG	34	26				
		AG+AA	461	468	1.33 (0.78 to 2.25)	ns	1.42 (0.83 to 2.46)	ns
	Allele	A	717	766				
		G	273	222	1.31 (1.07 to 1.61)	0.009†	1.78 (1.16 to 2.73)	0.008†
−366C/T	Genotype	CC	426	424				
no rs#		CT	68	69		ns		
		TT	1	1				
	Dominant model	CC	426	424				
		CT+TT	69	70	0.98 (0.69 to 1.40)	ns	0.96 (0.66 to 1.39)	ns
	Recessive model	TT	1	1				
		CT+CC	494	493	1.00 (0.06 to 16.00)	ns	1.24 (0.05 to 32.10)	ns
	Allele	C	920	917				
		T	70	71	0.98 (0.70 to 1.38)	ns	0.93 (0.45 to 1.91)	ns
7119C/T(His323His)	Genotype	CC	107	134				
rs28451040		CT	247	248		0.042†		
		TT	141	112				
	Dominant model	CC	107	134				
		CT+TT	388	360	1.35 (1.01 to 1.81)	0.044†	1.33 (0.98 to 1.80)	ns
	Recessive model	TT	141	112				
		CT+CC	354	382	1.36 (1.02 to 1.81)	0.036†	1.37 (1.02 to 1.85)	0.037†
	Allele	C	461	516				
		T	529	472	1.25 (1.05 to 1.50)	0.012†	1.57 (1.09 to 2.28)	0.016†
8590T/C(Phe434Ser)	Genotype	TT	325	326				
rs1126742		TC	157	153		ns		
		CC	13	15				
	Dominant model	TT	325	326				
		TC+CC	170	168	1.02 (0.78 to 1.32)	ns	1.01 (0.77 to 1.33)	ns
	Recessive model	CC	13	15				
		TC+TT	482	479	0.86 (0.41 to 1.83)	ns	0.81 (0.37 to 1.76)	ns
	Allele	T	807	805				
		C	183	183	1.00 (0.79 to 1.25)	ns	0.98 (0.60 to 1.58)	ns

*Data were adjusted for TG, FBS, and BMI. TG indicates triglyceride level; FBS, fasting blood sugar level; BMI, body mass index; ns, not significant.

†The *P* values of genotypes and alleles were calculated using the Fisher's exact test.

factor. The motif analysis extracted 1 transcription factor, cAMP-responsive element binding protein 1, which has 87% homology with the sequence including the −845G allele but 76% homology with the sequence including the −845A allele.

The third important finding of this study was the significant association between CYP4A11 gene polymorphisms and the prevalence of hypertension in a general Japanese population, warranting further investigation of the *CYP4A11* gene. The −845G allele and the 7119T allele were significantly associated with hypertension even after adjustment for confounding factors, as shown in Table 3. The haplotype analysis also showed that both alleles were associated with hypertension; however, after adjustment for confounding factors, only the G-C-T-T haplotype was significantly associated with hypertension, as shown in Table 5. In addition, both polymorphisms are in the same haplotype block, and 7119C/T is a nonsynonymous polymorphism. Thus, −845A/G might be considered the susceptible polymorphism for essential hypertension in the Japanese population.

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Table 4. Pairwise Linkage Disequilibrium for the 4 SNPs

		|D'|			
		−845A/G	−366C/T	7119C/T	8590T/C
r^2	−845A/G		1.0	0.989	0.975
	−366C/T	0.026		0.976	1.0
	7119C/T	0.319	0.071		0.966
	8590T/C	0.072	0.017	0.207	

$|D'|>0.5$, $r^2>0.5$.

In terms of the 8590T/C polymorphism, which has been reported as a susceptible polymorphism for hypertension,[19] the role of this SNP has been completely opposite in 2 different populations: the variant C allele is associated with hypertension in whites and some blacks; in contrast, the wild-type T allele associates with hypertension in a Japanese population from Tokyo. However, in the current study, 8590T/C is not associated with hypertension even by haplotype analysis in the Tanno-Sobetsu population. One of the reasons for this discrepancy is that the 8590T allele frequency is lower; in contrast, the −845G allele frequency is higher in Asians, including Japanese, than in whites. In the Tanno-Sobetsu population, which is characterized by a higher rate of high-salt intake (the average salt intake is ≈12.6±3.6 g/d), the −845G allele might be much more associated with hypertension. In addition, the A-C-C-T and A-T-T-T haplotypes were not associated either with normotension or hypertension, and the G-C-T-T was associated with hypertension. Thus, we can conclude that there is no role for the 8590T/C SNP in hypertension in this Japanese population.

Given previous results and the results of this study, we propose the following hypothesis for the mechanism by which the −845GG genotype of *CYP4A11* is involved in the etiology of hypertension. The −845G allele could result in decreased *CYP4A11* expression in renal tubules compared with the wild-type −845A allele, leading to attenuated 20-HETE production. The reduced levels of 20-HETE could promote sodium reabsorption, resulting in elevated BP.

