Reduced expression of regulator of G-protein signaling 2 (RGS2) in hypertensive patients increases calcium mobilization and ERK1/2 phosphorylation induced by angiotensin II

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Context **RGS2 (regulators of G-protein signaling) is a negative regulator of $G_{\alpha q}$ protein signaling, which mediates the action of several vasoconstrictors. RGS2-deficient mouse line exhibits a hypertensive phenotype and a prolonged response to vasoconstrictors.**

Objective **To compare RGS2 expression in peripheral blood mononuclear cells (PBMs) and cultured fibroblasts from normotensive subjects and hypertensive patients.**

Methods **PBMs were isolated from 100 controls and 150 essential hypertensives. Additionally, fibroblasts were isolated from skin biopsy of 11 normotensives and 12 hypertensives and cultured up to the third passage. Quantitative mRNA and protein RGS2 expression were performed by real-time quantitative reverse transcriptase-polymerase chain reaction and by immunoblotting, respectively. Free Ca^{2+} measurement was performed in monolayers of 24-h serum-deprived cells, using FURA-2 AM. Phosphorylation of the extracellular signal-regulated kinases ERK1/2 was measured by immunoblotting. Polymorphism (C1114G) in the 3′ untranslated region of the RGS2 gene was investigated by direct sequencing and real-time polymerase chain reaction (PCR).**

Results **RGS2 mRNA expression was significantly lower in PBM and in fibroblasts from hypertensives, in comparison to normotensives. C1114G polymorphism was associated with RGS2 expression, with the lowest values in GG hypertensives. The 1114G allele frequency was increased in hypertensives compared with normotensives. Angiotensin II-stimulated intracellular Ca^{2+} increase and ERK1/2 phosphorylation were higher in fibroblasts from hypertensive patients compared with control subjects, and in those with the G allele, independently of the blood pressure status. The angiotensin II-stimulated Ca^{2+} mobilization and ERK1/2 phosphorylation were negatively correlated with RGS2 mRNA expression.**

Conclusion **Low expression of RGS2 contributes to increased G-protein-coupled signaling in hypertensive patients. The allele G is associated with low RGS2 expression and blood pressure increase in humans.**

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Keywords: angiotensin II, arterial hypertension, fibroblasts, intracellular calcium, RGS2

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Introduction

Signaling by G-protein-coupled receptors (GPCRs) regulates blood pressure by controlling arterial tone, electrolyte and fluid balance [1]. GPCR signal transduction is triggered by dissociation of the GTP-bound G_{α} from the $G_{\beta\gamma}$ dimer. The dissociated subunits interact with effector molecules to propagate the intracellular signal [2]. The duration and intensity of the cellular response to external signals are largely limited by GTPase activity, intrinsic to G_{α} subunits, that catalyzes the conversion of active guanosine triphosphate (GTP)-bound into inactive guanosine diphosphate (GDP)-bound G_{α}, which, in turn, can reassociate with the $G_{\beta\gamma}$ and receptors [3].

A family of proteins called RGS (regulators of G-protein signaling) terminates G-protein signaling by accelerating the rate of GTP hydrolysis by G_{α} subunits [4]. Among the members of the RGS family, RGS2 displays regulatory selectivity for the $G_{\alpha q}$ subclass of G proteins. Many important cardiovascular hormones, such as angiotensin II (Ang II) and norepinephrine, activate receptors that

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couple to $G_{\alpha q}$ [5]. These hormones are potent vasoconstrictors and have been implicated in the pathogenesis of hypertension. In mice, disruption of the RGS2 gene increases blood pressure and prolongs vasoconstrictor responses of the peripheral resistance vasculature *in vivo* and of aortic vascular smooth muscle cells *in vitro* [6]. Furthermore, rare mutations in RGS2 have been associated with hypertension in a Japanese cohort [7].

It has been demonstrated that RGS2 regulates blood pressure by mediating the ability of nitric oxide (NO) pathway to relax the resistance vasculature and attenuate vasoconstrictor signaling in vascular smooth muscle cells [8]. A recent study showed that RGS2 mRNA expression is down-regulated in the vessel wall in a rat N^{ω}-nitro-L-arginine methyl ester model of hypertension [9]. However, to our knowledge, the role of RGS2 in human hypertension has scarcely been assessed. Aims of the present study were, therefore, to measure the expression of RGS2 in peripheral blood mononuclear cells (PBMs) *ex vivo* and in cultured fibroblasts *in vitro* in normotensive control and hypertensive patients, and to correlate it with Ca^{2+} mobilization and extracellular signal-regulated kinase (ERK1/2) phosphorylation induced by Ang II.

Methods

Subjects

We randomly selected 150 untreated patients aged > 18 years, with office diastolic blood pressure > 90 mmHg and/or office systolic blood pressure > 140 mmHg, among the patients evaluated for the first time between 1 January 2004 and 30 April 2005 at the hypertension outpatient clinic of the University of Padua Medical School. The diagnosis of essential hypertension was based on all the clinically required tests, which included measurements of plasma catecholamines, cortisol, and renal artery ultrasonography in selected cases. None of the patients had cardiac failure, evidence for coronary heart disease or renal failure. As a control group, we randomly recruited 100 healthy normotensive controls, without diabetes, cardiac failure, evidence for coronary heart disease or renal failure, among employees undergoing a regular medical check-up. The clinical characteristics of the normotensive controls and of the hypertensive patients are reported in Table 1. A randomly selected subgroup of 11 healthy normotensive volunteers (five men, six women, aged 45 ± 2 years), without a family history of hypertension and diabetes, and 12 patients with essential hypertension (five men, seven women, aged 38 ± 3 years) also gave separate consent to a skin biopsy for fibroblast studies.

