Protective Effect of the \textit{KCNMB1} E65K Genetic Polymorphism Against Diastolic Hypertension in Aging Women and Its Relevance to Cardiovascular Risk

Mariano Sentí, José M. Fernández-Fernández, Marta Tomás, Esther Vázquez, Roberto Elosua, Jaume Marrugat, Miguel A. Valverde

Abstract—The E65K polymorphism in the $\beta_1$-subunit of the large-conductance, Ca$^{2+}$-dependent K$^+$ (BK) channel, a key element in the control of arterial tone, has recently been associated with low prevalence of diastolic hypertension. We now report the modulatory effect of sex and age on the association of the E65K polymorphism with low prevalence of diastolic hypertension and the protective role of E65K polymorphism against cardiovascular disease. We analyzed the genotype frequency of the E65K polymorphism in 3924 participants selected randomly in two cross-sectional studies. A five-year follow-up of the cohort was performed to determine whether cardiovascular events had occurred since inclusion. Estrogen modulation of wild-type and mutant ion channel activity was assessed after heterologous expression and electrophysiological studies. Multivariate regression analyses showed that increasing age upmodulates the protective effect of the K allele against moderate-to-severe diastolic hypertension in the overall group of participants (odds ratio [OR], 0.35; $P=0.006$). The results remained significant when analyses were restricted to women (OR, 0.18; $P=0.02$) but not men (OR, 0.46; $P=0.09$). This effect was independent of the reported acute modulation of BK channels by estrogen. A five-year follow-up study also demonstrated a reduced age- and sex-adjusted hazard ratio of 0.11, 95% CI, 0.01 to 0.79 of K-carriers for “combined cardiovascular disease” (myocardial infarction and stroke) compared with EE homozygotes. Our study provides the first genetic evidence for the different impact of the BK channel in the control of human blood pressure in men and women, with particular relevance in aging women, and highlights the E65K polymorphism as one of the strongest genetic factors associated thus far to protection against myocardial infarction and stroke. (\textit{Circ Res.} 2005;97:1360-1365.)

Key Words: BK channel • estradiol • hypertension • \textit{KCNMB1} gene • cardiovascular risk • sex • age

Hypertension is not only a disease but also the most prevalent risk factor for heart, brain, and kidney diseases, present in \textasciitilde 30\% of adults.\textsuperscript{1,2} Augmented rates of hypertension are seen with increasing age,\textsuperscript{3} and the incidence of hypertension is higher in men 30 to 50 years of age compared with women of similar age and in postmenopausal compared with premenopausal women.\textsuperscript{4} The cause of hypertension cannot be identified in 90\% to 95\% of patients; this type of hypertension is named essential hypertension.\textsuperscript{5} Typically, essential hypertension is a multifactorial disorder involving abnormalities at different levels, particularly vascular volume homeostasis and vascular tone.\textsuperscript{6,7} Several association studies have found genetic variations in different genes involved in those homeostasis systems.\textsuperscript{8} We recently characterized a gain-of-function polymorphism (E65K) in the $\beta_1$-subunit of the large-conductance Ca$^{2+}$-dependent K$^+$ channel gene (\textit{KCNMB1}), which is associated with low prevalence of moderate-to-severe diastolic hypertension.\textsuperscript{9} Polymorphisms in the \textit{KCNMB1} gene have also been associated with the baroreflex function in humans.\textsuperscript{10} The vascular smooth muscle Ca$^{2+}$-dependent K$^+$ channel (BK), a key element in the control of vascular tone, is formed by an ion-conducting $\alpha$-subunit and a regulatory $\beta_1$-subunit, which couples local increases in intracellular Ca$^{2+}$ to augmented channel activity\textsuperscript{11–14} and vascular relaxation.\textsuperscript{15,16} Mutant E65K channels showed increased Ca$^{2+}$ sensitivity, which might result in a more efficient negative feedback on vascular smooth contractility.\textsuperscript{9}

Age and sex have been reported to regulate the expression or function of BK channels in vascular smooth muscle cells. A decreased expression of the $\alpha$-; and $\beta_1$ BK subunits\textsuperscript{18} with increasing age have been described. Among all sex hormones implicated in the control of vascular tone, estradiol has been linked to the regulation of the BK channel activity in vascular
smooth muscle cells. Estradiol increases the activity of BK channels by increasing NO availability and subsequent generation of intracellular signaling molecules such as cGMP\textsuperscript{39} or direct interaction with the BK channels.\textsuperscript{20} Therefore, the aims of the present study were to assess the potential modulatory effect of sex and age on the association of the E65K polymorphism with the prevalence of diastolic hypertension and to determine the protective role of E65K polymorphism against stroke and myocardial infarction.