The recent report on genome-wide associations did not reveal significant associations of essential hypertension with any single marker.[18] However, a positive association between *CYPA411* polymorphisms and hypertension or cardiovascular diseases has been reported, and this gene has been recognized as one of the susceptible genes of hypertension on the National Center for Biotechnology Information Web site (http://www.ncbi.nlm.nih.gov). More studies are required to elucidate further the involvement of this gene in the development of hypertension.

There are 3 limitations to address in the context of the current results. First, the effect on arachidonic acid metabolism or 20-HETE production was not well investigated. In fact, we measured urine 20-HETE levels in a healthy Japanese population, and there was no difference in 20-HETE excretion among the 3 genotypes of the −845A/G polymorphism (AA, AG, and GG), although 20-HETE excretion among those with the GG genotype had a tendency to be higher than that of the other genotypes (data not shown). Thus, some further experiments are needed to clarify the effect of this polymorphism on arachidonic acid metabolism or 20-HETE production. Second, in the current study, we investigated the positive association between −845A/G and hypertension only in 1 general Japanese population. These findings should be confirmed in other populations, and we plan further investigations in the near future. Finally, we expect to try to identify the transcription factor detected as the shifted band in the gel mobility shift assay in this study. Identifying this protein could be useful for analyzing the functional mechanism of this polymorphism in the development of hypertension.

In conclusion, this study describes a functional variant in the *CYP4A11*, the −845A/G polymorphism, as a new candidate polymorphism for genetic susceptibility to hypertension. Our study suggests that the −845A/G polymorphism of *CYP4A11* is a novel candidate for a predisposing genetic factor for hypertension via modulation of the arachidonate cascade.

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Table 5. Association Between the Haplotypes of CYP4A11 and Hypertension

	CYP4A11 Polymorphisms				Frequency		Unadjusted		Adjusted*	
Haplotypes	−845A/G	366C/T	7119C/T	8590T/C	Hypertensives	Normotensives	OR (95% CI)	P	OR (95% CI)	P
1	Mj	Mj	Mn	Mj						
	A	C	C	T	0.459	0.515	0.80 (0.67 to 0.96)	0.013†	0.74 (0.55 to 1.00)	0.051
2	Mn	Mj	Mj	Mj						
	G	C	T	T	0.274	0.223	1.31 (1.07 to 1.61)	0.009†	1.44 (1.11 to 1.87)	0.006†
3	Mj	Mj	Mj	Mn						
	A	C	T	C	0.183	0.177	1.04 (0.82 to 1.30)	ns	1.05 (0.80 to 1.38)	ns
4	Mj	Mn	Mj	Mj						
	A	T	T	T	0.070	0.072	0.97 (0.69 to 1.37)	ns	0.93 (0.64 to 1.35)	ns

Mj indicates major allele; Mn, minor allele; TG, triglyceride level; FBS, fasting blood sugar level; BMI, body mass index; ns, not significant. Haplotypes with frequency >0.01 were established using Haploview 4.1 software.

*Data were adjusted for TG, FBS, and BMI.

†The P values were calculated using χ^2 analysis.

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Disclosures

None.

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Original Article

Leptin-Receptor Polymorphisms Relate to Obesity through Blunted Leptin-Mediated Sympathetic Nerve Activation in a Caucasian Male Population

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Leptin plays a key role in the regulation of body weight through the sympathetic nervous system; however, the contributions of leptin-receptor polymorphisms to obesity and sympathetic nerve activity have not been fully clarified. In the present study, we examined the relationships between leptin-receptor polymorphisms, plasma leptin and whole-body norepinephrine (NE) spillover as an index of sympathetic nerve activity in a Caucasian male cohort. In 129 young healthy normotensive men with a wide range of body mass index (BMI) (19.4–39.5 kg/m²), we measured leptin-receptor polymorphisms (Gln223Arg, Lys656Asn, and Lys109Arg), plasma leptin levels, whole-body NE spillover, whole-body NE clearance, BMI and blood pressure (BP) levels in the supine position after overnight fasting. Overweight-obese (BMI≥25 kg/m²) subjects had significantly greater BMI, BP levels, plasma leptin and whole-body NE spillover compared to lean (BMI<25 kg/m²) subjects, but the NE clearance was similar. Overweight-obese subjects had significantly higher frequencies of the Arg223 allele and the Arg223 homozygous allele of Gln223Arg and the Asn656 allele of Lys656Asn compared to lean subjects. Subjects carrying the Arg223 homozygous or the Asn656 allele had higher levels of plasma leptin, BMI, waist circumference, and waist-to-hip ratio, but significantly less whole-body NE spillover, especially when they were also overweight-obese. BP levels and whole-body NE clearance were similar between subjects with and without the Arg223 homozygous or Asn656 allele. No differences were found in the distributions of the Arg109 allele of Lys109Arg polymorphism between nonobese and overweight-obese subjects. In addition, BMI, BP, plasma leptin levels, whole-body NE spillover and whole-body NE clearance were similar between those with and without the Arg109 allele. Together, these findings demonstrate that leptin-receptor polymorphisms were related to the incidence of obesity in a Caucasian male population. These polymorphisms were accompanied by high plasma leptin levels (leptin resistance) and lower whole-body plasma NE spillover (blunted sympathetic nerve activity). We therefore hypothesize that leptin-receptor play a role in the development of obesity through leptin resistance and blunted leptin-mediated sympathetic nerve activity. (*Hypertens Res* 2008; 31: 1093–1100)

