

Polymorphisms in human connexin40 gene promoter are associated with increased risk of hypertension in men

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Objective Gap junctions, formed by connexins (Cx), are important in the regulation of vascular tone. Previously, we reported two closely linked polymorphisms (–44G → A and +71A → G) within regulatory regions of the gene for Cx40, a major connexin in the vascular wall and the kidney. In the present study, we examined the hypothesis that these polymorphic variants are associated with hypertension and that they interact with blood pressure in healthy individuals.

Methods Cx40 genotypes were determined in 191 subjects with essential hypertension, 198 normotensive individuals, and a healthy control population (178 twin pairs, 108 monozygotic, 70 dizygotic).

Results We found a significant contribution of the minor Cx40 allele or genotype (–44AA/+71GG) to the risk of hypertension in men ($P = 0.013$ or $P = 0.035$; odds ratio, 1.87 or 2.10, respectively), but not in women. Moreover, in the healthy control population a significant effect of Cx40 genotype and sex on systolic blood pressure was found ($P < 0.05$ and $P < 0.0001$, respectively). Women carrying the minor Cx40 genotype had significantly higher systolic blood pressure compared with non-carriers ($P < 0.05$). In men, systolic blood pressure in carriers of the minor Cx40 genotype was not significantly different from the other two genotypes, possibly because of the small number of men in this group. However, men carrying the –44GA/+71AG genotype had higher standing systolic blood pressure

compared with the more common Cx40 genotype (–44GG; $P = 0.033$).

Conclusion These findings suggest that the Cx40 polymorphisms may form a genetic susceptibility factor for essential hypertension in men. *J Hypertens* 24:325–330 © 2006 Lippincott Williams & Wilkins.

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Introduction

Hypertension is a risk factor for a multitude of potentially life-threatening complications, such as myocardial infarction, congestive heart failure, renal failure, and stroke [1]. Although hypertension can affect anyone at any age, several recent studies have demonstrated that the pathogenesis of high blood pressure is multifactorial and involves both genetic and environmental factors [2–4].

Current evidence suggests that gap junctions may play a key role in the initial pathogenesis and eventual clinical manifestations of human cardiovascular disease, including hypertension [5]. Gap junctions are arrays of intercellular channels that connect the plasma membranes of neighbouring cells, forming cell-to-cell pathways, and thereby facilitating electrical and chemical communica-

tion between adjacent cells [6]. In cells of the vascular wall, gap junctions are believed to play a critical role in the coordination of vasomotor responses and the regulation of vascular tone [5,7,8]. Moreover, gap junctions have been implicated in the regulation of renin release in the kidney [9]. Connexins (Cx) are membrane-spanning protein subunits of gap junction channels. At least four different connexin isoforms, Cx37, Cx40, Cx43 and Cx45, are expressed in the endothelial and smooth muscle cells of the vascular wall [5,7,8,10] as well as in the kidney [5,9]. With the exception of Cx37, all these isoforms are also expressed in cardiomyocytes [11]. Recent studies in Cx40-deficient mice have revealed a key role for this protein in the control and regulation of blood pressure. Mice lacking Cx40 had significantly higher blood pressure levels than wild-type littermates, and displayed irregular arteriolar vasomotion as well as impaired

conduction of vasodilatory signals along their arterioles [12–14]. On the other hand, in spontaneously hypertensive rats the increase in blood pressure levels and the development of hypertension was reported to be significantly associated with a decreased expression of endothelial Cx40 protein but not Cx37 or Cx43 [15]. Furthermore, it has been shown that the inhibition of gap junctional communication with Cx40-mimetic peptides can antagonize the vasodilatory actions of the endothelium-derived hyperpolarizing factor in the kidney [16], suggesting that Cx40 is also involved in the long-term regulation of blood pressure.