Table 1 Characteristics of the study cohort

	Normotensive controls ($n = 100$)	Hypertensive patients ($n = 150$)	*P*
Sex (M/F)	39/61	76/74	NS
Age (years)	46.0 ± 1.5	48.0 ± 1.0	NS
BMI (kg/m^2)	23.4 ± 0.3	26.2 ± 0.4	0.0001
SBP (mmHg)	117.2 ± 1.4	152.3 ± 1.4	0.0001
DBP (mmHg)	76.3 ± 0.9	95.7 ± 1.0	0.0001
HR (bpm)	73.9 ± 1.4	75.3 ± 1.0	NS

BMI, body mass index; bpm, beats per minute; DBP diastolic blood pressure; F, female; HR, heart rate; M, male; SBP, systolic blood pressure. Values are means ± SEM.

All gave an informed consent to the study, which had been approved by the local ethical committee.

RGS2 expression in peripheral blood mononuclear cells

PBMs were collected from heparinized blood (10–15 ml) over Histopaque-1077 (Sigma-Aldrich, Milano, Italy), as described previously [10]. PBMs were washed three times with saline phosphate buffer (PBS) and suspended in OMNIzol (Euroclone, Lugano, Switzerland). Total RNA was extracted in accordance to the manufacturer's suggested protocol. RNA was resuspended in RNAse-free water and its quality and quantity were determined by LabChip technology (Agilent Bioanalyzer 2100; Agilent Technologies, Waldbroom, Germany) and by the ratio from the absorbance readings at 260 nm and 280 nm with a spectrophotometer (Perkin-Elmer, Foster City, California, USA). To eliminate DNA contamination, 1 μg of total RNA was treated with DNase I, Amp Grade (Invitrogen, Carlsbad, California, USA).

Primers and probes for gene expression analysis of RGS2 and of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with multiplex real-time quantitative reverse transcription-polymerase chain reaction (QRT-PCR) were designed from sequences derived from the GenBank database using Primer 3 (provided by the Whitehead Institute, Cambridge, Massachusetts, USA) and Operon's Oligo software (Operon Technologies Inc., Alameda, California, USA). The probe of RGS2 subunit was labeled with a reporter 5,6-carboxyfluorescein (FAM) dye at its 5′-end and a quencher Black Hole 1 at its 3′-end, while the probe of the housekeeping gene was labeled with Texas Red dye at its 5′-end and Black Hole 2 at its 3′-end. The sequence and amplicon length of both genes are shown in Table 2.

Equal amounts of cDNA were synthesized by reverse transcription in 20 μl reaction mixture using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, California, USA).

The reaction was allowed to proceed in the iCycler iQ thermal cycler (Bio-Rad) for 5 min at 25°C, followed by 45 min of heating at 42°C, and 5 min at 85°C. Two microliters of cDNA were amplified in a multiplex real-time PCR reaction using the iQ Supermix (Bio-Rad). All the reactions (samples) were performed in 96-well plates, in triplicate. A negative control containing all reagents except cDNA template was included in all runs. Real-time QRT-PCR was performed following the thermal protocol: 94°C for 3 min to denature, 45 cycles at

Table 2 Primers and probes for RGS2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

Oligonucleotide	Sequence	Genome localization
Forward primer RGS2	AAAAGCCACAAATCACCACAGA	631–652[a]
Reverse primer RGS2	TCCCCTCAGGAAAAAGAATG	702–722[a]
TaqMan probe RGS2	FAM-AGGGAGCCCAGAAATGGAGGACA-BH1[b]	677–699[a]
Forward primer GAPDH	CTCTACTGGCGCTGCC	687–702[c]
Reverse primer GAPDH	CACCACTGACAGTTGG	789–805[c]
TaqMan probe GAPDH	Texas Red-GCTCACTGGCATGGCCTTCCG-Dabcyl	741–761[c]

[a]Nucleotide positions are based on the sequence in GenBank Accession No. NM 002923. [b]5,6-Carboxyfluorescein attached to the 5′ terminus and Black Hole 1 to the 3′ terminus. [c]Nucleotide positions are based on the sequence in GenBank Accession no. M33197. Dabcyl, 4-(49-dimethylaminophenylazo) benzoic acid.

94°C for 30 s for denaturing and 60°C for 1 min for annealing and extension.

Relative quantification was performed by standard curves for RGS2 and GAPDH, total RNA preparation and comparative Ct (ΔΔCt) methods [11]. For each sample, Ct of the RGS2 gene was normalized with Ct of endogenous GAPDH, to compensate for inter-PCR variations. The normalized values were compared with a normalized calibrator (one normal control) to calculate RGS2 gene expression, expressed in arbitrary units as ΔΔCt. The efficiency of the amplification was close to 100% for both RGS2 and GAPDH genes.

Direct sequencing of RGS2

For the detection of single nucleotide polymorphisms (SNPs), total RNA from 10 normotensives and 10 hypertensives was reverse-transcribed using SuperScript RNase H^- II Reverse Transcriptase (RT) (Invitrogen) in a 20 μl reaction mixture. A mixture of 1 μg of RNA, 1 μl of oligo$(dT)_{12-18}$ (50 μmol/l) and 1 μl deoxynucleoside triphosphate (dNTP) mix (10 mmol/l each) was heated to 65°C and chilled on ice; 4 μl of 5× first-strand buffer and 1 μl (0.1 mol/l) of dithiothreitol were added and incubated at 42°C for 2 min. Finally 1 μl (200 units/μl) of the SuperScript II RT was mixed and the reverse transcription reaction was carried out in a iCycler iQ thermal cycler (Bio-Rad) at 42°C for 50 min. The enzyme was inactivated at 70°C for 15 min.

cDNA was amplified by two different primer pairs. The first primer pair flanked the coding sequence of RGS2 (GenBank: NM002923). They were designed as: ATGC AAAGTGCTATGTTCTTGG [sense, nucleotide position (nt) 33/55], TGTAGCATGAGGCTCTGTGG (antisense, nt 647/667).