Key Words: **leptin-receptor polymorphisms, plasma leptin levels, sympathetic nerve activity, obesity, hypertension**

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Introduction

Obesity is a growing health problem associated with a high risk of cardiac events. Obese humans have increased circulating plasma levels of the adipocyte hormone leptin (*1*), which is associated with heightened sympathetic nerve activity (*2*). Leptin increases thermogenesis and energy expenditure through stimulation of the sympathetic nervous system (*3, 4*). Leptin also increases norepinephrine (NE) turnover in brown adipose tissue (*5*) and white adipose tissue (*6*), mainly by enhancing the sympathetic nerve activity in these tissues. Thus, leptin plays a key role in the regulation of body weight through the sympathetic nervous system. The diminished effect of leptin on satiety is manifested as leptin resistance (*7, 8*). Leptin resistance might maintain obesity despite elevated leptin concentrations (*5, 7–9*).

Catecholamines are the major lipolytic hormones in human fat cells, and lipolytic catecholamine resistance has been observed in obesity. Studies on twins and in rare genetic disorders suggest a strong heredity component of catecholamine-induced lipolysis and resultant obesity (*10*). We have reported that obese men, especially those carrying the Gly16 allele of β2-adrenoceptor polymorphisms accompanied with heightened sympathetic nerve activity, show blunted leptin stimulation on sympathetic nerve activity (plasma NE) compared to lean subjects (*9*). Thus, one could speculate that blunted sympathetic nerve activity–induced lipolysis or blunted leptin-mediated sympathetic nerve activation determined by genes might contribute to the obesity associated with high plasma leptin levels (leptin resistance).

Several clinical and epidemiological studies have demonstrated leptin receptor polymorphisms associated with obesity (*3, 11–14*). However, there have been few investigations on the concurrent relationships between leptin-receptor polymorphisms, plasma leptin levels and sympathetic nerve activity (*15*). In the present study, three leptin-receptor polymorphisms (Lys109Arg in exon 4, Gln223Arg in exon 6, and Lys656Asn in exon 14) from buffy coats (white blood cells) that may contribute to obesity (*11–14*) were studied in a Caucasian male population with a wide range of body mass indices (BMI). Further, we examined the relationships between the polymorphisms, plasma leptin levels, and whole-body plasma NE spillover as an index of sympathetic nerve activity. To our knowledge, this is the first study to examine the relationships between leptin-receptor polymorphisms, obesity (BMI), plasma leptin levels and sympathetic nerve activity concurrently to evaluate the relationships between leptin-receptor polymorphisms, the leptin–sympathetic nerve activity axis, and obesity.

Table 1. Characteristics in Lean Subjects (BMI<25.0 kg/m^2) *vs.* Overweight or Obese Subjects (BMI≥25.0 kg/m^2)

Variables	Lean subjects	Overweight+ obese subjects
Subjects, *n*	40	89
Age, years	44.9±10.2	47.8±10.8
BMI, kg/m^2	22.0±1.9	30.3±3.5†
Systolic BP, mmHg	123±14	128±14
Diastolic BP, mmHg	74±11	76±9
Mean BP, mmHg	90±14	94±11*
Heart rates, bpm	61±13	62±11
Waist, cm	91±6	113±8†
Waist-to-hip ratio	0.81±0.04	0.91±0.06†
Whole-body NE spillover, ng/min	431.6±125.7	561.2±186.8*
NE clearance, L/min	1.87±0.38	2.01±0.76
Leptin, ng/mL	4.48±3.01	10.45±5.18†

BMI, body mass index; BP, blood pressure; NE, norepinephrine. *$p<0.05$, †$p<0.01$ *vs.* lean subjects.

Methods

Subjects

One-hundred and twenty-nine non-smoking Caucasian men aged 23–59 years, with a BMI ranging from 19.4–39.5 kg/m^2 were recruited through newspaper advertisements. They consisted of 40 lean subjects (BMI<25 kg/m^2), 45 overweight subjects (25≤BMI<30 kg/m^2) and 44 obese subjects (BMI≥30 kg/m^2) according to the overweight and obesity definitions by the Centers for Disease Control and Prevention (Atlanta, USA) (*16*). Subjects were excluded who had diabetes mellitus (fasting glucose level>6.0 mmol/L), hypertension (≥140/90 mmHg), secondary hypertension, obstructive sleep apnea, cardiovascular or cerebrovascular disease, renal dysfunction or liver dysfunction. None of the subjects were taking medication for diabetes, hypertension, hyperlipidemia or other illnesses. The goal of the present study was to clarify the relationships among the genetic variance of the leptin receptor polymorphisms, plasma leptin levels, sympathetic nerve activity (whole-body NE spillover) and obesity (BMI). Thus, to minimize the influence of weight changes on sympathetic nerve activity and leptin levels (*9, 17–19*), all subjects had been weight stable for the preceding 6 months. The study was approved by the Alfred Hospital Human Research Ethics Committee. All participants provided written informed consent.