Previously, we reported on two closely linked polymorphisms in the promoter region of the Cx40 gene, at nucleotides –44 (G → A) and +71 (A → G) compared with the transcription initiation site, which were associated with atrial-specific arrhythmias, familial atrial standstill [17] and atrial fibrillation [18]. Functional characterization revealed that the less common Cx40 haplotype (–44A/+71G) leads to a significant reduction (> 50%) in promoter activity *in vitro*. On the basis of the foregoing, we hypothesized that this haplotype would also lead to an increase in blood pressure. Therefore, the objectives of the present study were to identify the prevalence of the Cx40 polymorphisms in individuals with essential hypertension compared with normotensive individuals, as well as to investigate the effects of the genotypes on blood pressure in a healthy cohort without cardiovascular disease.

Methods

Study population

Sample I

To study the relationship between Cx40 polymorphisms and essential hypertension, a total of 389 individuals, 191 with essential hypertension and 198 normotensive controls, were included in the present study. The study population was randomly drawn from a larger longitudinal primary care study on cardiovascular risk. Subjects were age and sex-matched by the random selection of control subjects who matched the criteria of the cases. Participants were defined as being normotensive when their blood pressure was below 140 mmHg systolic and 90 mmHg diastolic and as hypertensive when they had a documented history of hypertension, treatment with antihypertensive medications or systolic blood pressure of 160 mmHg or greater or diastolic blood pressure of 95 mmHg or greater. Blood pressure was measured after 10 min rest in the sitting position using a sphygmomanometer. Blood samples were drawn for serum creatinine, uric acid, total cholesterol, glucose levels and DNA extraction. Creatinine clearance was determined using Cockcroft's formula [19]. Exclusion criteria for normotensive controls were myocardial infarction, angina pectoris, cerebrovascular disease, intermittent claudication, diabetes mellitus and the use of cardiovascular medica-

tion. At the time of recruitment, informed consent was obtained from each participant according to a protocol approved by the Medical Ethics Committee of the University Hospital Maastricht.

Sample II

To investigate the potential effect of the Cx40 polymorphisms on blood pressure levels, a second study population consisting of a total of 178 healthy twin pairs [108 monozygotic (MZ) and 70 dizygotic (DZ)] were recruited as previously described [20,21]. All subjects were German Caucasians. They underwent a medical history and physical examination. None had any chronic medical illness or were taking antihypertensive drugs. Blood was obtained for the determination of zygosity and molecular genetic studies. Blood pressure was measured after 5 min of rest by a sphygmomanometer in the sitting, standing, and recumbent positions by a trained physician who was unaware of the genotyping. The mean of two subsequent measurements in each position was used. The study protocol was approved by Berlin Humboldt University review committee, and all participants gave written informed consent.

Determination of Cx40 genotypes

Genomic DNA was extracted according to standard protocols. The Cx40 polymorphisms were genotyped by direct sequencing of polymerase chain reaction (PCR) products amplified from genomic DNA. The region encompassing both polymorphisms (–44G/A and +71A/G) in the Cx40 gene was amplified using the sense primer 5'–TTGGTGTGGGCATAGTGGAAT–3' and the anti-sense primer 5'–CCTTCCTCTGGCTACTTCATATC–3'. The samples were denatured at 95°C for 5 min, followed by 35 cycles at 95°C for 1 min, 62°C for 1 min and 72°C for 2 min, followed by a final elongation step at 72°C for 10 min. Amplified products (683 bp) were visualized on a 1.5% agarose gel by staining with ethidium bromide. One microlitre of five times diluted PCR products was subsequently used in a 25 µl nested PCR reaction using the primers 5'–AGGAAGAGACTTAGGGAGGT–3' and 5'–CCTTCCTCTGGCTACTTCATATC–3'. The samples were denatured at 95°C for 5 min, followed by 35 cycles at 95°C for 1 min, 62°C for 1 min and 72°C for 1 min, followed by one cycle at 72°C for 10 min. The nested PCR products were diluted 10 times and subjected (2 µl) to direct sequence analysis using the primer 5'–ACAAAAGAGAGAGAGGGGAGGGA–3' and a BigDye terminator kit (Applied Biosystems, Foster City, California, USA), in the presence of 5% dimethyl sulphoxide. Sequence reactions were run on an ABI Prism 3700 automated sequencer (96 capillary), and both polymorphisms were scored independently of each other.

