Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 303 (2003) 230–237
BBRC
www.elsevier.com/locate/ybbrc

Tissue-specific modulation of β-adducin transcripts in Milan hypertensive rats

Grazia Tripodi,[a,*] Rossana Modica,[a] Cristina Reina,[a] and Giuseppe Bianchi[b]

[a] *Prassis-Sigma Tau Research Institute, Via Forlanini, 1, 20029 Settimo Milanese, Milano, Italy*
[b] *Divisione di Nefrologia Dialisi e Ipertensione, Universita' Vita-Salute, Ospedale San Raffaele, Milano, Italy*

Received 6 February 2003

Abstract

Genetic variants in Adducins, a family of cytoskeleton proteins (α, β, and γ) encoded by three genes, have been associated with primary hypertension in humans and in Milan hypertensive (MHS) rats. The present paper describes the identification of a rat β4 alternative splicing isoform differing from β subunit for an in-frame insertion of 18 amino acids and showing a polymorphic site (R592W) between MHS and its normotensive control (MNS). Furthermore, we established a quantitative real-time PCR assay for analyzing the tissue expression of adducin gene family and determining whether any subunit transcript demonstrates altered expression during the development of MHS hypertension, especially in tissues relevant for the control of cardiovascular phenotypes (i.e., kidney, left ventricle, and large arteries). Among the three adducins only β transcripts were modulated, in a tissue-specific manner, during the development of hypertension in MHS, compared to age-matched MNS controls. A 43% decrease in renal outer medulla was already present at the prehypertensive phase; a 70% decrease in femoral artery and 66% increase in left ventricle were observed after the development of hypertension. Surprisingly β4-Add, which is a minor component of total β transcripts, is drastically reduced up to 88% in all MHS tissues. Alteration in β-Add expression levels may account, at least in part, for the observed phenotypic changes in MHS hypertension.

Keywords: Adducin; Real-time quantitative RT-PCR; Hypertension; Genetic; Alternative splicing; Rats; Inbred MHS

Experimental evidences including kidney cross-transplantation, body fluid and renal function changes, and ion transport across erythrocyte membranes showed many similarities between the Milan hypertensive rats (MHS) and a subset of patients with primary hypertension [1]. By different approaches it was demonstrated that Adducin (ADD) polymorphisms are involved in determining the functional characteristics underlying hypertension in these two species. ADD is a membrane-skeletal protein ubiquitously expressed as combination of α/β and α/γ oligomers, which plays an important role in determining cellular morphology and motility [2,3]. The α-Add (F316Y) polymorphism alone or in combination with those of β (Q529R) and γ-Add (Q572K) was shown to explain a portion of the blood pressure difference between MHS and its control Milan normotensive strain (MNS) [4,5] and to modulate cell ion transport through the activation of the Na–K pump [6,7]. Our recent results on congenic rats indicate that β and γ-Add can control left ventricle mass and glomerulosclerosis ([8] and unpublished data). Also the polymorphism identified in human α-ADD (G460W), although occurring in a different position within the protein, was found linked to hypertension [9] and ion transport [10] and shown to activate the Na–K pump [7]. In spite of some subsequent negative association studies in human [11,12], the most recent data are now concordant with the involvement of ADD in a variety of human cardiovascular affections. In fact, α-ADD "hypertensive variant," alone or in interaction with those of β-ADD (C1797T) or other genes, was recently found associated to the increased risk of hypertension ([13], and Lanzani C. unpublished data 2002), coronary heart disease [14], left ventricular mass [15], stiffness, and thickness of femoral artery [16,17], progression of renal

* Corresponding author. Fax: +39-2-33500408.
E-mail address: grazia.tripodi@prassis.it (G. Tripodi).

0006-291X/03/$ - see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S0006-291X(03)00330-9

failure [18,19], and a selective beneficial effect of diuretics on the incidence of stroke and myocardial infarction [20]. The data so far accumulated clearly indicate that ADD polymorphisms similarly affect various cardiovascular and renal phenotypes either in humans and rats.