Another primer pair (sense: TGGCCTTAGGTAGCTG GT, nt: 1009/1026; and antisense: AGCATTACATGAG ACAACAGTAC, nt: 1146/1169) flanking the 3′ untranslated region (UTR), was selected on the basis of the report in GenBank of a described polymorphism (refSNP ID: rs4606), mapping in position 1114 in the 3′UTR.

The PCR was carried out in a final volume of 50 μl using Platinum *Taq* DNA polymerase High Fidelity Kit (Invitrogen), with 2 μl of cDNA in 5 μl of 10× High Fidelity PCR buffer, 2 μl of $MgSO_4$ (50 mmol/l), 1 μl dNTP mix (10 mmol/l), 1 μl of the sense primer and 1 μl of the antisense primer (each 10 μmol/l), 0.2 μl Platinum *Taq* High Fidelity and sterile water. The thermal protocol followed was: 94°C for 2 min for denaturation and 40 cycles at 94°C for 30 s for denaturation, at 60°C for 30 s for annealing and 1 min at 68°C for extension.

The products were subsequently purified using the ExoSAP-IT PCR Clean-Up method (USB, Ohio, USA): 5 μl of PCR product was mixed with 2 μl of ExoSAP-IT and incubated at 37°C for 15 min. ExoSAP-IT was inactivated by heating to 80°C for 15 min. The purified cDNA was quantified by spectrophotometric reading at 260 nm. The samples were dried at 65°C for 5 min in duplicate, each containing an equal amount of primer sense or antisense (3.2 pmol).

The products were sequenced directly in an automated sequencer (ABI PRISM 3100 DNA Sequencer; Applied Biosystems, Foster City, California, USA) at the Biomolecular Research Center (University of Padova). For sequence analysis the free version of the ABIView software (http://bioinfo.weizmann.ac.il/pub/software/abiview/abiview.html) was used. The resulting sequences were compared with the reference GenBank sequence through the BLAST software (http://www.ncbi.nlm.nih.gov/blast/).

C1114G genotyping

Cells were collected from the buffy coat obtained from heparinized blood (2 ml) from all patients and controls by centrifugation (400 *g* for 30 min at 4°C). Genomic DNA was obtained through the NucleoSpin blood kit (Macherey-Nagel, Düren, Germany).

Primers and probes for allelic discrimination analysis of SNP C1114G (ref SNP: rs4606) were designed from sequences derived as described above.

The probe for the G allele was labeled with a reporter 5,6-carboxyfluorescein (FAM) dye at its 5′-end and a quencher 4-(4′-dimethylaminophenylazo)benzoic acid (Black Hole 1) dye at its 3′-end, while the probe of the allele C was labeled with Texas Red dye at its

Table 3 Primers for single nucleotide polymorphism C1114G genotyping

Oligonucleotide	Sequence	Genome localization
Forward primer	TCCATGTTACCACATAGTAG	1049–1068[a]
Reverse primer	GCATTACATGAGACAACAG	1150–1168[a]
TaqMan probe allele C	Texas Red-TCAATACCGTTGCACATA-BH2	1105–1122[a]
TaqMan probe allele G	FAM-TTCAATACCCTTGCACATA-Dabcyl[b]	1105–1123[a]

[a]Nucleotide positions are based on the sequence in GenBank Accession No. NM 002923. [b]5,6-Carboxyfluorescein attached to the 5′ terminus and 4-(49-dimethylaminophenylazo) benzoic acid attached to the 3′ terminus.

5′-end and Black hole 2 at its 3′-end. The sequence and amplicon length of both genes are shown in Table 3.

Two microliters of purified DNA were amplified in a real-time PCR reaction in the iCycler iQ system (Bio-Rad). All the reactions were performed in 96-well plates, using the iQ Supermix (Bio-Rad), as described above. Positive controls, genotyped by direct sequencing, were included in each run, together with a negative control containing no DNA template. The amplification was performed with the following thermal protocol: 94°C for 3 min to denature, 40 cycles at 94°C for 30 s for denaturing and 53°C for 1 min for annealing and extension.

Messenger RNA and protein RGS2 expression in cultured fibroblasts

Fibroblasts were derived from a skin biopsy taken from the anterior surface of the left forearm by excision, under topical anesthesia with ethyl chloride, and cultured in nutrient mixture F-10 HAM medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and 4 mmol/l glutamine, as described previously [12]. Briefly, the cells were seeded on to a 75-cm^2 flask, incubated at 37°C, and the medium was changed every 2–3 days. Fibroblasts obtained from each subject and grown separately were used for the experiments after the third passage. At confluence, the cells were left in a quiescent medium for one night before RNA and protein extraction. They were identified morphologically, and there were no morphological differences in fibroblasts from hypertensives and normotensives. The cells were used in the plateau phase of growth and, in the same experimental conditions, they produce collagen, as shown by [^{3}H]proline incorporation and mRNA expression studies [13,14].

Messenger RNA RGS2 expression was determined by RT-PCR in real time as described above. For RGS2 protein expression cultured fibroblasts were scraped into ice-cold lysis buffer [12.5 mmol/l Tris, 2 mmol/l EGTA, 1 mmol/l dithiothreitol, 2 mmol/l Na_3VO_4, 10 μmol/l phenylmethylsulfonyl fluoride (PMSF), 1 μmol/l leupeptin, 5 μmol/l aprotinin] and sonicated. Proteins were separated by electrophoresis through a 12% polyacrylamide gel and were electroblotted on to nitrocellulose membrane (Hybond ECL, Amersham Biosciences Europe, Freiburg, Germany). Membranes were incubated with RGS2 antibody (Santa Cruz Biotechnologies, Santa Cruz, California, USA), as described previously [10]. Membranes were then incubated with 1:5000 goat anti-rabbit antibody conjugated to horseradish peroxidase (Amersham Biosciences Europe). Detection was made using SuperSignal West Pico Chemiluminescent Substrate (Perbio Science, Erembodegem, Belgium). The density of the protein bands was analyzed by Versa-Doc 1000 Chemiluminescence Molecular Imaging Systems (Bio-Rad), and the results expressed as ratio to GAPDH protein expression.