**Materials and Methods**

**Study Population and Measured Variables**

The representative population sample was composed of 3924 participants 25 to 74 years of age: 1973 (50.3\%) women and 1951 (49.7\%) men. They were randomly selected in two cross-sectional studies performed to establish the prevalence of cardiovascular risk factors in the province of Girona (Spain), in 1995 and in 2000 (the REGICOR study). Full details of recruitment and measured variables are provided by Masia et al.\textsuperscript{21} Participants were considered hypertensive when their diastolic blood pressure (DBP) was $>90$ mm Hg or they were under antihypertensive drug therapy. Diastolic hypertensive patients receiving antihypertensive therapy were 38.4\% men and 60.1\% women, although no individual information on the therapeutic protocol was available. Subjects with DBP $<90$ mm Hg not receiving antihypertensive therapy constituted the diastolic normotensive group. All participants gave written informed consent. The study was approved by the local ethical committee.

**Five-Year Follow-Up Study**

A five-year follow-up of the 1995 cohort with a personal contact was organized to obtain an ECG and to administer a structured questionnaire to determine whether myocardial infarction events had occurred since inclusion. Finally, it was possible to obtain data on the follow-up and genotype in 1282 participants (73.3\% of initial). All ECGs were blindly interpreted and compared with the baseline recording of intracellular signaling molecules such as cGMP\textsuperscript{19} and the bath solution contained (in mmol/L): 140 KCl, 0.7 MgCl\textsubscript{2}, 3.5 CaCl\textsubscript{2}, 5 EDTA, and 10 HEPES (300 mmol/L, pH 7.3; 100 mmol/L free Ca\textsuperscript{2+}, calculated using EqCal; Biosoft). A total of 10 mmol/L 17β-estradiol was added to the pipette solution. Currents were recorded immediately after seal formation ($t=0$ minutes; control) and 10 minutes later (effect of 17β-estradiol). Normalized conductance versus voltage (G/Gmax-V) curves were obtained from tail currents and fitted to Boltzmann equations from which $V_{1/2}$ were calculated.

**Clinical Characteristics and E65K Genotypes in Men and Women**

<table>
<thead>
<tr>
<th></th>
<th>Men (n=1951)</th>
<th>Women (n=1973)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>50.7±13.8</td>
<td>50.7±13.3</td>
<td>NS</td>
</tr>
<tr>
<td>Body mass index, kg/m$^2$</td>
<td>27.4±4.3</td>
<td>27.1±5.5</td>
<td>0.022</td>
</tr>
<tr>
<td>Diastolic hypertension*, n (%)</td>
<td>594 (30.4)</td>
<td>469 (23.7)</td>
<td>0.001</td>
</tr>
<tr>
<td>Diabetes mellitus, n (%)</td>
<td>273 (13.9)</td>
<td>198 (10.8)</td>
<td>0.001</td>
</tr>
<tr>
<td>E65K genotypes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K carriers, n (%)</td>
<td>414 (21.2)</td>
<td>439 (22.2)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Continuous variables are shown as mean±SD.

*Subjects under antihypertensive treatment with DBP $<90$ mm Hg were also included.

**Effect of Dibutyryl cGMP on BK Channels**

Single-channel recordings were obtained from cell-attached patches clamped at several voltages (+40, +60, +80, and +100 mV) before and 20 minutes after exposure to 500 μmol/L dibutyryl cGMP (db-cGMP). The solution filling the patch pipettes (10 mol/L db-cGMP) had the bath solution contained (in mmol/L): 140 KCl, 0.7 MgCl\textsubscript{2}, 0.25 CaCl\textsubscript{2}, 0.5 EGTA, and 10 HEPES (304 mmol/L, pH 7.26, 100 mmol/L free Ca\textsuperscript{2+}, calculated using EqCal; Biosoft). Average BK channel activity (number of channels×single channel open probability [NPo]) was determined from 20-s continuous recordings by fitting the sum of Gaussian functions to an all-points histogram plot at each voltage tested.

Experiments were performed at room temperature on enhanced green fluorescent protein–positive cells. Currents were sampled at 10 kHz and low-pass filtered at 1 kHz.

