ORIGINAL ARTICLE

Intra-erythrocyte cation concentrations in relation to the C1797T β -adducin polymorphism in a general population

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Genetic variability in the ADD1 (Gly460Trp) and ADD2 (C1797T) subunits of the cytoskeleton protein adducin plays a role in the pathogenesis of hypertension, possibly via changes in intracellular cation concentrations. ADD2 1797CC homozygous men have decreased erythrocyte count and hematocrit. We investigated possible association between intra-erythrocyte cations and the adducin polymorphisms. In 259 subjects (mean age 47.7 years), we measured intra-erythrocyte Na⁺ [iNa], K⁺ [iK] and Mg²⁺ [iMg], serum cations and adducin genotypes. Genotype frequencies (ADD1: GlyGly 61.5%, Trp 38.5%; ADD2: CC 80.4%, T 19.6%) complied with Hardy–Weinberg proportions. In men, ADD2 CC homozygotes ($n=100$) compared to T-carriers ($n=23$) had slightly lower iK (85.8 versus 87.5 mmol/l cells; $P=0.107$), higher iMg (1.92 versus 1.80 mmol/l cells; $P=0.012$), but similar iNa (6.86 versus 6.88 mmol/l cells;

$P=0.93$). In men, iK, iMg and iNa did not differ according to ADD1 genotypes. In men, iK ($R^2=0.128$) increased with age and serum Na⁺, but decreased with serum total calcium and the daily intake of alcohol. iMg ($R^2=0.087$) decreased with age, but increased with serum total calcium. After adjustment for these covariates ($P\leq 0.04$ for all), findings in men for iK (CC versus T: 85.8 versus 87.3 mmol/l; $P=0.14$) and iMg (1.91 versus 1.82 mmol/l; $P=0.03$) remained consistent. In 136 women, none of the phenotype–genotype relations reached significance. Changes in intra-erythrocyte cations in ADD2 1797CC homozygous men might lead to osmotic fragility of erythrocytes, but to what extent they reflect systemic changes or are possibly involved in blood pressure regulation remains unknown.

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Introduction

The membrane-skeleton protein adducin stimulates the assembly of the spectrin–actin network, which determines the structural integrity of the cell membrane. Adducin is composed of either α - and β - or α - and γ -subunits, which are encoded by different genes. In erythrocytes, α - and β -adducin are abundantly present, whereas most other tissues predominantly express α - γ -heterodimers. β -Adducin

knockout mice show a phenotype characterised by mild anemia and compensated hemolysis.^{1,2} In humans, a common polymorphism (C1797T) occurs in exon 15 of the β -adducin gene. We have demonstrated previously in cross-sectional and prospective studies that genetic variability in the subunits of the cytoskeleton protein adducin plays a role in the pathogenesis of hypertension, possibly via alteration of the intracellular cation concentrations.^{3,4} In addition, we have recently noticed that in men consuming alcohol, β -adducin CC homozygosity was associated with lower erythrocyte count, haemoglobin level and haematocrit.⁵ The osmotic stability of erythrocytes decreases with lower intracellular potassium concentration.⁶ We therefore measured the intra-erythrocyte concentration of cations in a sub-sample of our previous study population⁵ to test possible association with the α - and β -adducin polymorphisms.

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Methods

Study population

The Ethics Committee of the University of Leuven approved the protocol of the Flemish study on Environment, Genes and Health Outcomes (FLEMENGHO). All subjects gave informed consent. From August 1985 to November 1990, we recruited a random sample of the households living in a geographically defined area of Northern Belgium.⁷ We stratified the sample by sex and age (20–39, 40–59 and ≥ 60 years) to recruit equal numbers of participants in each of the six strata. From June 1996 to December 2000, we enrolled nuclear families, using the former participants (1985–1990) as index persons. The participation rate among the 2310 subjects contacted was 66.1%. Our previous study⁵ included 1870 subjects with haematological phenotypes and the C1797T β -adducin genotype. Of these, 277 subjects were randomly selected and underwent additional measurements of their intra-erythrocyte cation concentrations and the concentration of serum ionised calcium. We excluded 18 subjects, because of missing values.