Statistical analysis

Differences in quantitative variables were analysed using Student's *t*-test. The chi-squared and Fisher's exact tests

Table 1 Characteristics of sample I study population

Variable	Men (n = 177)			Women (n = 212)			P* total
	Normotensive (n = 88)	Hypertensive (n = 89)	P	Normotensive (n = 110)	Hypertensive (n = 102)	P	
Age (years)	67.1 ± 1.2	66.2 ± 1.3	0.589	69.2 ± 1.0	69.9 ± 1.3	0.628	0.925
Smoking (%)	66	65	0.631	62	56	0.231	0.595 [†]
Height (cm)	174.5 ± 0.7	175.7 ± 1.1	0.323	165.0 ± 0.6	163.5 ± 0.9	0.186	0.920
Weight (kg)	77.7 ± 1.5	84.3 ± 1.8	0.006	68.7 ± 1.1	80.0 ± 2.3	< 0.001	< 0.001
BMI (kg/m ²)	26.9 ± 0.5	27.7 ± 0.8	0.344	26.7 ± 0.5	29.8 ± 1.0	0.002	0.020

BMI, Body mass index. Data are presented as mean ± SEM or number (%) of subjects. *P value for comparison between the overall (including both sexes) normotensive and hypertensive groups. Statistical significance was determined by Student's *t*-test or chi-squared test ([†]).

were used to test deviations of genotype distribution from Hardy–Weinberg equilibrium and to compare allele and genotype frequencies between the hypertensive and normotensive groups (sample I). Multiple logistic regression analysis [with age, weight, body mass index (BMI), and smoking status used as covariates] was also performed to assess the independent effects of Cx40 genotypes on the risk of hypertension. Odds ratios (approximating relative risk) with 95% confidence intervals (CI) were estimated (by 2 × 2 contingency tables). The blood pressure values of the MZ twins (sample II) were converted into singleton values by calculating the average per twin pair. The effect of genotype on blood pressure was determined by mixed model analysis of variance as described previously [22]. For this analysis, the DZ twins were treated as repeated measures within the random factor 'family'. The effect of genotype was tested with sex as an independent factor between groups, and age was used as a covariate. When a significant effect of genotype was found, the analyses were further repeated for both sexes separately by one-way analysis of variance, followed by Student–Newman–Keuls post-hoc test for pairwise comparisons between genotypes. SPSS statistical software (version 11.5; SPSS Inc., Chicago, Illinois, USA) was used for all analyses. Statistical differences were judged significant at *P* < 0.05.

Results

Consistent with our previously published results [17,18], we found that in both sample populations the Cx40

polymorphisms were in complete linkage disequilibrium: all subjects with G at position –44 had A at +71, and vice versa. Therefore we further analysed the data for the –44 bp polymorphism.

Allelic and genotypic frequency of Cx40 polymorphisms in essential hypertension

Table 1 shows the characteristics of subjects with essential hypertension when compared with normotensive subjects. There were no significant differences in age, sex distribution, height, or smoking frequency between the hypertensive and normotensive subjects. Subjects with hypertension had greater body weight than normotensive participants (82.1 ± 1.5 versus 72.9 ± 1.0 kg, *P* < 0.001). This association remained significant when men and women were analysed separately. Moreover, BMI was significantly associated with hypertension in women (26.7 ± 0.5 versus 29.8 ± 1.0, *P* = 0.002), but not in men (26.9 ± 0.5 versus 27.7 ± 0.8, *P* = 0.344). There were no significant differences in biochemical profile such as serum creatinine, uric acid, total cholesterol, glucose levels or creatinine clearance between the two groups (data not shown).