**Statistical Analysis**

Deviation from Hardy–Weinberg equilibrium was assessed using a $\chi^2$ test with 1 df. $\chi^2$ or Fisher’s exact tests were used as appropriate to compare categorical variables between groups. Continuous variables were compared between groups with the Student’s $t$ test. Adjusted odds ratios (ORs) of moderate-to-severe diastolic hypertension and their 95\% CIs were estimated for K-carriers versus EE genotype by unconditional logistic regression analysis. These models were also used to test for interactions between genotype and age, and between genotype and sex.

The association between the E65K polymorphism and cardiovascular events (myocardial infarction and stroke) was evaluated by a standard age- and sex-adjusted Cox proportional hazards model. Electrophysiological data are presented in graphs as mean±SEM. ANOVA was used to test the effect of 17β-estradiol or db-cGMP on different combinations of α and β\textsubscript{1}-subunits. $P$ values $<0.05$ were considered statistically significant.

**Results**

The frequency of the K allele was 0.22 and the genotype frequencies were 78.3\%, 20.2\%, and 1.5\% for the EE,EK, and KK genotypes, respectively. The observed genotype frequencies of the E65K polymorphism were consistent with Hardy–Weinberg equilibrium. The clinical characteristics of the participants grouped by sex are shown in the Table. Men were more overweight and more hypertensive, with greater
prevalence of diabetes than women. The genotype frequencies for the E65K polymorphism were similar in both sexes.

The E65K genotype frequencies of total, moderate-to-severe, and severe diastolic hypertensive subjects were compared with genotype frequencies of the diastolic normotensive group in both sexes (Figure 1). The frequency of the K allele decreased with increasing DBP values in both sexes; however, the differences were statistically significant only in women with moderate-to-severe diastolic hypertension (Figure 1B). In this group, only 10.7% were K-carriers compared with 22.3% in the normotensive group (P = 0.047). Moreover, from 12 severe hypertensive women (DBP ≥110 mm Hg), only one was K-carrier.

To assess the influence of age on the relationship between the E65K polymorphism and diastolic hypertension, normotensive and moderate-to-severe hypertensive (DBP ≥100 mm Hg) participants were stratified by sex and age, and 55 years was the cutoff. In subjects <55 years of age, no association between the E65K polymorphism and moderate-to-severe diastolic hypertension was observed. In hypertensive men >54 years of age, there was a decreasing trend of the E65K polymorphism frequency that was not statistically significant. In women >54 years of age the polymorphism frequency shifted from 29.3% in normotensive to 4.8% in moderate-to-severe hypertensive women (P = 0.003). Interestingly, there were 10 women >54 years of age with severe diastolic hypertension, but none of them was K-carrier. The ORs adjusted for body mass index and diabetes mellitus in the overall population, and in both sexes stratified by age, all of them with moderate-to-severe diastolic hypertension were estimated for the K allelic variant compared with the EE genotype (Figure 2). Interactions of genotype by age and genotype by sex were also tested. In the overall group of participants (OR, 0.35; 0.17 to 0.72 95% CI; P = 0.004) as well as in women >54 years of age (OR, 0.18; 0.04 to 0.77 95% CI; P = 0.02), the magnitude of the association was consistent with a protective effect of the K allele against moderate-to-severe diastolic hypertension. Similar logistic regression analysis applied to men >54 years of age showed a relationship between the E65K polymorphism and moderate-to-severe diastolic hypertension that did not reach statistical significance (OR, 0.46; 0.18 to 1.11 95% CI; P = 0.08). Analyses of interactions in the overall group of participants demonstrated that the effect of age (P = 0.006) on the protective effect of the K allele was more pronounced than sex (P = 0.38). The interaction of genotype by age was also significant in women (P = 0.02) and nonsignificant in men (P = 0.09). Our data shows an age-dependent association between the E65K polymorphism and protection against moderate-to-severe diastolic hypertension, which is only present in women. Because our hypertensive group included subjects under antihypertensive therapy, we set out to discard a possible confounding effect of treatment on the association of E65K polymorphism with DBP. In this respect, we compared the mean values of DBP in hypertensive men and women ≥55 years of age with and without antihypertensive therapy, stratified by genotype (Figure 3). The K genetic variant was associated with a significant reduction in DBP in untreated (P < 0.001) and treated (P < 0.01) hypertensive women >54 years of age compared with the EE genotype. On the other hand, no significant differences in DBP were observed between genotypes in women <55 years of age and in men of any age, both treated and untreated (data for subjects <55 years of age are not shown).