The three ADD genes map on different chromosomes and show a very complex pattern of expression with alternative spliced isoforms identified in human, rat, and mouse [21–27]. Among all these isoforms we put our attention on β4-Add, since the only polymorphism (C1797T) localized on the human β-ADD coding region is localized on this alternative spliced isoform. A comprehensive assay of tissue expression shows that α and γ-ADD are expressed in all tissues, whereas expression of β-ADD is restricted, with the highest levels in brain and erythropoietic tissues [28].

In an attempt to further elucidate the molecular basis of the ADD-associated dysfunctions, we have analyzed in the present work, the expression profiling of the ADD gene family in relevant rat tissues, to give an additional contribution in the assessment of ADD role in humans. The objective of the present investigation was 3-fold: (i) identify the rat β4 isoform, (ii) give an accurate quantification of transcripts for Add subunits in a panel of rat tissues applying a powerful method, the real-time quantitative PCR, to detect and quantify mRNAs even if present in very low amounts, and (iii) determine which, if any subunit of adducin gene expression, is differently modulated in relevant tissues (i.e., kidney, left ventricle, and large arteries) before and after the development of hypertension.

Methods

Inbred rat strains. The studies were performed in 27 or 150-day-old inbred male MNS and MHS rats derived from our original stock colony (Prassis Research Institute, Settimo Milanese, Italy). Rats were anesthetized with ether and killed by decapitation. Tissues from liver, spleen, bone marrow, kidney cortex, kidney outer medulla, adrenal gland, left ventricle, aorta, femoral artery, carotid artery, brain, and axolemma were dissected, immediately frozen in liquid nitrogen, and stored at −70 °C.

Eighteen normotensive and three hypertensive inbred rat strains used in the present work were the same as previously described [25]. The other rat strains were SBN (Sabra hypertension-resistant rats) and SBH (Sabra hypertension-prone rats) kindly provided by Dr. Yagil (Barzilai Medical Center, Ashkel, Israel).

RNA extraction and cDNA synthesis. Total RNA was isolated by using RNeasy Kit from Qiagen (Hilden Germany) according to the manufacturer's protocols. RNA samples were further purified by on-column DNAse digestion using RNase-Free DNase set from Qiagen. Agarose gel electrophoresis and spectrophotometic absorbance determined RNA quality and verified its concentration. Two micrograms of total RNA was reverse transcribed in a 100 μl reaction mixture with RT-PCR kit and random hexamer as primers (ABI P/N N808-0234). For each comparative experiment, all samples were reverse transcribed at the same time and stored at −70 °C.

Real-time quantitative PCR. The amount of mRNA for α, γ, β, and β4-Add was measured by real-time quantitative RT-PCR with fluorogenic probes (Taqman) using Applied Biosystems prism model 7700 Sequence Detection Instrument. The optimal primer and probe sequences used for amplification as designed by Primer-Express and the base pair size of the corresponding PCR products are listed in Table 1. Primer pairs and probe for each Add subunit have been selected spanning exon junctions and no coamplification or interference of residual contaminating genomic DNA was verified (data not shown). The coding region cDNA for α, γ, and β4-Add was cloned in pBSK+ vector and plasmid was quantified as the number of molecules. The plasmid was serially diluted 5-fold to obtain a standard curve of seven points with a mean concentration similar to the target in RNA samples. All curve points and samples were tested in triplicate. The β4-Add.pBSK+ recombinant vector was used for both β and β4-Add RNA quantitation. To compare the sample tissues with each other, the mRNA levels for Add subunits were corrected for 18S ribosomal RNA which was measured using a kit (ABI P/N 4308310).

Isolation and characterization of β4-Add clone and polymorphism genotyping. The complete coding region of β-Add was amplified from bone marrow cDNA with specific primer pairs with synthetic restriction sites.

5′-CCGGAATTCGCTAGCATGAGTGAGGACACGGTC/5′-CCGCTCGAGACCCACCTGAGCCACTAATCA and cloned in *Eco*RI/*Xho*I sites of pBSK+ vector. Two of 20 clones, corresponding to the β4 isoform, were sequenced in both strands.