Measurement of intracellular calcium in cultured fibroblasts

Intracellular Ca^{2+} (Ca_i^{2+}) was determined in cultured fibroblasts from eight control subjects and 10 hypertensive patients by fluorescent spectrophotometric analysis with FURA-2 AM, as described previously [12]. Briefly, the cells (5×10^5) were seeded on to coverslips (3 cm × 1 cm) and allowed to grow to confluence. Then the medium was changed to a quiescent medium without serum and the cells used after 24 h. Before starting the experiments, the cells were loaded with 3 μmol/l FURA-2 AM for 1 h at room temperature. The coverslip was placed into a quartz cuvette inside a fluorescent spectrophotometer (Shimadzu RF-1501, Kyoto, Japan). The baseline fluorescence was obtained by rapidly alternating the excitation wavelength between 340 and 380 nm and recording the 510 nm emission intensity. Intracellular Ca^{2+} levels were calculated from the fluorescence ratio recordings according to the standard formula: $[Ca^{2+}] = K_d [(R - R_{min})/(R_{max} - R)](Sf_2/Sb_2)$. K_d was taken as 224 nmol/l, R_{max}, R_{min} and Sf_2/Sb_2 were calculated by a calibration curve with buffers containing different Ca^{2+} concentrations [15].

Ang II (100 nmol/l) was added to the perfusion solution when baseline fluorescence was stable, and the fluorescence measurements continued until Ca_i^{2+} recovered to basal level. Ang II-induced Ca_i^{2+} mobilization was rapid and transient, with a peak between 60 and 120 s.

Immunoblot analysis of ERK1/2 expression and phosphorylation

ERK1/2 expression and phosphorylation were determined in fibroblasts from seven normotensives and seven hypertensives at baseline and after Ang II (1 μmol/l for 2 min)

stimulation, as described previously [16]. The cells, grown in 10% FBS F10-HAM medium until confluence, were made quiescent by incubation in serum-free F10-HAM for 24 h.

Statistical analysis

Analysis was carried out using the SPSS software package (version 10.0.1; SPSS Inc., Chicago, Illinois, USA). Relations between variables were assessed using Spearman's ρ correlation for continuous variables and χ^2 or Fisher exact test for categorical variables. Student's *t*-test and univariate analysis of variance with Bonferroni correction for multiple comparisons were used to compare means among alleles. The significance level was set to $\alpha = 0.05$. Results are given as means ± SEM.

Results

RGS2 expression in peripheral blood mononuclear cells

The mRNA expression for RGS2 was significantly reduced in PBMs from hypertensive patients in comparison with normotensive subjects (0.75 ± 0.04, $n = 150$, versus 1.14 ± 0.08, $n = 100$, $\Delta\Delta C_t$, $P < 0.001$). In the whole cohort, mRNA expression for RGS2 was negatively correlated with systolic ($\rho = -0.232$, $P = 0.001$) and diastolic blood pressure ($\rho = -0.210$, $P = 0.002$), but not with body mass index (BMI) ($\rho = -0.134$, NS) or heart rate ($\rho = -0.001$, NS). No difference was observed with regards to sex distribution.

RGS2 C1114G polymorphism

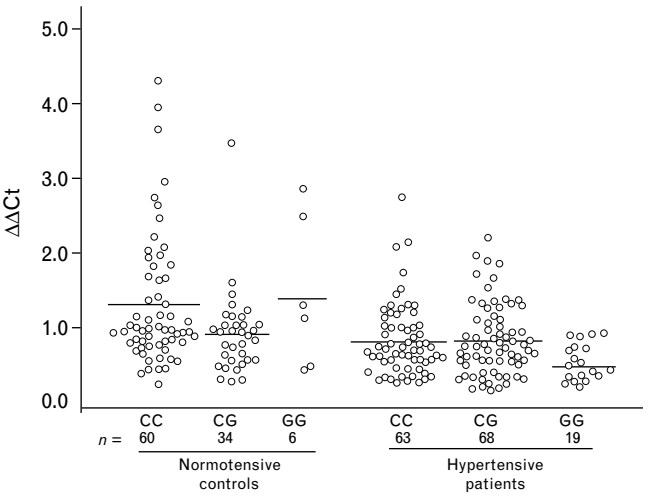
The direct sequencing of the coding region of RGS2 in 10 normotensives and 10 hypertensives did not show evidence of polymorphism. Therefore, untranslated regions of mRNA were explored for single nucleotide polymorphisms (SNPs), as they are known to play crucial roles in the post-transcriptional regulation of gene expression, including modulation of the transport of mRNA out of the nucleus, translation efficiency, subcellular localization and stability [17].

A polymorphism (1114C/G) in region 3′UTR of the RGS2 gene has recently been described [GenBank ref SNP ID: rs4606]. C1114G polymorphism was present in normotensive subjects and hypertensive patients (Table 4). The genotype frequency of hypertensives was significantly different from that of normotensives ($\chi^2 = 8.507$, $P = 0.014$) and the 1114G allele frequency increased in hypertensive patients in comparison with normotensive subjects (0.35 versus 0.23, $P = 0.005$).