Measurements

Investigations were performed in a quiet, temperature-controlled room at the same time of the day (from 9:00 AM). After an overnight fast of >12 h, BMI, blood pressure (BP),

Table 2. Comparisons of the Frequencies of Genotype of the Leptin-Receptor Polymorphisms between Lean Subjects (BMI < 25.0 kg/m²) and Overweight or Obese Subjects (BMI ≥ 25.0 kg/m²)

	Genotype	Lean subjects (*n* (%))	Overweight or obese subjects (*n* (%))	χ^2 test	
				For genotype	For allele
Lys109Arg	Arg/Arg	2 (5.0)	1 (1.1)	$\chi^2=5.31, p=0.379$	$\chi^2=2.92, p=0.405$
	Arg/Lys	12 (30.0)	26 (29.2)		
	Lys/Lys	26 (65.0)	62 (69.7)		
Gln223Arg	Gln/Gln	18 (45.0)	25 (28.1)	$\chi^2=10.52, p=0.062$	$\chi^2=10.96, p=0.012$
	Gln/Arg	17 (42.5)	38 (42.7)	(*vs.* Arg223 homozygous, $\chi^2=11.84, p=0.008$)	
	Arg/Arg	5 (12.5)	26 (29.2)		
Lys656Asn	Lys/Lys	21 (52.5)	32 (36.0)	$\chi^2=8.11, p=0.1505$	$\chi^2=8.303, p=0.040$
	Lys/Asn	15 (37.5)	39 (43.8)		
	Asn/Asn	4 (10.0)	18 (20.2)		

BMI, body mass index.

heart rate, and venous sampling for the extraction of genomic DNA from leukocytes were taken after 30 min rest in the supine position. Subjects abstained from caffeine for 12 h, alcohol for 24 h, and exercise for 36 h. BP and heart rate were measured ≥3 times in the supine position using an automated sphygmomanometer (DINAMAP 1846-SX Blood Pressure Monitor; Critikon Corp., Tampa, USA) with a cuff size that was adjusted for arm circumference, and were averaged. The accuracy of the automated sphygmomanometer was tested against a mercury sphygmomanometer before use.

Laboratory Determinations

Whole-body sympathetic nerve activity was assessed by measurement of the apparent rate of appearance of endogenous NE in plasma (NE spillover rate) using the isotope dilution technique (*20, 21*). The plasma concentrations of neurochemicals were determined by HPLC with electrochemical detection. The intra-assay coefficients of variations in our laboratory were 3% for NE and 7% for [^{3}H]NE; the inter-assay coefficient of variations were 11% and 6%, respectively. Plasma leptin levels were determined by RIA (Linco Research, Inc., St. Charles, USA).

Genotyping

Three single-nucleotide polymorphisms (SNPs) (rs1137100, rs1137101, rs8179183) were genotyped by pre-developed TaqMan allelic discrimination assays (Applied Biosystems, Foster City, USA). The PCR was carried out in a total reaction volume of 5 µL using the following amplification protocol: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 60 s. Allelic discrimination was measured automatically on an ABI Prism HT7900 (Applied Biosystems) using the Sequence Detection System Version 2.1 software.

Statistical Analysis

The genotype distribution of the polymorphisms was tested for Hardy-Weinberg equilibrium by χ^2 analysis. Values are shown as the mean±SD. Differences among groups were examined by an unpaired *t*-test. All data analyses were performed with the program SPSS 8.0 for Windows (SPSS Inc., Chicago, USA). Values of $p<0.05$ were considered significant.

Results

Characteristics of Lean Subjects (BMI < 25.0 kg/m²) *vs.* Overweight or Obese Subjects (BMI ≥ 25.0 kg/m²)

The overweight or obese group had significantly greater BMI, BP levels, waist circumference, waist-to-hip ratio, whole-body NE spillover and plasma leptin compared to the lean group. The NE clearance rate, however, was similar between the two groups (Table 1).

Comparisons of the Frequencies of the Leptin-Receptor Polymorphisms (Lys109Arg, Gln223Arg, and Lys656Asn) between Lean Subjects (BMI < 25.0 kg/m²) *vs.* Overweight or Obese Subjects (BMI ≥ 25.0 kg/m²)

Overweight or obese subjects had significantly higher frequencies of the Arg223 homozygous allele of the Gln223Arg polymorphism and the Asn656 allele of the Lys656Asn polymorphism. However, the allele distributions of Lys109Arg were similar between lean and overweight or obese subjects (Table 2).

The Hardy-Weinberg equilibriums were $p=0.6398$ for Lys109Arg, $p=0.1122$ for Gln223Arg, and $p=0.2055$ for Lys656Asn.