A rat genomic DNA fragment of 1045 bp containing the β4 specific exon and the flanking introns was amplified with primer pairs 5′-

Table 1
Fluorogenic probe and primer sets

Target gene	Primer/probe	Sequence	Length (bp)
α-Add	Forward	CACCTCCCAAGTGGCAGATT	81
	Reverse	AGCCAGTTCTGTAGCCCAGATT	
	Probe	AATTTGAAGCTCTTATGCGGATGCTCGA	
γ-Add	Forward	TCCTTCACCGGTTTTTCTTCA	74
	Reverse	TCTGCACCAGGAAGGTCATTAA	
	Probe	CTCCCCTGAGTCTTGGCATGGTCACT	
β-Add	Forward	GCACCTCCCACGCAGTCT	69
	Reverse	GGAGGTCATTGATGGGTGTCA	
	Probe	CCCAGCTTCCTCCATGAACTTCTCCA	
β4-Add	Forward	GGAAAGGAAGAAGCTAGAACAGGA	77
	Reverse	GAGAGCTGCAGGCCCTCC	
	Probe	AGGAAGGAGCAGAGGCAAACCTGAGC	

CAACTCACTGACCAGGAGCTG/5′-GTGTGGACTTTACAGGT GAAC and direct sequenced in both strands (GenBank Accession No. AY226987).

The polymorphic site of β4-Add was amplified in MNS, MHS, and 23 inbred rat strains with the primer pairs 5′-CAGCCATGTGGTA GACAAGAC/5′-ACCTTAGTACACAAGTCACAG and the 187 bp PCR product was applied to duplicate nylon filters and hybridized with P32 end-labeled ASO probe (5′-TGAGCTACGGAGGGC/5′-TG AGCTATGGAGGGC).

Statistical analysis. All data are presented as means ± SE. Comparisons between MNS and MHS values were performed by Student t test with StatView Software. All significant data reported reach a value of $P < 0.01$.

Results

Identification of β4-Add mRNA with a polymorphism between MHS and MNS

Cloning of β-Add cDNA revealed the existence of a new variant in the precursor RNA, differing by the presence of 54-nt encoding amino acid between codons 583 and 584 (Fig. 1A). The insertion maintained the β-Add reading frame with the addition of 18 amino acids in the corresponding translational product. The exon–intron structure of the rat β-Add gene is unknown but, assuming a similarity between rat and human gene, this new variant represents the rat counterpart of human β4-ADD that includes exon 15 in its transcription product. The rat DNA region encompassing the correspondent exon 15 with flanking introns was amplified and sequenced. The acceptor and donor sites are conforming to mammalian consensus splice sites (Fig. 1B). In spite of the high similarity of gene structure, nucleotide and amino acid sequences for β-ADD among species, the rat β4 isoform shows striking divergence. In fact the human variant is a truncated carboxyterminus peptide as a result of an out of frame insertion of 86 nucleotides [27]. Comparative sequence analysis between MNS and MHS revealed an Arginine to Tryptophane substitution in the codon 592 of β4-Add isoform. This polymorphism was investigated in additional 19 normotensive and four hypertensive inbred rat strains. A different allelic frequency was found in normotensive $R = 0.45$, $W = 0.55$ with respect to hypertensive strains $R = 0.2$, $W = 0.8$ (Fig. 1C).

Quantitative analysis of Add gene family

Several Add variants, due to alternative splicing of the corresponding gene, are known for each subunit. The α-Add primer pairs and probe were selected to anneal to a sequence conserved among all the known rat

A

```
CAA GAA G |GA AGG AGC AGA GGC AAA CCT GAG CTA CGG |
 Q   E   G|  R   S   R   G   K   P   E   L   R*   |
        583
|AGG GCC TGC AGC TCT CCC GCC AGA A|GG GAG AAG GAC
| R   A   C   S   S   P   A   R   R|  E   K   D
                                     584
```