Table 4 Genotype frequencies of C1114G polymorphism

	Normotensive controls ($n = 100$)	Hypertensive patients ($n = 150$)
CC genotype	60 (60.0%)	63 (42.0%)
CG genotype	34 (34.0%)	68 (45.3%)
GG genotype	6 (6.0%)	19 (12.7%)

Fig. 1



Regulators of G-protein signaling (RGS2) gene expression in human peripheral blood mononuclear cells (PBMs), according to C1114G polymorphism (rs4606), by quantitative reverse transcriptase-polymerase chain reaction (QRT-PCR). For each sample, the Ct value of the RGS2 gene was normalized with the Ct value of endogenous glyceraldehyde-3-phosphate dehydrogenase (GAPDH), to compensate for inter-PCR variations. These normalized values were compared to the normalized values of a calibrator (a normal control), to calculate the RGS2 gene expression, according to the ΔΔCt method [11]. The data are expressed in arbitrary units (ΔΔCt).

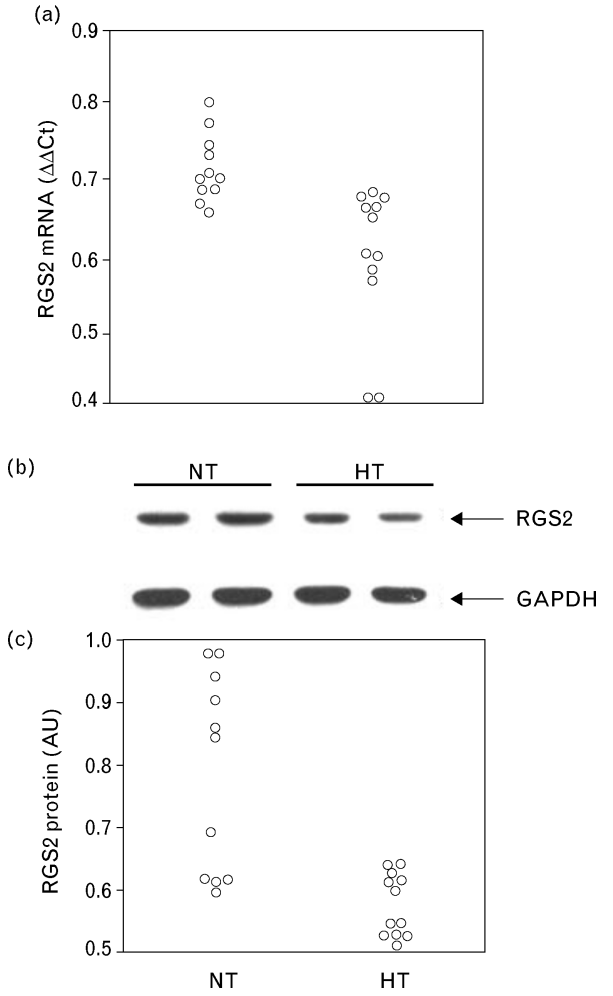
RGS2 mRNA expression was significantly correlated with the C1114G polymorphism ($\sigma = 0.196$, $P = 0.002$), with the lowest values in GG carriers. Hypertensive carriers of the GG genotype had a reduced mRNA expression for RGS2 in comparison with hypertensive carriers of CG genotype and of CC genotype (0.48 ± 0.06, $n = 19$, versus 0.78 ± 0.06, $n = 68$, versus 0.80 ± 0.07, $n = 63$, $\Delta\Delta C_t$, $P = 0.025$), but the same was not observed in normotensive controls (Fig. 1).

RGS2 expression and effect of angiotensin II on intracellular Ca^{2+} and ERK1/2 phosphorylation in fibroblasts

The mRNA expression for RGS2 was significantly reduced in fibroblasts from hypertensive patients in comparison with normotensive subjects (0.61 ± 0.03, $n = 12$, versus 0.72 ± 0.01 $\Delta\Delta C_t$, $n = 11$, $P < 0.01$) (Fig. 2a). Western blot analysis of extracts of fibroblasts confirmed the lower expression for RGS2 protein expression in hypertensives in comparison with normotensive controls (0.58 ± 0.01, $n = 12$, versus 0.78 ± 0.05 AU, $n = 11$, $P < 0.01$) (Fig. 2b and c).

Ang II (100 nmol/l)-stimulated Ca_i^{2+} peak was increased in fibroblasts from hypertensives compared to normotensives (246 ± 27, $n = 10$, versus 149 ± 19 nmol/l, $n = 8$, $P < 0.05$), while basal Ca_i^{2+} levels were similar (87 ± 8, $n = 10$, versus 71 ± 9 nmol/l, $n = 8$, NS) (Fig. 3). The percentage increase from baseline elicited by Ang II