Table 3. Characteristics of the Subjects Carrying the Arg223 Homozygous and Carrying the Gln223 Allele of the Gln223Arg Polymorphism

Variables	Gln223/Gln223	Gln223/Arg223	Arg223/Arg223	With Gln223 allele (without Arg223 homozygous)	With Arg223 allele
Subjects, *n*	43	55	31	98	86
Age, years	46.5±8.2	48.3±11.1	44.7±10.2	47.5±9.8	47.0±10.4
BMI, kg/m²	24.8±5.7*	27.6±4.8	31.9±8.9‡	26.4±7.4*	29.2±6.9‡
Systolic BP, mmHg	128±14	125±13	126±15	126±14	126±14
Diastolic BP, mmHg	76±11	75±9	76±8	75±10	76±9
Mean BP, mmHg	90±11	91±12	94±10	91±13	92±12
Heart rates, bpm	63±15	61±11	60±10	62±13	61±11
Waist, cm	93±13†	110±9‡	119±10§	103±10*	113±10§
Waist-to-hip ratio	0.85±0.06*	0.87±0.05	0.92±0.06‡	0.86±0.07*	0.89±0.07
Whole-body NE spillover, ng/min	568.7±123.5*	523.1±131.2	451.3±105.3‡	543.1±116.1*	497.3±124.3
NE clearance, L/min	2.26±0.90	1.90±0.55	1.69±0.58	2.06±0.72	1.82±0.51
Leptin, ng/mL	5.32±2.63†	7.74±3.73	14.68±3.70§	6.68±4.79†	10.21±4.81‡

BMI, body mass index; BP, blood pressure; NE, norepinephrine. *p<0.05, †p<0.01 *vs.* Arg223/Arg223; ‡p<0.05, §p<0.01 *vs.* Gln223/Gln223.

Table 4. Characteristics of the Subjects with and without the Gln223 Allele of Gln223Arg Polymorphism in Lean Subjects (BMI<25.0 kg/m²) *vs.* Overweight or Obese Subjects (BMI≥25.0 kg/m²)

Variables	Lean subjects		Overweight+obese subjects	
	With Gln223 allele	With Arg223 homozygous	With Gln223 allele	With Arg223 homozygous
Subjects, *n*	35	5	63	26
Age, years	44.4±8.7	48.4±7.0	49.2±8.9	44.0±10.1
BMI, kg/m²	21.9±2.0	22.7±0.9	28.9±2.5†	33.7±3.3†‡
Systolic BP, mmHg	123±13	123±14	129±12*	127±11
Diastolic BP, mmHg	74±9	74±7	76±9	77±10
Mean BP, mmHg	90±12	90±11	94±10*	94±9
Heart rates, bpm	61±11	61±9	62±11	60±10
Waist, cm	90±8	92±9	110±9†	123±7†‡
Waist-to-hip ratio	0.80±0.05	0.87±0.05‡	0.88±0.09†	0.95±0.08†‡
Whole-body NE spillover, ng/min	457.6±102.3	249.6±101.6‡	590.6±152.0*	490.1±100.7*‡
NE clearance, L/min	1.79±0.33	2.43±0.60	2.21±0.91	1.55±0.81
Leptin, ng/mL	4.02±3.25	7.70±3.45	8.15±3.77†	16.01±6.67†§

BMI, body mass index; BP, blood pressure; NE, norepinephrine. *p<0.05, †p<0.01 *vs.* lean subjects; ‡p<0.05, §p<0.01 *vs.* subjects with Gln223 allele.

The findings that overweight or obese subjects had significantly higher frequencies of the Arg223 allele (Arg223 homozygous) of Gln223Arg and the Asn656 allele of Lys656Asn, but not the Arg109 allele of Lys109Arg, indicates that the Arg223 allele of Gln223Arg and Asn656 allele of Lys656Asn (but not Lys109Arg) are closely linked to overweight-obesity. Thus, we compared the parameters between subjects with and without the Arg223 allele (especially Arg223 homozygous) of Gln223Arg or the Asn656 allele of Lys656Asn.

Characteristics of the Subjects Carrying the Arg223 Allele of Gln223Arg Polymorphism

Subjects carrying the Arg223 allele had significantly greater BMI, waist circumference and plasma leptin levels, and slightly lower whole-body NE spillover compared to those carrying the Gln223 homozygous allele (those without the Arg223 allele) (Table 3). Subjects carrying the Arg223 homozygous allele of Gln223Arg had significantly greater BMI, waist circumference, waist-to-hip ratio, and plasma leptin levels compared to subjects carrying the Gln223 allele

Table 5. Characteristics of the Subjects Carrying the Asn656 Allele of the Lys656Asn Polymorphism

Variables	Lys656/Lys656	Lys656/Asn656	Asn656/Asn656	With Asn656 allele
Subjects, *n*	53	54	22	76
Age, years	47.2±10.1	46.7±9.8	45.3±9.1	46.1±9.6
BMI, kg/m^2	25.8±4.9‡	28.4±2.4	30.6±3.7*	29.5±4.9*
Systolic BP, mmHg	127±11	127±12	123±15	125±14
Diastolic BP, mmHg	75±11	75±5	80±8	77±7
Mean BP, mmHg	90±14	92±7	94±10	90±13
Heart rates, bpm	61±10	63±14	60±10	62±15
Waist, cm	95±9‡	112±9*	121±14*	115±12†
Waist-to-hip ratio	0.83±0.11‡	0.88±0.09	0.97±0.10*	0.91±0.08*
Whole-body NE spillover, ng/min	561.1±194.4‡	503.4±111.3‡	457.8±103.0*	487.9±111.7*
NE clearance, L/min	2.00±0.72	1.93±0.54	1.99±0.27	1.95±0.39
Leptin, ng/mL	6.97±4.33‡	9.28±3.02	10.09±4.61*	9.74±2.61*