B

Exon n°	3′ splice acceptors	Exon sequence	5′ splice donors	Intron sizes
14		AAG	GTaaacctgtgtgagccatagc	836 bp
15	attgcccccttttggtcccccAG	GAA--GAA	GTaactaagtagaacagcactg	92 bp
16	taaggtgggcttgttgtcttcAG	GGG		

C

Normotensive strains				Hypertensive strains	
MNS	R	AUG	W	MHS	W
ACI	R	BN	W	LH	W
BUF	R	F344	W	SBH	W
CBH	R	LN	W	SS/JR	W
COP	R	LOU	W	SHR	R
DA	R	LOU-C	W		
LEW	R	LUDW	W		
PVG	R	MW	W		
WKY	R	SBN	W		
WF	W	SR/ JR	W		

Fig. 1. Sequencing of rat cDNA and genomic DNA reveals β4-Add isoform and a polymorphic site in inbred rat strains. (A) The 54 nt encoding amino acids in β4 isoform (boxed) are inserted in frame between codons 583 and 584 of β-Add. The amino acid polymorphism between MNS and MHS is indicated (asterisk). (B) 3′ Acceptor and 5′ acceptor splice sites of the rat genomic clone encompassing the alternative spliced exon for β4-Add and the flanking introns. Exon numbers are deduced by the corresponding human ones. (C) β4-Add amino acid polymorphism in 20 normotensive and 5 hypertensive inbred rat strains.

α-Add (α1, α2, and α3) alternative splicing variants [24]. Similarly β and γ-Add primers/probes were selected to recognize the known β (β1, β2, and β4), and γ-Add (γ1 and γ2) rat isoforms, respectively ([24,25] and present data).

The relative abundance of Add family mRNAs has been determined by real-time quantitative RT-PCR in twelve tissues of two young (27-day-old) and two old (150-day-old) MNS rats. For each subunit quantification, all the tissues were included in the same experiment and the results were corrected for 18S ribosomal RNA and expressed as arbitrary units relative to liver values defined as 1. Fig. 2 shows the differential expression of α, β, and γ-Add mRNAs found in the tissues analyzed. The α-Add and γ-Add relative amounts ranged from 0.5 to 7 both in young and old animals. A strong expression of β-Add mRNA was found in bone marrow, brain, and axolemma whose relative amounts were, respectively, 93, 45, and 17 both in young and old animals, and in spleen (57 in young and 9.8 in old animals). We were able to quantify very low amount of β-Add RNA also in all the other tissues whose relative amounts are reported in Fig. 1.

The absolute amount of mRNAs for individual Add subunits was determined in young and old MNS rats ($n = 2$–6 rats in each group). Table 2 shows the quantitative proportions of α, β, and γ-Add for each tissue, expressed as a percentage of the sum of mRNAs for all three subunits taken together. The results indicate that the mRNA for α-Add exceeded, almost ubiquitously, the concentrations of β-Add and γ-Add subtypes, ranging from 62% to 83% in young animals and from