Trained nurses measured each participant's blood pressure (BP) five times consecutively by conventional sphygmomanometry after the subjects had rested for at least 5 min in the sitting position. These five readings were averaged for analysis. Hypertension was defined as a BP of at least 140 mm Hg systolic or 90 mm Hg diastolic, or as use of anti-hypertensive drugs. Body mass index (BMI) was body weight in kilograms divided by height in metres squared. We used a standardized and validated⁸ questionnaire to collect information on medical history, smoking habits, intake of alcohol, use of medications and the menstrual cycle of women. From the type and number of alcoholic beverages used each day, we calculated alcohol consumption in grams per day.

Measurement of phenotypes

Venous blood samples were collected into ethylenediamine-tetraacetic acid-containing tubes. Erythrocyte count, haemoglobin, and haematocrit were measured in whole-blood specimens with a SYS-MEX HST 403XE model automated cell counter (Systmex Corporation, Chuku, Kobe, Japan). Serum ionised calcium and pH were determined using an ICA2 ionised calcium analyser (Radiometer, Copenhagen, Denmark).

Erythrocytes (1.5 ml) were washed three times with ice-cold 140 mM choline chloride. After centrifugation at 5500 r.p.m. at 4°C for 3 min, the supernatant and the top layer of the cells were discarded by aspiration. The cells were lysed with double-distilled water and the Na⁺, K⁺ and Mg²⁺ concentrations were measured by atomic absorption spectrometry. The intra-assay ($\pm$ s.e.) variation determined in 10 blood samples was $1.63 \pm 0.31\%$ for

iNa⁺ and $2.12 \pm 0.12\%$ for iK⁺. The inter-assay variation determined in five blood samples was $2.37 \pm 1.03\%$ for iNa⁺ and $3.22 \pm 1.18\%$ for iK⁺.

Determination of genotypes

Genomic DNA was extracted from peripheral blood. Allelic discrimination of the ADD1 460 glycine (Gly)/460 tryptophan (Trp) and the ADD2 C1797 T polymorphism was carried out using the 5'-nuclease assay⁹ on an ABI Prism 7700 apparatus (Perkin Elmer, Foster City, CA, USA). The forward and reverse primers and the 460Gly and 460Trp probes employed in the TAQMan2 assay were 5'-CGTCCAC ACCTTAGTCTTCGACTT-3' 5'-GGAGAAGACAAGA TGGCTGAACTC-3', 5'-FAM-TTCCATTCTGCCCTTC CTCGGATAMRA-3' and 5'-TET-TTCCATTCTGCCA TTCCTCGGAA-TAMRA-3', respectively (where FAM, TAMRA and TET are dyes used with the TAQMan system). For the ADD2 C1797T polymorphism, the forward and reverse primers and the 1797C and 1797T probes employed in the TAQMan2 assay were 5'-AGG AACGAGAGCCAGGCTCT-3' 5'-TTCATCAAAACAC ACACCTACCAAT-3', 5'-VIC2-TTCTTCAGCGTTGC CCTCCACATTAMRA2-3' and 5'-FAM2-TCTTCAGT GTTGGCCCTCCACATCTG-TAMRA2-3' (where VIC2, TAMRA2 and FAM2 are dyes used with the TAQMan system). Per 25 μ l, the polymerase chain reaction (PCR) fluid contained 50 ng of DNA, 200 nmol of primers, 50 nmol of FAM probe and 100 nmol of VIC probe. The amplification conditions were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 62°C for 1 min. The genotyping procedure was established after we had confirmed the polymorphism (C1797T, starting from ATG; dbSNP number rs4984; URL: <http://www.ncbi.nlm.nih.gov/SNP>) by sequencing 17 individuals using the ABI Prism Big Dye Terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA, USA). We tested the frequency of the polymorphism in a random population of 250 blood donors from North Italy, and found that the minor allele had a frequency of 15%. For quality control, 10% of the DNA samples in our study were genotyped in duplicate in a blinded fashion. Duplicate genotypes were confirmatory in all samples.