Estradiol has been linked to the regulation of the BK channel activity in vascular smooth muscle cells via an indirect pathway involving the generation of NO and cGMP19 and via direct binding to the channel.20 To evaluate whether sex hormones modulate the protective effect of the E65K polymorphism, we analyzed both direct and indirect estradiol-mediated mechanisms of channel regulation in

Figure 1. E65K genotype distribution by DBP and sex. The number of subjects is shown in parentheses. *Subjects under antihypertensive treatment with DBP <90 mm Hg were also included in this group. ND indicates not determined.

Figure 2. Interaction between E65K genotype and sex, and E65K genotype and age. Body mass index and diabetes-adjusted ORs and their 95% CIs of moderate-to-severe diastolic hypertension for the presence of the β2-subunit of the large-conductance KCNMB1 E65K rare variant in age-stratified groups in the whole sample and in men and women separately. The P values for the interaction between the E65K genetic variant and age on moderate-to-severe diastolic hypertension were 0.006 in the overall group of participants and 0.091, and 0.020 in men and women, respectively.
HEK-293 cells expressing the pore-forming α-subunit and different combinations of wild-type and mutant β1-subunits. Changes in the activity of the BK channels were evaluated by the analysis of the conductance–voltage relationship of the ionic currents generated in cell membrane macropatches expressing the α, α + β1WT, α + βE65K, or α + β1WT + βE65K subunits in the absence or presence of 10 μmol/L 17β-estradiol. From these curves, the voltage necessary to half activate the channel (V1/2) can be calculated (Figure 4A). This is a convenient measure to evaluate the effect of modulators of BK channels because V1/2 is directly related to the energy to open the channel.22 In accordance with previous reports,20 addition of 17β-estradiol did not modify the V1/2 of channels formed by just α-subunits but reduced the V1/2 (∼10 mV) of channels including the β1-subunit, although no significant differences were observed between β1WT- and βE65K-containing BK channels.

As mentioned above, estradiol also increases the bioavailability of NO via the activation or increased expression of endothelial23 or inducible (smooth muscle) NO synthases.24 Subsequent activation of NO-dependent guanylate-cyclase will result in increased intracellular levels of cGMP and phosphorylation of BK channels via a cGMP-dependent protein kinase.19 The β1-subunit is necessary for the cGMP-dependent activation of BK channels,25 therefore, we evaluated whether the mutant βE65K subunit behaves differently in response to the membrane-permeant db-cGMP.

Single BK channel activity obtained from cell-attached patches of HEK-293 cells expressing α, α + β1WT, α + βE65K, or α + β1WT + βE65K subunits was measured at different voltages (only results at +80 mV are shown) and represented as the number of channels×Npo, Figure 4B through 4E). No significant differences (ANOVA) in Npo were observed between different BK channels before cGMP application, as reported previously for wild-type and mutant BK channels at intracellular Ca2+ levels in the low nanomole range.29 BK channels formed by α-subunits alone (Figure 4B) did not respond to 500 μmol/L db-cGMP, whereas the presence of β1WT (Figure 4C), βE65K (Figure 4D), or β1WT + βE65K (Figure 4E) determined a similar increase in channel activity at each voltage tested. Altogether, our electrophysiological analysis of the BK channel response to estradiol and db-cGMP showed no difference between β1WT and βE65K expressing patches.

In the five-year follow-up study of the first cohort, participants were personally contacted to assess possible cardiovascular events (myocardial infarction and stroke) in this period. The follow-up study showed that 17 of them had experienced a myocardial infarction event and 16 a stroke. Interestingly, only one case of myocardial infarction and none of the 16 strokes reported during the follow-up were K-carriers. Therefore, the proportion of K-carriers presenting a cardiovascular event was significantly lower compared with those free of cardiovascular disease (3% versus 24.0%; P=0.003). Compared with EE carriers, K-carriers had a reduced hazard ratio of cardiovascular risk (0.11; 0.01 to 0.79 95% CI; P=0.029) in a Cox regression model adjusted for age and sex. Further adjustment for hypertension status had a negligible effect on the hazard ratio (0.11; 0.01 to 0.82 95% CI; P=0.031).
Discussion

Essential hypertension is an example of a complex, multifactorial, and polygenic disease that is sexually dimorphic and with higher prevalence in the elderly population. Sexual dimorphism also applied to the pathophysiology of hypertension. Experiments performed in animal models revealed that blood pressure control in males is more dependent on the renin-angiotensin system than females, an observation corroborated in a recent study demonstrating the association of the angiotensin-converting enzyme gene polymorphisms with hypertension in males but not in females. Other observations offer further support to the view that the pathophysiology of hypertension may differ in women and men. We recently identified a genetic variation (E65K) in the β1-subunit (KCNMB1) of the large conductance, Ca2+-dependent potassium (BK) channel that is associated with low prevalence of diastolic hypertension. Functional analysis of the mutant channel revealed a gain-of-function consistent with a more efficient feedback mechanism for the control of the vascular tone, compatible with a protective effect of this mutation against the severity of diastolic hypertension. No association between the E65K polymorphism and systolic hypertension was found. In the present study, we estimated the impact of the K allele in the population stratified by sex and age.