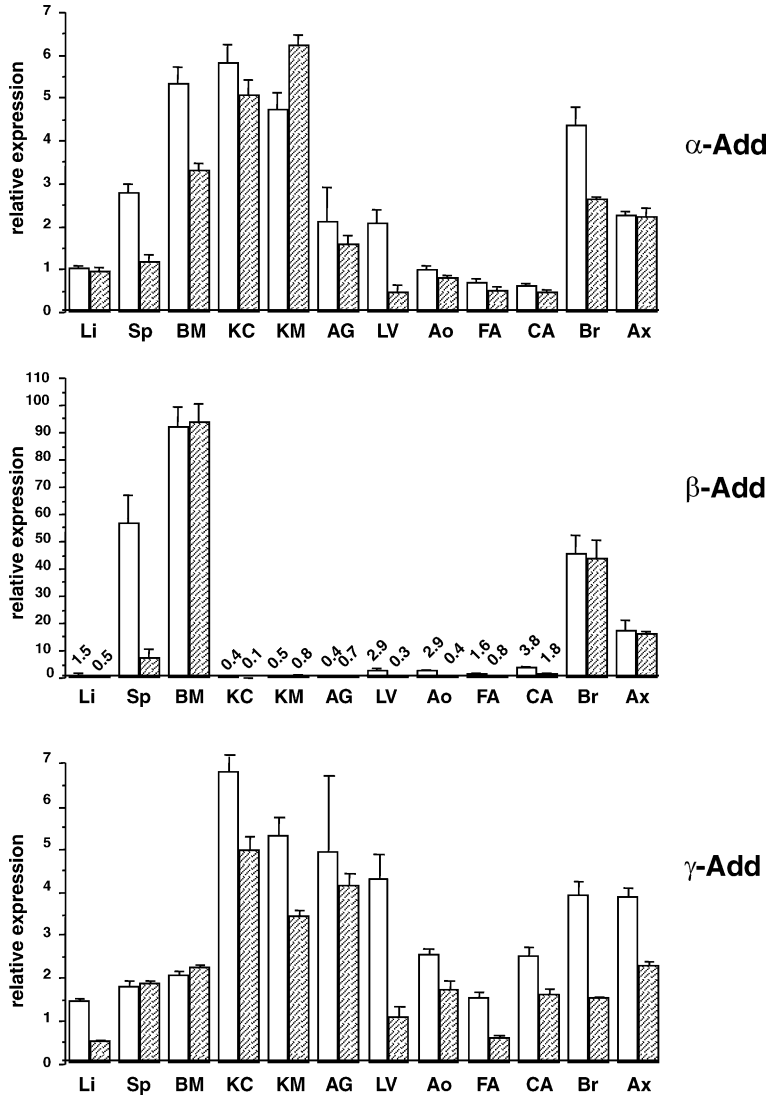

Fig. 2. Quantitative RT-PCR analysis of α, β, and γ-Add (mRNAs abundance in rat tissues). Data are normalized for 18S ribosomal RNA and expressed as arbitrary units relative to liver values defined as 1. Values are means ± SE of two young (27 days, white bars) and two old (5 months, hatched bars) rat tissues each tested in triplicate. Li, liver; Sp, spleen; BM, bone marrow; KC, kidney cortex; KM, kidney outer medulla; AG, adrenal gland; LV, left ventricle; Ao, aorta; FA, femoral artery; CA, carotid artery; Br, brain; and Ax, axolemma. The lower mean values for β-Add are reported.

Table 2
Relative representation of mRNAs for Add family in rat tissues

Tissue	No.	Young rats				Old rats			
		α-Add	β-Add	γ-Add	β4/β-add	α-Add	β-Add	γ-Add	β4/β-add
Li	2	74.8 ± 4.0	9.8 ± 0.3	15.4 ± 0.8	2.2 ± 0.2	84.7 ± 2.8	4.6 ± 0.3	10.7 ± 1.0	3.4 ± 0.9
Sp	2	66.1 ± 2.3	30.1 ± 2.4	3.9 ± 4.1	3.4 ± 0.3	78.1 ± 1.2	10.2 ± 2, 8	11.7 ± 1.6	4.4 ± 0.1
BM	6	61.8 ± 1.8	34.9 ± 0.7	3.2 ± 0.2	10.6 ± 0.7	46.0 ± 1.4	51.1 ± 2.2	2.9 ± 0.1	16.7 ± 0.7
KC	6	70.0 ± 2.4	0.31 ± 0.03	29.7 ± 1.3	2.9 ± 0.5	84.8 ± 4.7	0.71 ± 0.07	14.4 ± 1.2	2.2 ± 0.4
KM	6	73.6 ± 6.6	0.28 ± 0.04	26.1 ± 3.3	2.1 ± 0.4	90.4 ± 6.4	0.87 ± 0.1	8.7 ± 0.55	2.1 ± 0.2
AG	2	77.4 ± 0.4	1.8 ± 0.2	20.8 ± 0.2	3.2 ± 0.2	75.8 ± 5.2	1.9 ± 0.3	22.3 ± 4.9	2.7 ± 0.4
LV	5	65.9 ± 1.1	9.6 ± 0.9	24.5 ± 0.5	1.7 ± 0.1	68.1 ± 0.7	5.4 ± 0.6	26.5 ± 0.7	2.3 ± 0.2
Ao	2	83.2 ± 2.1	1.9 ± 0.4	14.8 ± 1.8	5.4 ± 0.1	88.1 ± 1.3	1.1 ± 0.1	10.7 ± 1.3	8.4 ± 0.9
FA	5	72.0 ± 2.4	21.2 ± 0.8	6.7 ± 0.3	1.99 ± 0.1	82.1 ± 2.0	11.7 ± 0.2	6.1 ± 0.2	2.2 ± 0.24
CA	5	61.8 ± 1.4	29.4 ± 0.7	8.8 ± 0.2	2.4 ± 0.1	76.7 ± 1.3	10.6 ± 0.3	12.7 ± 0.3	4.3 ± 0.2
Br	6	70.9 ± 3.8	20.4 ± 1.4	8.7 ± 1.1	3.0 ± 0.25	64.0 ± 2.9	27.4 ± 2.8	8.6 ± 0.6	5.9 ± 0.8
Ax	2	73.2 ± 0.7	15.9 ± 0.2	10.9 ± 0.9	1.3 ± 0.1	75.2 ± 0.9	13.9 ± 0.3	10.9 ± 1.2	1.4 ± 0.1