BMI, body mass index; BP, blood pressure; NE, norepinephrine. $^{*}p<0.05$, $^{\dagger}p<0.01$ *vs.* Lys656/Lys656; $^{\ddagger}p<0.05$ *vs.* Asn656/Asn656.

Table 6. Characteristics of the Subjects with and without the Asn656 Allele of Lys656Asn Polymorphism in Lean Subjects (BMI<25.0 kg/m^2) *vs.* Overweight or Obese Subjects (BMI≥25.0 kg/m^2)

Variables	Lean subjects		Overweight+obese subjects	
	Without Asn656 allele	With Asn656 allele	Without Asn656 allele	With Asn656 allele
Subjects, *n*	21	19	32	57
Age, years	46.1±9.0	48.5±5.0	47.9±9.0	47.6±8.7
BMI, kg/m^2	21.7±2.1	22.3±0.8	28.5±6.1†	31.3±3.5†‡
Systolic BP, mmHg	123±15	123±7	130±9*	127±10*
Diastolic BP, mmHg	73±9	75±7	76±9	77±10
Mean BP, mmHg	89±13	91±9	94±9*	96±10
Heart rates, bpm	61±10	61±9	61±12	62±10
Waist, cm	89±10	99±5‡	101±9*	122±7†§
Waist-to-hip ratio	0.81±0.06	0.83±0.04	0.88±0.09*	0.94±0.07†
Whole-body NE spillover, ng/min	445.0±115.6	416.8±125.7	628.5±159.9†	518.5±137.5*‡
NE clearance, L/min	1.83±0.31	1.91±0.57	2.11±0.88	1.96±0.91
Leptin, ng/mL	4.10±2.78	4.92±2.22	8.85±3.95†	11.35±6.01†‡

BMI, body mass index; BP, blood pressure; NE, norepinephrine. $^{*}p<0.05$, $^{\dagger}p<0.01$ *vs.* lean subjects; $^{\ddagger}p<0.05$, $^{\S}p<0.01$ *vs.* subjects without Asn656 allele.

(those without the Arg223 homozygous allele), but whole-body NE spillover was significantly less in subjects carrying the Arg223 homozygous allele. Subjects carrying the Arg223 homozygous allele had similar whole-body NE clearance rates, BP levels and heart rates to those carrying the Gln223 allele (Table 3).

Subjects carrying the Arg223 homozygous allele of Gln223Arg, especially within the overweight or obese group, had significantly greater BMI, waist circumference, waist-to-hip ratio, and plasma leptin levels compared to subjects carrying the Gln223 allele. Whole-body NE spillover was significantly lower in subjects carrying the Arg223 homozygous allele, either within the lean or the overweight or obese group, compared to those with the Gln223 allele (Table 4).

Characteristics of the Subjects Carrying the Asn656 Allele of the Lys656Asn Polymorphism

BMI, waist circumference and plasma leptin levels were significantly greater in subjects carrying the Asn656 allele of Lys656Asn, but whole-body NE spillover rates were significantly lower. NE clearance rates, BP levels and heart rates were similar between subjects with and without the Asn656 allele (Table 5).

In both the lean and overweight or obese subjects carrying the Asn656 allele, BMI, waist circumference and plasma leptin levels were greater than in those without the Asn656 allele, but whole-body NE spillover was significantly lower in overweight or obese subjects carrying the Asn656 allele (Table 6).

Discussion

The main findings in the present study were that overweight or obese subjects had significantly higher frequencies of the Arg223 homozygous allele of the Gln223Arg leptin-receptor polymorphism and the Asn656 allele of the Lys656Asn leptin-receptor polymorphism, although the number of subjects studied was relatively small (n=129). Further, those subjects carrying the leptin-receptor polymorphisms had significantly higher levels of plasma leptin, BMI, and waist circumference (central obesity), but significantly lower sympathetic nerve activity shown as significantly lower whole-body NE spillover. It should be noted that this is the first study to examine the relationships between leptin-receptor polymorphisms, obesity (BMI), plasma leptin levels and sympathetic nerve activity concurrently to evaluate the relationships between leptin-receptor polymorphisms and the leptin-sympathetic nerve activity axis in obesity.