Allelic and genotypic distribution for the Cx40 polymorphisms are shown in Table 2. The normotensive group and the hypertensive group were in Hardy–Weinberg equilibrium. When the whole population was considered, there was no significant association between alleles or genotypes of the Cx40 polymorphisms

Table 2 Comparison of allele and genotype frequencies of connexin 40 polymorphisms in hypertensive and normotensive subjects in sample I study population

	Allele frequencies n (%)		P* allele frequency	Genotype frequencies n (%)			P* genotype frequency
	–44G	–44A		–44GG	–44GA	–44AA	
All subjects (n = 389)							
Hypertensives (n = 191)	291 (76)	91 (24)	0.220	111 (58)	69 (36)	11 (6)	0.420
Normotensives (n = 198)	316 (80)	80 (20)		128 (65)	60 (30)	10 (5)	
Men (n = 177)							
Hypertensives (n = 89)	128 (72)	50 (28)	0.013	45 (50)	38 (43)	6 (7)	0.035
Normotensives (n = 88)	146 (83)	30 (17)		60 (68)	26 (30)	2 (2)	
Women (n = 212)							
Hypertensives (n = 102)	163 (80)	41 (20)	0.511	66 (65)	31 (30)	5 (5)	0.765
Normotensives (n = 110)	170 (77)	50 (23)		68 (62)	34 (31)	8 (7)	

Only –44 polymorphism data are presented, data for +71 polymorphism are complementary. *The chi-squared test was used to compare the connexin (Cx) 40 allele and genotype frequencies between the hypertensive and normotensive subjects.

and the risk of hypertension ($P = 0.220$ and $P = 0.420$, respectively). However, when subjects were stratified according to sex, the prevalence of the minor Cx40 allele ($-44A$) and $-44AA$ genotype was significantly higher in men with hypertension compared with normotensive men ($P = 0.013$ and $P = 0.035$, respectively, Table 2). In women, there was no association between Cx40 polymorphisms and hypertension. The association between the Cx40 polymorphisms and the presence of essential hypertension was further investigated by logistic regression analysis in both sexes separately. The regression model was examined for the risk of hypertension as a dependent variable and for age, weight, BMI, smoking status, and Cx40 polymorphisms as independent variables. The model showed that the Cx40 polymorphisms were the only predictors of hypertension in men ($P = 0.024$), whereas in women, BMI was the only significant determinant of hypertension ($P = 0.003$). The odds ratios for hypertension in men carrying the minor Cx40 allele or the $-44AA$ genotype, compared with non-carrier men, were 1.87 (95% CI 1.13–3.12) and 2.10 (95% CI 1.14–3.68), respectively. In normotensive subjects, either in the overall group or after stratification according to sex, no association was found between the Cx40 polymorphisms and systolic or diastolic blood pressure levels (not shown).

Effect of Cx40 polymorphisms on blood pressure in healthy subjects

Allelic and genotypic frequencies of Cx40 polymorphisms in the sample II population did not differ from those we reported previously [17] in a control population. The -44 polymorphism was found in Hardy–Weinberg equilibrium. There was no significant difference in age between different genotype groups. Compared with women (mean age 33.9 ± 1.0 years), men (33.8 ± 1.5 years) had significantly higher systolic blood pressure in the three positions: recumbent, sitting and standing ($P < 0.001$ for all three positions) as well as standing diastolic blood pressure ($P = 0.007$, Table 3). Mixed model analysis indicated a significant effect of the Cx40 genotype on systolic blood pressure in all three positions ($P = 0.042$, $P = 0.035$, and $P = 0.001$, respectively for recumbent, sitting and standing positions), but not on diastolic blood pressure ($P = 0.253$, $P = 0.618$, and

Table 3 Comparison of blood pressure levels between men and women in sample II study population