Data for individual α-Add, β-Add, and γ-Add mRNAs are reported as percentage over the sum of the three adducin mRNA levels. The relative amount of the β4-Add mRNA is expressed as percentage of the β-Add mRNA. Li, liver; Sp, spleen; BM, bone marrow; KC, kidney cortex; KM, kidney outer medulla; AG, adrenal gland; LV, left ventricle; Ao, aorta; FA, femoral artery; CA, carotid artery; Br, brain; and Ax, axolemma. Values are means ± SE.

46% to 90% in old animals. The quantitative proportion of β-Add showed the lowest expression in kidney and the highest expression in bone marrow; the opposite result was seen for the quantitative proportion of γ-Add with the lowest expression in bone marrow and the highest expression in kidney.

The absolute amount of mRNA for β4-Add subunit was also determined and the calculated percentage out of the total β-Add mRNA in young and old rat tissues is shown in Table 2. It is seen that the abundance of β4-Add of the total β-Add mRNA was in the range of 1.3–16.7%.

Add gene family expression in MHS and MNS

To test differences in Add gene family expression between MHS and MNS rats two groups of 5–6 animals were compared both at the pre-hypertensive (young rats) and hypertensive stage (old rats). The relative abundance of α, γ, β, and β4-Add mRNAs was investigated by real-time quantitative RT-PCR in kidney cortex and outer medulla, left ventricle, femoral artery, carotid artery, brain, and bone marrow. For each tissue the two groups of MHS and MNS animals were compared in parallel, the results were corrected for 18S