Fig. 2

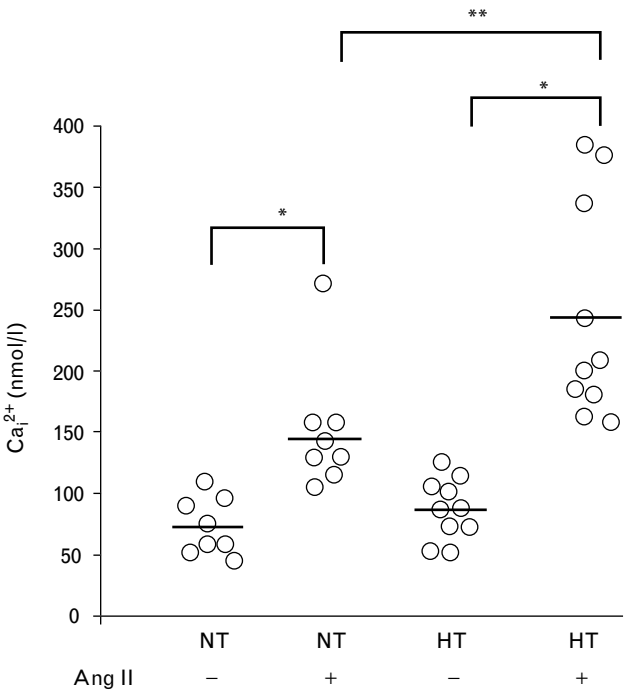


RGS2 gene and protein expression in fibroblasts from normotensive subjects (NT, $n = 11$) and from hypertensive patients (HT, $n = 12$). Skin fibroblasts were cultured in normal growth medium until confluence and made quiescent in serum-free medium for 24 h. (a) Quantitative analysis of gene expression calculated by ΔΔCt methods. mRNA was extracted as described in the Methods section and quantitative mRNA RGS2 expression was performed by quantitative reverse transcriptase-polymerase chain reaction (QRT-PCR). (b) A representative immunoblot for RGS2 and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in two NT (left-hand lanes) and two HT (right-hand lanes). Proteins were extracted as described in the Methods section and immunoblots for RGS2 and GAPDH were performed. (c) Quantitative analysis of protein expression (each point represents the RGS2/GAPDH ratio of a single patient).

exposure was negatively correlated with mRNA RGS2 expression ($\rho = -0.476$, $n = 18$, $P < 0.05$).

ERK1 phosphorylation stimulated by Ang II (1 μmol/l, for 2 min) was significantly higher in fibroblasts from hypertensives than from normotensives (1.19 ± 0.25, $n = 7$, versus 0.50 ± 0.07 AU, $n = 7$, $P < 0.05$), whereas basal ERK1 phosphorylation and basal and Ang II-stimulated ERK2 phosphorylation did not differ. Ang II-stimulated ERK1 and ERK2 phosphorylation were negatively

Fig. 3



Free intracellular Ca^{2+} (Ca_i^{2+}) in quiescent fibroblasts at baseline and after acute angiotensin II (Ang II) stimulation (100 nmol/l, for 2 min) in normotensive subjects (NT, $n = 8$) and hypertensive patients (HT, $n = 10$). $^*P < 0.01$ versus unstimulated fibroblasts, $^{**}P < 0.01$ versus NT.

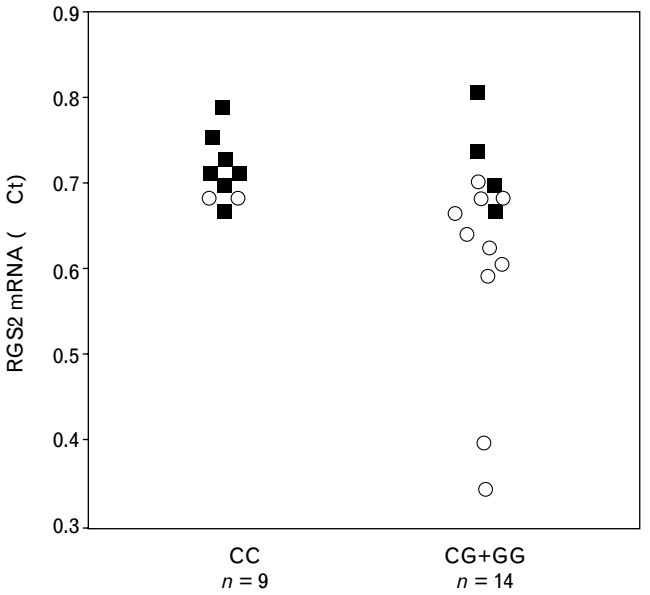
correlated with mRNA expression for RGS2 ($\rho = -0.71$, $n = 14$, $P < 0.01$, and $\rho = -0.65$, $n = 14$, $P < 0.05$, respectively).

Patients and controls were pooled to study the influence of the RGS2 C1114G polymorphism on gene and protein expression, as well as on Ang II-induced Ca_i^{2+} mobilization and ERK1/2 phosphorylation in fibroblasts, independently of the blood pressure category. As there was only one GG genotype (one hypertensive patient), it was pooled with CG. RGS2 gene expression was significantly reduced in fibroblasts carrying the G allele in comparison with those with CC genotype (0.63 ± 0.03, $n = 14$, versus 0.71 ± 0.01 $\Delta\Delta C_t$, $n = 9$, $P < 0.05$) (Fig. 4).The difference of RGS2 protein expression was similar, but it did not reach statistical significance (0.64 ± 0.04, $n = 14$, versus 0.73 ± 0.05 AU, $n = 9$).

Ca_i^{2+} mobilization induced by Ang II (100 nmol/l) was higher in fibroblasts with CG + GG genotype compared to those with CC genotype (143 ± 20, $n = 13$, versus 72 ± 10 nmol/l, n = 5, $P < 0.01$), while basal Ca_i^{2+} level was similar (82 ± 7 versus 74 ± 13 nmol/l, NS).

ERK1/2 expression was similar in fibroblasts with CG + GG genotype, compared to those with CC

Fig. 4



RGS2 mRNA and protein expression in fibroblasts from normotensive subjects (squares, $n = 11$) and hypertensive patients (dots, $n = 12$), by C1114G polymorphism. Patients and controls were pooled to study the influence of this polymorphism on gene expression. Since there was only one GG genotype (one hypertensive patient), he was pooled with CG. $P < 0.05$ CC versus CG + GG.

genotype (data not shown). Basal and Ang II-stimulated ERK1 phosphorylation were higher in fibroblasts with the G allele than in fibroblasts with CC genotype (0.72 ± 0.15, $n = 9$, versus 0.31 ± 0.04 AU, $n = 5$, $P < 0.05$, in basal conditions, and 1.10 ± 0.20, $n = 9$, versus 0.39 ± 0.04 AU, $n = 5$, $P < 0.01$, after Ang II). Similarly, ERK2 phosphorylation was higher in fibroblasts with CG + GG genotype compared to patients with CC genotype (0.62 ± 0.07, $n = 9$, versus 0.42 ± 0.04 AU, $n = 5$, $P < 0.05$, in basal conditions; and 0.95 ± 0.11, $n = 9$, versus 0.63 ± 0.08 AU, $n = 5$, $P < 0.05$, after Ang II).