Blood pressure (mmHg)	Men ($n = 82$)	Women ($n = 166$)	P
Systolic BP recumbent	131.4 ± 1.4	122.4 ± 1.1	< 0.001
Systolic BP sitting	129.0 ± 1.4	120.9 ± 1.1	< 0.001
Systolic BP standing	129.5 ± 1.3	119.5 ± 1.0	< 0.001
Diastolic BP recumbent	72.4 ± 1.3	70.3 ± 0.8	0.150
Diastolic BP sitting	74.5 ± 1.2	72.2 ± 0.7	0.09
Diastolic BP standing	81.8 ± 1.1	78.5 ± 0.7	0.007

BP, Blood pressure. Data are mean \pm SEM. Statistical significance was determined by Student's t -test.

Table 4 Effect of connexin 40 polymorphisms on blood pressure in sample II study population

Blood pressure (mmHg)	Cx40 genotype			P
	$-44GG$	$-44GA$	$-44AA$	
Men, n	44	34	4	
Systolic BP recumbent	129.7 ± 1.8	134.4 ± 2.4	124.4 ± 3.9	0.146
Systolic BP sitting	127.1 ± 1.8	132.4 ± 2.3	120.1 ± 3.6	0.067
Systolic BP standing	127.1 ± 1.9	133.0 ± 1.9	126.1 ± 3.9	0.087
Women, n	90	59	17	
Systolic BP recumbent	121.9 ± 1.7	120.7 ± 1.6	131.0 ± 3.4	0.041
Systolic BP sitting	120.5 ± 1.5	119.5 ± 1.5	128.5 ± 3.3	0.046
Systolic BP standing	118.3 ± 1.4	118.6 ± 1.6	128.9 ± 3.3	0.009

BP, Blood pressure. Data are presented as mean \pm SEM. Only -44 polymorphism data are presented, data for $+71$ polymorphisms are complementary. Statistical significance was determined by one-way analysis of variance analysis (see Methods, sample II population).

$P = 0.431$, respectively, for recumbent, sitting and standing positions). In the same analysis, sex was strongly associated with systolic blood pressure in all three positions ($P < 0.0001$ for all three positions). When the population was stratified according to sex, further analysis by one-way analysis of variance revealed that the $-44AA$ genotype was more predictive of systolic blood pressure in women than in men (Table 4). In men, although there was a trend towards a significant association with genotype, systolic blood pressure in carriers of the $-44AA$ genotype was not significantly different from the other two genotypes, presumably because of the small number of men carrying this genotype in the sample population (four men versus 17 women $-44AA$ carriers). However, male carriers of the $-44GA$ genotype had higher standing systolic blood pressure compared with carriers of the more common Cx40 genotype ($-44GG$; $P = 0.105$, $P = 0.066$, and $P = 0.033$, respectively, for recumbent, sitting and standing systolic blood pressure). Female carriers of the $-44AA$ genotype (17/166) had a significantly higher systolic blood pressure in all three positions compared with non-carriers ($P = 0.041$, $P = 0.046$, and $P = 0.009$, respectively, Table 4).

Discussion

The present study was conducted to investigate the association between Cx40 polymorphisms and hypertension. We also investigated the interaction between the Cx40 promoter polymorphisms and blood pressure levels in a clinically healthy population. We found that the minor Cx40 allele ($-44A/+71G$) was associated with higher systolic blood pressures in healthy individuals. Moreover, we report a significant association between the minor Cx40 allele and hypertension in men.

The molecular mechanism by which the Cx40 polymorphisms may be associated with elevated blood pressure is unknown. Our previous findings indicated that the minor Cx40 haplotype leads to a major reduction in promoter activity consistently in different Cx40-expressing cell types of vascular and cardiac origin (Firouzi *et al.*,

unpublished data). Reduced promoter activity could potentially result in reduced Cx40 expression *in vivo*, and as such may enhance the risk of hypertension through the impaired control and coordination of vasomotor responses along the vessel wall, as supported by studies in Cx40-deficient mice [12–14]. Moreover, besides its presence in the systemic vasculature, Cx40 has also been implicated in the regulation of renin release [9]. De Vriese *et al.* [16] recently reported that the Cx40-mimetic peptide 40Gap27 interferes with Cx40-mediated gap junctional communication through binding to Cx40 docking sites. The acute administration of 40Gap27 was shown to reduce renal blood flow and increase systemic blood pressure in rats. These studies indicate that Cx40 may also be involved in long-term blood pressure regulation by affecting renal haemodynamic function.