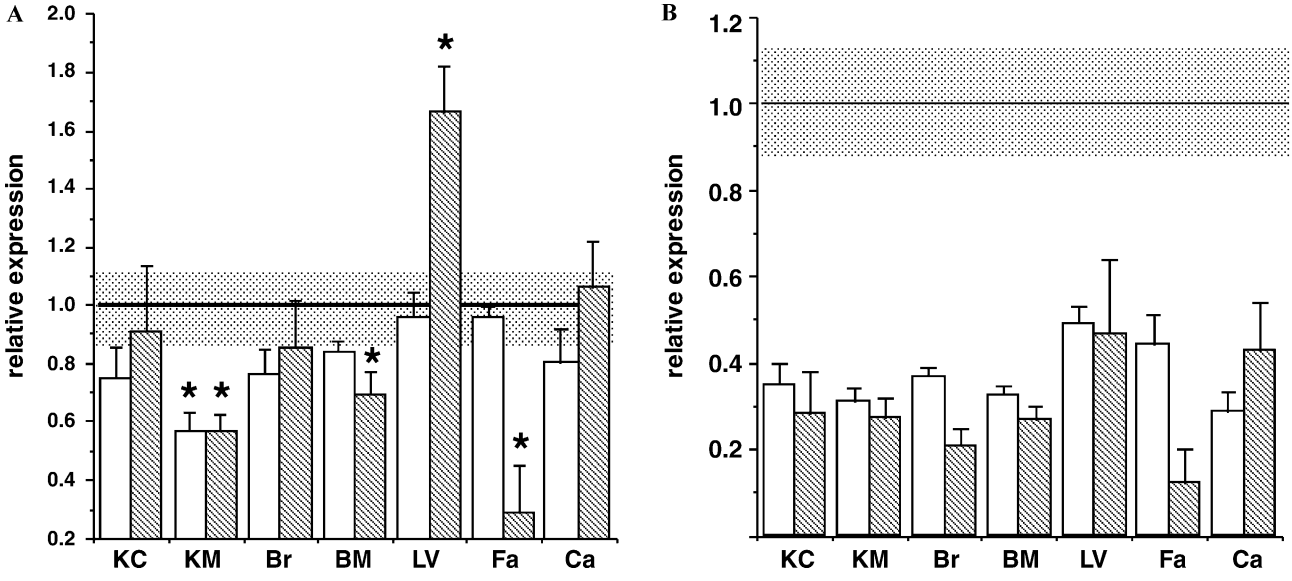

Fig. 3. Quantitative RT-PCR analysis of β-Add (A) and β4 (B) in MHS rat tissues. Data are normalized for 18S ribosomal RNA and expressed as arbitrary units relative for each tissue to value of the corresponding MNS tissue defined as 1. Values are means ± SE of six young (27 days, white bars) and six old (5 months, hatched bars) rats tissues each tested in triplicate. KC, kidney cortex; KM, kidney outer medulla; Br, brain; BM, bone marrow; LV, left ventricle; FA, femoral artery; and CA, carotid artery. The MNS values (horizontal solid line) ± the mean value of SE for all tissues (dotted area) are shown. All the β4-Add data are significant different from the MNS ones while the significance for β-Add is indicated (asterisk).

ribosomal RNA, and the MHS value was expressed as arbitrary units relative to the corresponding MNS value defined as 1. No significant differences in the expression levels of α-Add and γ-Add mRNAs were found either in young or in old animals in all tissues examined, also if a γ-Add reduction to 0.76 (24%) was seen in brain (data not shown). In contrast β and β4-Add showed a wide tissue-specific differential expression (Fig. 3). In MHS kidney, β-Add mRNA was significantly reduced in renal outer medulla but not in renal cortex of both young and old animals (0.57 and 0.56, respectively, Fig. 3A). In the other MHS tissues, β-Add mRNA was differentially modulated after the development of hypertension. Among the tissues relevant for cardiovascular disease, a significant reduction in femoral artery (0.29) but not in carotid artery and an increment in left ventricle (1.67) of old animals were observed (Fig. 3A). The parallel comparison in brain and bone marrow reveals a reduction of β transcript in bone marrow (0.66) of old animals. The β4-Add mRNA was drastically reduced in all MHS tissues examined ranging from 0.28 to 0.49 in young rats and from 0.12 to 0.47 in old rats (Fig. 3B).

Discussion

The most important findings of the present study are: (i) the identification of rat β4-Add that shows striking sequence divergences from the human counterpart in spite of the high degree of ADD sequence conservation among species; (ii) the detection and accurate quantitation of β-Add transcripts, also in those tissues where their contribution to the total pool of Add mRNAs is minor; and (iii) the tissue-specific modulation of β-Add expression in relevant tissues during the development of hypertension and associated phenotypes. We also confirm the already known expression of α and γ Add in all tissues examined, providing a precise quantitation of transcripts for each subunit.