Statistical analysis

For database management and statistical analysis, we used SAS software, version 9.1.3 and JMP, version 6 (SAS Institute, Cary, NC, USA). Measurements with a skewed distribution were normalised by logarithmic transformation. Comparisons of means and proportions were performed using Student's *t*-test and Fisher's exact test, respectively. We computed Pearson's correlation coefficients between the cation concentrations in erythrocytes and serum and compared these correlation coefficients between women and men using Fisher's *z*-transformation.

To examine the relationship of the intra-erythrocyte cation concentrations with serum and urinary cations, anthropometric characteristics, and various environmental and lifestyle factors, we used step-wise linear regression analysis with the *P*-value for covariates to enter and stay in the model set at 0.15. We considered as possible explanatory variables: age, BMI, the serum concentrations of sodium, potassium, magnesium and ionised and total calcium, the 24-hour urinary excretion of sodium, potassium, magnesium and calcium and their excretion ratios, current smoking (0, 1) and the quantity of alcohol consumed per day. Because of our previously reported sex-specific findings on the association between hematological phenotypes and genetic polymorphisms, we analysed women and men separately.^{5,10} We performed retrospective power calculations, using the PROC POWER procedure.

Results

Anthropometric characteristics and cation measurements

The 259 participants included 136 women (52.3%) and 32 hypertensive patients (12.3%) of whom only one was taking antihypertensive drugs. Age ranged from 20 to 76 years. Table 1 lists the characteristics of the study population by gender. Systolic and diastolic BP, blood haemoglobin concentration, haematocrit and erythrocyte count were lower in women. iNa was higher in men than women, whereas the

opposite was true for iK (Table 2). As expected, the serum concentrations of sodium, potassium and magnesium and the 24-hour urinary excretion of sodium, potassium and calcium were lower in women than men (Table 2).

Smoking was equally prevalent among women and men. The median number of cigarettes smoked per day was 15 (interquartile range (IQR) 10–25). Compared to women, more men reported alcohol intake of at least 5 g daily (Table 1). Among regular drinkers, the median daily alcohol consumption was 20 g (IQR 10–23.2) in women and 20 (IQR 10–33.5) in men. Of the premenopausal women, 15 (17.9%) took oral contraceptives. None of the 52 postmenopausal women was on hormone replacement therapy.

In men, we found positive and significant correlations between iMg and serum total calcium ($r = 0.22$, $P = 0.013$) and between iK and serum Mg^{2+} ($r = 0.27$, $P = 0.029$; Figure 1). In women, the corresponding correlation coefficients were -0.08 ($P = 0.32$) and 0.09 ($P = 0.32$), respectively. The *P*-values for the sex differences in the strength of these associations were 0.14 and 0.013, respectively.

Phenotype–genotype associations

The frequencies of the α -adducin (ADD1) genotypes (GlyGly 61.5%, Trp 38.5%) and the β -adducin (ADD2) genotypes (CC 80.4%, T 19.6%) did not deviate from Hardy–Weinberg equilibrium ($P > 0.15$). Sex distribution, anthropometric characteristics and BP did not differ according to the α - and β -adducin genotypes.