In the present study, the Cx40 polymorphisms were not associated with essential hypertension in women. As male sex is a risk factor for cardiovascular disease and as men have higher blood pressure than women, which was also the case in the present study, it has been suggested that female sex hormones may confer protection against a genotypic predisposition in women [23,24]. This notion is certainly supported by experimental findings [25,26]. Nevertheless, the actions of oestrogen on blood pressure are complex. On the one hand, oestrogens have been shown to promote endothelium-dependent vasodilatation [24], and on the other hand, they have been associated with high blood pressure, presumably by stimulating renin release [27]. Furthermore, clinical studies have shown that oestrogen may modulate blood pressure responses to stressful stimuli [28]. Less is known regarding the role of androgens in the pathogenesis of hypertension. In spontaneously hypertensive rats, castration in males was reported to ameliorate the hypertension, whereas testosterone treatment of ovariectomized females caused an exacerbation of hypertension [29].

The findings in the present study are consistent with several other studies [23,30,31], which demonstrated the sex-specific effects of genetic variations on blood pressure, reporting positive associations with hypertension only in men. Of note is a study of the C1019T polymorphisms in the Cx37 gene by Boerma *et al.* [32], which reported a significant association with borderline hypertension in Swedish men. Interestingly, a recent large-scale study by Yamada *et al.* [33], involving 112 polymorphisms in 71 candidate genes, revealed this polymorphism in the Cx37 gene as the only predictor of myocardial infarction risk in men but not in women. As with Cx40, Cx37 is expressed in the endothelial and smooth muscle cells of the vascular wall.

In the current study, significance was obtained in independent samples from two different populations, and the frequencies of Cx40 polymorphisms are remarkably

similar in both populations. The selection criteria adopted to recruit individuals with hypertension and normotensive controls in the sample I population (i.e. blood pressure systolic ≥ 160 mmHg or diastolic ≥ 95 mmHg) were deliberately chosen to avoid the unintentional inclusion of individuals with borderline elevated blood pressure in the hypertensive group, thereby exclusively recruiting patients with 'true' hypertension in this group. Furthermore, the mean age in the sample II population was only 34 years, rendering the possibility of age-related influences on blood pressure in these subjects unlikely. These important characteristics of our study design should have minimized the chance of spurious results, a problem inherent in association studies.

Our results indicated that a high BMI was the only predictor of essential hypertension in women. Although the association between obesity and hypertension has been firmly established, there is evidence that body weight has a greater impact on blood pressure in women than in men [34]. Obesity is significantly more common in middle-aged women than men, and a significant amount of hypertension in women is attributable to obesity.

In the present study, we demonstrated that the minor allele of the Cx40 gene promoter polymorphisms is associated with essential hypertension in men, suggesting that this allele may be a genetic susceptibility factor for essential hypertension. Further studies will be necessary to confirm whether these variants can be used as an early genetic marker for the risk of essential hypertension. In addition, the data may be a starting point for studying the interaction between the Cx40 gene promoter polymorphisms and environmental factors such as sodium intake. The latter is of particular relevance in view of the fact that the polymorphisms have also been linked to renal function and the regulation of renin release. Another option may be to study this polymorphism in relation to atrial fibrillation in different hypertensive subgroups. However, as there was only a small effect of the genotype on men in the sample 1 population but not in men in the twin population, we cannot entirely exclude the possibility that our findings arose by chance. Therefore, there is a clear need for the replication of our results in other populations.

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