Leptin was initially touted as a major suppressor of appetite based on the finding that total deficiency of leptin or its receptor leads to hyperphagia and obesity in mice and humans (*22, 23*). Leptin links adipose tissue mass to food intake and energy expenditure through a negative feedback loop by binding and activating leptin-specific receptors in the hypothalamus (*24–26*). Further, leptin receptors are expressed in the nervous system and peripheral tissue, including adipose tissue, skeletal muscle, pancreatic β-cells, and the liver (*22, 23, 25*). Exogenous administration of leptin has been reported to decrease body weight, body fat, and food intake, and to increase energy expenditure accompanied with sympathetic nervous activation in animals and humans (*27–31*). However, as body fat determines the circulating leptin concentrations, obese individuals generally have increased levels of leptin (*32*), prompting the hypothesis that "leptin resistance" plays an important role in the development of obesity in humans (*7, 8*). Importantly, it has been suggested that leptin responsiveness to energy restriction is affected by the functionality of the leptin receptor.

Several investigators have demonstrated that leptin-receptor polymorphisms predict obesity (*3, 14, 33*) and weight change (*12, 34*), and that leptin-receptor polymorphisms might play a role in the acute leptin reduction after energy restriction (*35*). The contributions of the leptin-receptor polymorphisms Lys109Arg, Gln223Arg, and Lys656Asn on obesity were analyzed (*36*). On the other hand, other investigators have observed no linkages between leptin-receptor polymorphisms and obesity in Pima Indians (*37*) and in French subjects (*38*). The contributions of leptin-receptor polymorphisms to obesity are thus controversial. A Caucasian population was reported to have lower frequencies of the Arg223 and Arg109 alleles and higher frequency of the Asn656 allele compared to an Asian population (*36*), demonstrating that the allele distributions of Lys109Arg, Gln223Arg, and Lys656Asn are different among ethnic groups. Thus, it is possible that these conflicting results may be due in part to differences in the ethnicity (*14*), gender (*14, 39*), severity of obesity, and dietary status (*35*) of the studied populations. Further, a weak association might have been dismissed due to the relatively limited numbers of subjects studied (*36*). In the present study we evaluated the relationships between three leptin-receptor polymorphisms (Lys109Arg, Gln223Arg, and Lys656Asn) and obesity (BMI) in 129 middle-aged Caucasian men with a wide range of BMI. Interestingly, our findings are in good accordance with the HERITAGE Family Study, which has shown that the Arg223 allele of Gln223Arg is associated with heavier BMI, fat mass, percent fat mass, and leptin levels only in the middle-aged male Caucasian population, but not in blacks or female Caucasian populations (*14*).

Obesity is considered to result from reduced energy expenditure, which results in a reduction of resting metabolic rate, and a negative energy balance (thermogenesis) through diminished sympathetic nerve activity. Sympathetic nerve activity plays an important role in stimulating lipolysis by catecholamines in white adipose tissue in humans and in the development of obesity. However, many clinical and epidemiological studies have shown heightened sympathetic nerve activity in obesity as well as higher levels of plasma leptin (*2, 9, 17–19*). In the present study, sympathetic nerve activity in subjects without the Arg223 homozygous allele and Asn656 allele was significantly higher than in those carrying the Arg223 homozygous allele or Asn656 allele, although those without the Arg223 homozygous allele or Asn656 allele had lower levels of plasma leptin, BMI and waist circumference. These results demonstrate that subjects carrying the Arg223 homozygous or Asn656 allele, which are closely linked to obesity, have relatively blunted leptin-mediated sympathetic nerve activity (observed as lower whole-body NE spillover) leading to lower thermogenesis, which may result in obesity (central obesity). Blunted leptin-mediated sympathetic nerve activity might play an important role in obesity, as we have previously reported (*9, 40*). However, we could not discuss the relationship of leptin-receptor polymorphisms in thermogenesis or energy expenditure because we did not measure resting metabolic rates or energy expenditure in the present study. One could speculate that subjects carrying leptin-receptor polymorphisms might be prone to obesity because of reductions in energy expenditure preceded by low sympathetic nerve activity (*12*).

Leptin resistance, which is defined as an impairment in the action of leptin accompanied by hyperleptinemia, is a common feature of obesity in animal models and in humans (*7, 41, 42*). Therefore, hyperleptinemia has been used as a surrogate index for leptin resistance to the adipose tissue mass (*42*). Experimental results show that chronic systemic and intracerebral administration of leptin increases BP levels or heart rates in animal models (*4, 41*). Leptin produces sympathetic nerve activation in the kidneys, adrenal glands and brown adipose tissue, indicating that the obesity-associated

increases in sympathetic nerve activity could be due in part to these various sympathetic effects of leptin (*4, 5, 8*). In other words, leptin resistance selectively affects the metabolic actions of leptin, but spares leptin-mediated sympathetic activation. In the present study, the findings that obese subjects, irrespective of whether or not they had the leptin-receptor polymorphisms, had higher whole-body NE spillover and BP levels might be linked to "selective leptin resistance" (*7, 8*). On the other hand, the subjects carrying the Arg223 homozygous or Asn656 allele had higher leptin levels, BMI and waist circumference compared to those without these polymorphisms, but BP levels and heart rates were similar. The absence of any linkage between leptin-receptor polymorphisms, plasma leptin levels, and BP levels might have been caused by the relatively lower sympathetic nerve activity associated with these polymorphisms, because higher BP levels associated with high sympathetic nerve activity were observed in overweight or obese subjects compared to lean subjects, even in those carrying these polymorphisms. These findings might help to explain why not all obese subjects have hypertension.