Table 1 Characteristic of the study participants

Characteristics	Women (n = 136)	Men (n = 123)	P-value
Age (years)	46.8 ± 13.3	48.7 ± 13.4	0.25
BMI (kg/m ²) ^a	25.2 ± 3.9	25.9 ± 3.3	0.11
SBP (mm Hg) ^b	119.7 ± 14.5	126.0 ± 18.5	0.0042
DBP (mm Hg) ^b	71.4 ± 8.5	74.5 ± 8.7	0.0034
Smokers (n%)	39 (28.7)	38 (30.7)	0.73
Alcohol intake ≥ 5 g/day (n%)	12 (8.8)	37 (29.8)	<0.0001
Contraceptive pill use (n%)	15 (11)		
Postmenopausal status ^c (n%)	52 (38)		
Haemoglobin (mmol/l)	8.28 ± 0.71	9.37 ± 1.00	<0.0001
Haematocrit (%)	38.3 ± 2.9	42.7 ± 2.7	<0.0001
Serum erythrocyte count (10 ¹² cells/l)	4.30 ± 0.38	4.85 ± 0.34	<0.0001

Abbreviations: BMI, body mass index, DBP, diastolic blood pressure; SBP, systolic blood pressure.

Plus-minus values are mean ± s.d.

P-values are for the differences between women and men.

^aBody mass index is weight in kilograms divided by the square of height in metres.

^bOnly one participant was on anti-hypertensive drug treatment.

^cNone of the postmenopausal women was on hormonal replacement therapy.

Table 2 Intra-erythrocyte, serum and urinary cation concentrations

Characteristics ^a	Women (n = 136)	Men (n = 123)	P-value [†]
<i>Intra-erythrocyte</i>			
Sodium (mmol/l cells)	6.42 ± 1.01	6.86 ± 1.03	0.0005
Potassium (mmol/l cells)	87.60 ± 4.32	86.12 ± 4.58	0.0078
Magnesium (mmol/l cells)	1.86 ± 0.20	1.90 ± 0.22	0.15
<i>Serum</i>			
Sodium (mmol/l)	139.9 ± 1.7	141.0 ± 1.8	<0.0001
Potassium (mmol/l)	4.02 ± 0.33	4.12 ± 0.31	0.0209
Magnesium (mmol/l)	0.80 ± 0.07	0.83 ± 0.07	0.0009
Total calcium (mmol/l)	2.35 ± 0.09	2.37 ± 0.10	0.10
Ionised calcium at pH 7.4 (mmol/l)	1.27 ± 0.52	1.27 ± 0.54	0.98
<i>Urine</i>			
Sodium (mmol/24 h)	141 ± 54	175 ± 72	<0.0001
Potassium (mmol/24 h)	67 ± 22	78 ± 29	0.0008
Calcium (mmol/24 h)	4.29 ± 2.45	5.10 ± 2.51	0.0087
Magnesium (mmol/24 h)	4.76 ± 4.83	5.24 ± 4.42	0.40
Na/K	2.31 ± 1.89	2.35 ± 0.82	0.81

[†]*P*-values are for the differences between women and men.

^aPlus-minus values are mean ± s.d.

Figure 1 Correlations between cations in erythrocytes and serum in 123 men. Open symbols denote β -adducin (ADD2) 1797CC homozygotes and closed symbols T allele carriers.

Figure 2 Intra-erythrocyte cation concentrations in 123 men by β -adducin (ADD2) genotype. Horizontal lines in the scatterplot represent means.

In men, BAD CC homozygotes ($n=100$) compared to T-carriers ($n=23$) had slightly lower iK (85.8 versus 87.5 mmol/l cells, $P=0.107$), higher iMg (1.92 versus 1.80 mmol/l, $P=0.012$), but similar iNa (6.86 versus 6.88, $P=0.93$; Figure 2). In men iK, iMg and iNa did not differ according to the α -adducin genotypes or the interaction between the α - and β -adducin polymorphisms. In 136 women, none of the phenotype-genotype relations reached significance (Table 3, $P>0.49$). A sensitivity analysis, from which we excluded hypertensive patients, produced results similar to those reported above, in both men and women. Retrospective power calculations suggested that 120 subjects provided approximately 70 and 60% power to demonstrate significant ($P<0.05$) differences on one- and two-sided tests, respectively.