Discussion

This study reports the first evidence that: (i) RGS2 expression is reduced in PBMs *ex vivo* and in cultured fibroblasts from hypertensive patients in comparison with normotensive subjects; (ii) there is a different frequency distribution of the C1114G polymorphism in hypertensives in comparison to normotensives; (iii) C1114G polymorphism is associated with RGS2 expression, with the lowest values in GG hypertensives; and (iv) the mobilization of Ca^{2+} and ERK1/2 phosphorylation induced by Ang II are higher in fibroblasts from hypertensive patients and associated with low RGS2 gene and protein expression and with the 1114G allele. These data, therefore, suggest that RGS2 is a candidate gene in human essential hypertension because it can increase the cell responses to Ang II and other vasoconstrictive agents.

Ang II is a potent vasoconstrictor and regulates several physiological responses, such as salt and water balance, and vascular tone, and thus plays a critical role in the regulation of blood pressure [18]. Ang II activates several effectors, such as phospholipase C, protein kinase C, and regulates intracellular levels of second messengers, such as diacylglycerol and inositol phosphate [19]. The homeostasis of intracellular Ca^{2+} is regulated mainly through AT_1R, which is a $G_{\alpha q}$-coupled receptor [18]. Intracellular Ca^{2+} mobilization induced by Ang II is increased in various cell lines from animal models of hypertension and from human hypertensive patients [18,19].

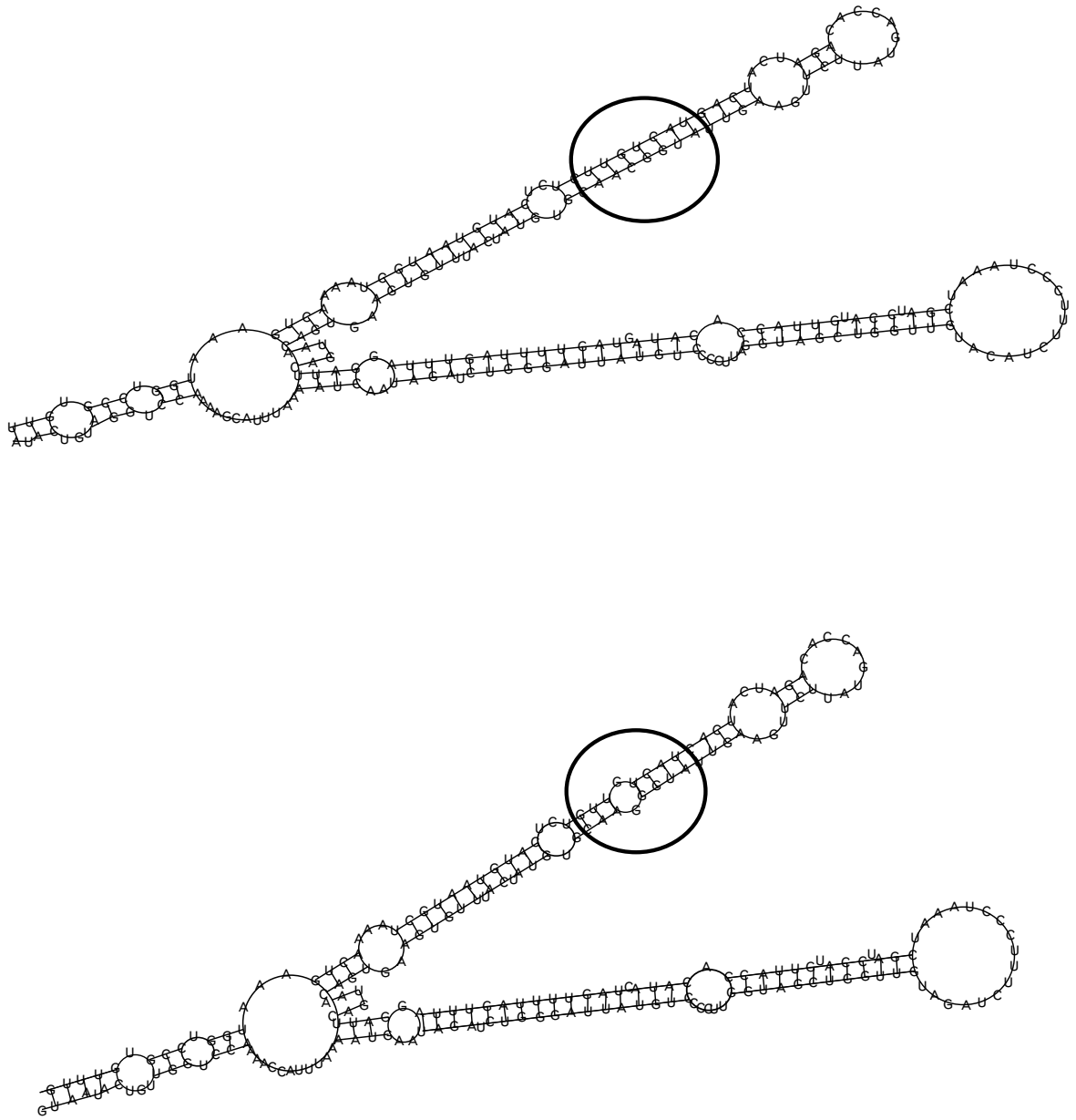
Alterations of the signaling system of Ang II, affecting both long and short-term related effects of this peptide, contribute to the vascular structural and functional changes leading to hypertension. RGS2, one of the members of the RGS family, has a selective inhibitory effect for $G_{\alpha q}$-mediated signal [4]. It can interact also with $G_{\alpha i}$, albeit with a lower specificity [20]. Once activated, RGS2 increases the GTPase activity of $G_{\alpha q}$, leading to a diminished cellular activation by the $G_{\alpha q}$-coupled receptor [5]. Recently, it has been demonstrated that the levels of blood pressure are markedly increased in the RGS2 knockout mouse [6]. Furthermore, we demonstrated that RGS2 is increased and maximally stimulated in patients with Bartter's and Gitelman's syndromes, which are characterized by salt wasting, chronic hypotension, high Ang II levels and resistance to its vasoconstrictive action [21].