Our epidemiological data showed an age-dependent association between the E65K genotype and protection against moderate-to-severe diastolic hypertension, which is only present in women, although a not significant trend is also seen in men. Furthermore, a significant reduction in DBP levels was observed in untreated and treated hypertensive women >54 years of age carrying the K allele, suggesting that the protective effect of the genetic variant is not affected by antihypertensive therapy.

It has been described that in systolic hypertensive women, DBP is positively and linearly correlated with cardiovascular mortality; therefore, it would be interesting to evaluate the cardiovascular protective effect conferred by the K allele in systolic hypertensive patients. At the end of our five-year follow-up study, in the first cohort, we found a markedly reduced age- and sex-adjusted and hypertension-adjusted hazard ratio of K-carriers for myocardial infarction and stroke compared with EE homozygotes. Despite the limited numbers of events, we consider our results (only one cardiovascular event among 33 was documented in K-carriers) of special relevance for the early detection of susceptible subjects enabling prevention and tailored treatment.

Sexual dimorphism in arterial blood pressure appears in adolescence and persists throughout adulthood until the fifth decade. Accordingly, the incidence of cardiovascular diseases, including hypertension, is greater in men 30 to 50 years of age compared with women of similar age. This dimorphism could be attributed to the differences in sex hormones, environmental factors, and the sex chromosomes. On the other hand, the prevalence of hypertension in women match or cross over that observed for men after women enter menopause, a fact that has been attributed to the loss of the protective effect of female sex hormones. Our multivariate analysis also suggests a sexual dimorphism of the K allele protective effect. The analysis of DBP values in hypertension patients further emphasizes that the protective effect of E65K is mainly present in women >54 years of age.

Most of the sex hormones are able to modify cardiovascular responses, although the vascular relaxant effects of estrogen significantly surpass those of progesterone or testosterone. BK channel function or expression is modified by the presence of estradiol. We tested whether estradiol might regulate differently the wild-type and mutant BK channels, hence providing a clue on the different effect of the E65K polymorphism in aging (postmenopausal; ≥55 years of age) women compared with premenopausal women (<55 years of age) or men >54 years of age. Our electrophysiological analysis showed no difference in either the direct or indirect (cGMP-mediated) acute effects of estradiol on wild-type and mutant BK channels, discarding that such acute effects underlie the sexual dimorphism observed in our study.

There is cumulative evidence that increased rates of hypertension exist with increasing age and that expression and overall function of the BK channel decrease with age, leading to a decreased vascular relaxation. In this respect, oxidative stress is an important factor to take into account when age-dependent phenomena is considered. Moreover, increased oxidative stress underlies the pathophysiology of hypertension. The link between oxidative stress and BK channel function may occur at two independent levels: (1) BK channel activity is impaired (reduced Ca2+ sensitivity) in the presence of reactive oxygen species, an effect related to the alteration of cysstein-mediated calcium sensing in the α-subunit; and (2) the expression of BK β-subunit is downregulated under hypoxia conditions. Altogether, these evidences suggest that under oxidative stress conditions, BK channel activity, in terms of Ca2+ sensitivity, is diminished. Therefore, it is conceivable that the age-dependent association of the E65K polymorphism to protection against hypertension might be attributable, at least in part, to the gain-of-function of the mutated BK channel, which would compensate the loss of channel expression and function with age or as a result of increasing oxidative stress conditions. Further studies are necessary to dissect the molecular mechanisms involved in the sexual dimorphism of the protective effect of the K allele against diastolic hypertension. Such mechanisms could involve sex hormone induced changes in activity or expression level of key proteins in the vascular bed that in turn might affect BK channel function, such as L-type calcium channel or α-protein kinase C. We acknowledge that the pathophysiology of hypertension is also sexually dimorphic. Therefore, it could be possible that women pathophysiology is more related to the control of the vascular tone, although no conclusive evidence exists on this hypothesis.

In summary, our study provides the first genetic evidence for the different impact of the BK channel in the control of human blood pressure in men and women, with particular relevance in aging women, and highlights the E65K polymorphism as one of the strongest genetic factors associated thus far to protection against myocardial infarction and stroke.
Acknowledgments

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References


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