In stepwise regression analysis involving men, iK ($R^2=0.128$) increased with age ($P=0.029$) and serum sodium ($P=0.0667$), but decreased with serum total calcium ($P=0.0456$) and the amount of alcohol consumed per day ($P=0.0163$). iMg ($R^2=0.087$) decreased with age ($P=0.0272$), but increased with serum total calcium ($P=0.0217$). After adjustment for these covariates, findings in men for iK (CC versus T, 85.8 versus 87.3 mmol/l, $P=0.14$) and iMg (1.91 versus 1.82 mmol/l, $P=0.03$) remained consistent.

Discussion

The key finding of our study was that in men intra-erythrocyte magnesium was on average 0.12 mmol/l cells lower in β -adducin 1797T-allele carriers compared to CC homozygotes, with an opposite trend in the intra-erythrocyte potassium concentration. In women, these associations with the ADD2 C1797T polymorphism did not reach statistical significance. In both sexes, none of the phenotype-genotype relations with the α -adducin Gly460Trp polymorphism reached significance. In men, there was also no interaction between the ADD1 and ADD2 genotypes in relation to the intra-erythrocyte cation concentrations.

Investigations in rats,¹¹ interventions in never-treated hypertensive patients and epidemiological studies^{12–14} have shown that the Trp point mutation in the α -adducin subunit is associated with a phenotype characterised by higher activity of the sodium pump, hence increased tubular sodium reabsorption in the kidney and ultimately hypertension. Alterations in the erythrocyte membrane mirror those in renal tubular cells. Glorioso and co-workers¹⁵ investigated erythrocytes from 268 never-treated North Sardinian patients with essential hypertension (34.7% women). They noticed that at V_{max} , the Na,K-pump, the Na,K,Cl-cotransport and the Li, Na-countertransport were faster in α -adducin Trp allele carriers than GlyGly homozygotes.¹⁵ The current study included only 12.3% hypertensive patients and had a sample size, which was insufficient to confirm the well-known associations of BP with the α -adducin Gly460Trp polymorphism.

We have demonstrated previously that in men consuming alcohol β -adducin 1797CC homozygosity was associated with lower erythrocyte count, haemoglobin level and haematocrit.⁵ We hypothesised that male CC homozygotes compared with T allele carriers might have a more fragile erythrocyte membrane and that alcohol intake might unveil this innate greater fragility of erythrocytes.⁵ In other words, the β -adducin CC genotype might invigorate the structural and functional haematological disturbances associated with excessive alcohol intake. Higher intra-erythrocyte potassium concentration increases the hydration and osmotic stability of

Table 3 Intra-erythrocyte electrolyte concentrations by genotype (unadjusted)

Characteristic ^a	α -Adducin allele			β -Adducin allele		
	GlyGly	Trp	P-value [†]	CC	T	P-value [†]
<i>Women</i>						
Sodium (mmol/l cells)	6.45 $\pm$ 0.11	6.37 $\pm$ 0.13	0.66	6.40 $\pm$ 1.04	6.49 $\pm$ 0.86	0.91
Potassium (mmol/l cells)	87.60 $\pm$ 0.49	87.59 $\pm$ 0.57	0.99	87.53 $\pm$ 4.50	87.88 $\pm$ 3.59	0.93
Magnesium (mmol/l cells)	1.87 $\pm$ 0.02	1.85 $\pm$ 0.21	0.49	1.86 $\pm$ 0.20	1.88 $\pm$ 0.22	0.73
<i>Men</i>						
Sodium (mmol/l cells)	6.82 $\pm$ 1.01	6.95 $\pm$ 1.07	0.49	6.86 $\pm$ 1.04	6.88 $\pm$ 1.00	0.93
Potassium (mmol/l cells)	85.94 $\pm$ 0.51	86.45 $\pm$ 0.70	0.56	85.80 $\pm$ 4.33	87.47 $\pm$ 5.41	0.11
Magnesium (mmol/l cells)	1.90 $\pm$ 0.22	1.90 $\pm$ 0.22	0.85	1.92 $\pm$ 0.22	1.80 $\pm$ 0.19	0.01

Abbreviations: Gly, glycine; Trp, tryptophan.