RGS2 expression is stimulated by NO [7], through phosphorylation by cyclic guanosine S-monophosphate-dependent protein kinase I-alpha [22], and by Ang II [23]. Therefore, we measured RGS2 expression in circulating PBMs *ex vivo* and in cultured fibroblasts, after many passages in standard conditions, to avoid the possible influences of circulating NO, Ang II and other agonists *in vivo*.

Our data demonstrate, for the first time, a reduction of both protein and mRNA expression of RGS2 in PBMs and in cultured fibroblasts from hypertensive patients. PBMs are relevant for the pathogenesis of atherosclerosis [24] and are abnormal in hypertension [25], while fibroblasts are actively involved in the process of renal and cardiovascular fibrosis and in the development of target organ damage in hypertensive patients [26].

Skin fibroblasts express a variety of different receptors, including G-protein-coupled Ang II type 1 [27], and can be cultured for several passages in standardized conditions, offering a useful model for the investigation of intrinsic (possibly genetic) defects of cell function,

Fig. 5



Secondary structures of a portion of RGS2 mRNA predicted by the Vienna Package Software. The sequence of the C1114G is highlighted. The first result is referred to the sequence carrying the C allele and the second to the sequence carrying the G allele. The presence of the G allele creates an internal loop in the structure that may cause a destabilization of the mRNA molecule.

independent of the environmental abnormality caused by hypertension *in vivo*. In fibroblasts, we demonstrated that the expression of RGS2 correlates with calcium response and ERK1/2 phosphorylation induced by Ang II, thereby providing further experimental evidence for a role of RGS2 in the modulation of Ang II signaling. In our cohort, Ang II-induced Ca_i^{2+} mobilization and ERK1/2 phosphorylation were enhanced in hypertensives in comparison with normotensives. These differences can be attributed, at least partially, to lower RGS2 expression.

The present study provides one possible explanation for the different RGS2 expression in normotensives and hypertensives, by showing that one polymorphism of 3′UTR mRNA is involved in RGS2 expression. RGS2 expression was related to this polymorphism and the lowest RGS2 expression was present in the hypertensive patients homozygous for 1114G allele. Untranslated regions of mRNA were explored for SNPs, as they are known to play crucial roles in the post-transcriptional regulation of gene expression, including modulation of mRNA transport out of the nucleus, translation

efficiency, subcellular localization and stability [17]. Unlike DNA-mediated regulatory signals, whose activity is essentially mediated by their primary structure, the biological activity of regulatory motifs at the RNA level relies on a combination of primary and secondary structure. The C1114G polymorphism maps within one of the two conserved AU-rich elements (ARE) in the 3′UTR of RGS2. It has recently been demonstrated that the ARE regions of RGS2 are involved in mRNA decay in T lymphocytes after inflammatory stimuli [28]. The analysis of the secondary structures of mRNA, predicted by the Vienna Package Software [29], results in different conformations of the sequence according to the allele. In particular, the G allele creates an internal loop in the structure that may cause a destabilization of the mRNA molecule (Fig. 5). The fact that this SNP, mapping in a regulatory sequence of mRNA, is associated with lower gene expression is in agreement with its role in the post-transcriptional regulation of gene expression, while the higher frequency of the 1114G allele among the hypertensives than among the normotensives can be explained by the acceleration of the age-dependent blood pressure rise, due to increased sensitivity to vasoconstrictors, caused by reduced RGS2 expression.

Yang *et al.* [7] investigated other SNPs of RGS2 for an association with hypertension in a population-based sample. They showed that 1026T > A and 1891–1892delTC were associated with hypertension in women and with a borderline significance [7]. Only longitudinal studies may clarify whether any such SNPs are correlated with blood pressure increase and increased risk of developing hypertension. The fact that our study in a relatively small cohort shows an association between RGS2 and hypertension suggests that RGS2 is an interesting candidate gene for hypertension studies and encourages further investigations.

The RGS2 gene is localized in chromosome 1, position q31. Genome-wide scan studies have already identified loci of chromosome 1 that are associated with blood pressure regulation and hypertension in humans. Perola *et al.* [30] found a link between hypertension and the marker D1S1679, which maps to position 1q21–q22 (at 170,84 cM according to the Marshfield Human Genetic Map), in north-east Finnish sibpairs. Mitchell *et al.* [31] demonstrated a region of chromosome 1 at 192 cM, corresponding to 1q23, linked with mean arterial pressure in the Framingham Study offspring cohort. Other markers associated with hypertension have been identified in 1p35 [32], 1p13 [33] and 1p36.32 [34]. None of the above-mentioned loci is close to 1q31. Therefore, we propose that C1114G polymorphism does not affect blood pressure regulation directly, but, more likely, through the modulation of RGS2 gene expression and, therefore, by modulating the hypertensive action of other genes, such as those of the renin–angiotensin system. We exclude a role of obesity in the link between low RGS2 expression and hypertension, as we did not find any correlation between RGS2 expression and BMI.

In conclusion, reduced RGS2 expression may account for an exaggerated G-protein signaling and a larger Ca^{2+} release and ERK1/2 phosphorylation induced by Ang II and, probably, by other vasoconstrictive agents, and it may contribute to the pathogenesis of essential hypertension and its associated cardiovascular damage.

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