The Hardy-Weinberg equilibrium demonstrated the subjects in this study had a normal distribution ($p > 0.05$) of the three polymorphisms studied, but we cannot rule out the possibility of a chance variation due to the small number of subjects ($n = 129$). The findings that Lys656Asn polymorphism was not associated with overweight-obesity or blunted sympathetic nerve activity may have been related to the small size of the cohort. The distribution of the Arg109 homozygous allele was very low ($n = 3$) in this cohort.

In summary, a strong association of leptin-receptor polymorphisms with obesity, but not BP levels, was observed in a middle-aged Caucasian male population. Blunted leptin-mediated sympathetic nerve activity (observed as whole-body NE spillover) related with leptin-receptor polymorphisms may play a major role in the maintenance of obesity through lower thermogenesis. Further, the absence of a link between leptin-receptor polymorphisms and BP levels may have been related with the relatively lower sympathetic nerve activity. Further study will be needed to clarify the differences in the contributions of leptin-receptor polymorphisms to BMI (obesity) and BP levels (hypertension).

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A haplotype of the CYP4A11 gene associated with essential hypertension in Japanese men

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Objective CYP4A11, a member of the cytochrome P450 family, acts mainly as an enzyme that converts arachidonic acid to 20-hydroxyeicosatetraenoic acid, a metabolite involved in blood pressure regulation in humans. Disruption of the murine cyp4a14 and cyp4a10 genes, homologues of human CYP4A11, was reported recently to cause hypertension. The gene-disrupted male mice had higher blood pressure than the gene-disrupted female mice. The present study aimed to assess the association between the human CYP4A11 gene and essential hypertension, using a haplotype-based case–control study including separate analysis of the gender groups.

Methods The 304 essential hypertension patients and 207 age-matched control individuals were genotyped for three single-nucleotide polymorphisms of the human CYP4A11 gene (rs2269231, rs1126742, rs9333025). Data were assessed for three separate groups: total participants, men and women.

Results For total participants, the genotypic distribution of rs1126742 differed significantly between the two groups ($P = 0.005$). For total participants, men and women, the recessive model (CC versus TC + TT) of rs1126742 differed significantly between the two groups ($P = 0.007$, $P = 0.043$, and $P = 0.045$, respectively). Logistic regression analysis showed the TC + TT genotype was significantly higher in essential hypertension patients than in control individuals for total participants and men ($P = 0.022$ and $P = 0.043$, respectively). The A-T-G haplotype frequency (established by rs2269231, rs1126742, rs9333025) was significantly higher in essential hypertension men than in control men ($P = 0.043$).

Conclusions Essential hypertension is associated with the TC + TT genotype of rs1126742 in the human CYP4A11 gene. The A-T-G haplotype appears a useful genetic marker of essential hypertension in Japanese men. *J Hypertens* 26:453–461 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Keywords: androgen, case–control study, CYP4A11, haplotype, single-nucleotide polymorphism

Abbreviations: 20-HETE, 20-hydroxyeicosatetraenoic acid; BMI, body mass index; CYP, cytochrome pigment; DBP, diastolic blood pressue; EH, essential hypertension; EM, expectation maximization; HDL, high density lipoprotein; HL, hyperlipidmia; LD, linkage disequilibrium; MAF, minor allele frequencies; MGB, minor groove-binding group; NCBI, National Center for Biotechnology Information; NT, normotensive; PCR, polymerase chain reaction; rs, Reference SNP ID; SBP, seated systolic BP; SDS, Sequence Detection System; SNPs, single nucleotide polymorphisms; JSNP, Japanese Single Nucleotide Polymorphisms

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Introduction

Essential hypertension is a major risk factor for many common causes of morbidity and mortality including stroke, myocardial infarction, congestive heart failure, and end-stage renal disease [1–3]. The etiology and pathogenesis of essential hypertension are likely to comprise a multifactorial disorder resulting from inheritance of several susceptibility genes, as well as multiple environmental determinants. A variety of gene variants have been shown to be associated with essential hypertension [4,5]. The use of knockout mice in which specific genes are disrupted, and which exhibit hypertensive phenotypes, has been a very informative approach for the researching of essential hypertension susceptibility genes [6,7].

Cytochrome P450 is a superfamily of cysteinato-heme enzymes that are key mediators of oxidative transformation of exogenous molecules. Cytochrome P450 enzymes are classified into families, subfamilies, and individual isoenzymes, based on similarities in their amino acid sequence [8]. The CYP4A family is expressed at high levels in cardiovascular and renal tissue, and is involved in the metabolism of arachidonic acid [9]. In a study by Holla *et al.* [10], disruption of the cyp4a14 gene was found to cause hypertension in mice and could be used as a model of human hypertension. They found that the difference in systolic blood pressure between cyp4a14$^{-/-}$ and cyp4a14$^{+/+}$ male mice was 30 mmHg, and that the difference between cyp4a14$^{-/-}$ and cyp4a14$^{+/+}$ female

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