[†]P-values are for the significance of differences between genotypes.^aPlus-minus values are mean $\pm$ s.d.

circulating erythrocytes.⁶ We therefore investigated in a subsample of our previous study⁵ possible association between the intra-erythrocyte cation concentrations and the β -adducin C1797T polymorphism. In the current study, male β -adducin CC homozygotes compared with T allele carriers had a slightly, albeit not significantly lower intra-erythrocyte potassium concentration. These findings are in line with the above hypothesis.⁵

The intra-erythrocyte potassium concentration is regulated via the K_{ATP}-cotransport and the Ca²⁺-activated K-channel, also known as the Gardos-channel. Opening of the channel increases K⁺ efflux through the membrane by three orders of magnitude.^{16,17} Magnesium inhibits the opening of the Gardos channel. Intra-erythrocyte magnesium represents only 0.5% of the total body content of magnesium. Intra-erythrocyte magnesium and potassium are tightly regulated.¹⁸ This may explain why, in the current study, we did not find any association between the intra-erythrocyte concentrations of magnesium and potassium. However, the positive correlation between the intra-erythrocyte potassium concentration and serum magnesium is consistent with the notion that magnesium inhibits the Gardos channel¹⁹ and might increase the intra-erythrocyte potassium concentration. Another potential mechanism affecting cytosolic magnesium is the energy-requiring extrusion, which might be coupled to the movement of other cations. To what extent such ion exchange might underlie the positive correlation between intra-erythrocyte magnesium and serum total calcium remains to be elucidated. Irrespective of the mechanism involved, the difference among men in the intra-erythrocyte magnesium concentration between CC homozygotes and T-allele carriers is unlikely to contribute to the hypothesised higher innate fragility of erythrocytes of CC homozygotes.

Several factors might explain the absence of any phenotype-genotype association in women in our previous⁵ as well as in our current study. Haematological phenotypes such as erythrocyte count,

haemoglobin level and haematocrit are influenced by menstrual blood loss and by the use of oral contraceptives or hormone replacement therapy. Fluctuations in female sex hormones owing to menopausal status or menstrual cycle may mask haematological phenotypes associated with genetic mutation of the β -adducin gene.

The present study has to be interpreted within the context of its limitations. We investigated a 13.9% subsample of our previous study population.⁵ Our study lacked the statistical power to demonstrate the effects of the α -adducin Trp allele on BP and the intra-erythrocyte sodium concentration and the effects of alcohol intake *per se* on erythrocyte count, haemoglobin concentration and haematocrit.⁵ Although the P-value for the genotypic difference in intra-erythrocyte magnesium associated with the β -adducin polymorphism was 0.01, we cannot exclude with certainty a type 1 error. Our present findings are therefore only hypothesis-generating and are currently being tested in a larger group of never-treated hypertensive patients.

What is known about the topic

- Genetic variability in the ADD1 (Gly460Trp) and ADD2 (C1797T) subunits of the cytoskeleton protein adducin plays a role in the pathogenesis of hypertension, possibly via changes in intracellular cation concentrations.^{1,3–5,13}
- ADD2 1797CC homozygous men have decreased erythrocyte count and haematocrit.⁵
- Intra-erythrocyte cation concentrations affect the osmotic stability of red blood cell membrane.^{6,16,17}

What this study adds

- ADD2 1797CC homozygous men exhibit slightly lower concentrations of intra-erythrocyte potassium, higher magnesium but similar sodium compared with T-allele carriers.
- In 136 women, none of the phenotype-genotype relations reached significance.
- Changes in intra-erythrocyte cations in ADD2 1797CC homozygous men might lead to osmotic fragility of erythrocytes, but to what extent they reflect systemic changes or are possibly involved in blood pressure regulation remains unknown.

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