The adducins are known as a large family of alternative spliced isoforms derived from three closely related genes, mapping on different chromosomes [21–27]. We report here the identification of rat β4-Add, a novel alternative spliced isoform, differing from β-subunit for an in-frame insertion of 54 nucleotides. The corresponding exon, flanked by consensus splice sequences, has been verified at the genomic level and is inserted in the alternative spliced mRNA in the same position of exon 15 of the correspondent human β4-ADD [27]. This novel identified isoform represents the rat counterpart of the human one also considering the high degree of gene structure conservation with alternative spliced isoforms that show similar RNA organization among human rat and mouse [21–27]. Noteworthy, while ADD polypeptide sequences are more than 90% conserved across species, sometimes their alternative spliced isoforms show striking species divergence [21–27]. The rat β4-Add variant, that encodes for a 18 amino acid longer protein, is highly divergent from the human counterpart, a truncated carboxy-terminus isoform with novel 63 amino acids, thus suggesting a specie-specific functions. We focused our attention on this alternative spliced isoform, among all the known, since we found on it the only SNP in human β-ADD coding sequence found associated to hypertension ([13], and C. Lanzani et al. 2002, unpublished data). Interestingly, also in rat we identified a polymorphism (R592W) that shows a different allelic distribution between hypertensive and normotensive inbred rat strains. In MHS hypertension we previously reported one missense mutation for each Add subunit [4,5,25] but this new polymorphism is the only identified in an alternative spliced isoform and its functional consequence should be clarified.

The use of a very sensitive method of quantitative PCR to quantify the Add gene family expression allows us to report two different and equally important informations (i) the relative proportion of a single subunit among tissues and (ii) the relative proportion of the three subunits in each tissue. The former data verified the presence of α and γ-Add transcripts in all tissues examined, with the 7-fold highest expression in kidney, supporting published data of dot and northern blot analyses [28–30]. We also confirmed a particularly strong but not exclusive expression of β-Add in brain and hematopoietic tissues with the 90-fold highest value in bone marrow and we were able to detect and quantify small amount of β-Add transcripts also in the other tissues. The latter data give the exact percentage of single subunit relative to the total amount of Add transcripts in each tissue. A divergence occurs among message levels with, almost ubiquitously, 2–3-fold higher levels of α-Add relative to β or γ-Add, even if the stoichiometry of the heterodimeric of heterotetrameric protein is 1:1 [31]. Otherwise the reciprocal proportion of β and γ transcripts should reflect the presence of specific α/β and α/γ dimers at tissue level. Further studies will be required to clarify whether Add transcripts differ for their stability or translation efficiency and whether a rapid turnover of β and γ mRNAs control the synthesis of the assembled protein.

We also demonstrated that the contribution of β4 transcript to the total amount of β-Add is minor (lower that 10% in all tissues, with the only exception of bone marrow). However, also a poorly detectable β and β4 expression in these tissues may be representative of the protein localization in a specific cell type (i.e., situ hybridization analysis revealed the selective presence of β-Add in glomerular podocytes of kidney, unpublished data) and may contribute to a defined cell function.

The observations of this study establish an additional link between Add gene(s) and hypertension. The expression analysis of the three Add subunits, before and

after the development of hypertension in relevant tissues of MHS, compared to age-matched MNS control, revealed a profound modulation only for β and β4-Add. β-Add was up-regulated by 66% in left ventricle and down-regulated by 70% in femoral artery of adult rats; it was also down-regulated by 43% in renal outer medulla of both young and adult animals. β4-Add was drastically reduced in all MHS tissues. The absence of significant changes in α and γ-Add expression indicates that the influence of their polymorphisms on blood pressure, previously demonstrated [4,5], is not mediated by changes in their expression pattern, at least between MHS and MNS rats. In fact in other rat models of hypertension a reduction of γ-Add mRNA levels has been recently linked to hypertension through a modulatory effect on central nervous system [32]. Conversely, in MHS hypertension the regulation of β-Add gene expression may be involved in some phenotypic alterations. In particular, the selective lowering of β transcripts in kidney outer medulla at both ages can be associated to MHS abnormalities in renal Na handling while the increase in left ventricle of old rats may be related to the development of cardiac hypertrophy. An involvement of β-Add in favoring cardiac hypertrophy and renal damage has been supported by our recent data in congenic strains for β-Add ([8] and unpublished data). Moreover, the selective reduction of β expression in femoral artery, but not in carotid artery, of old rats may be in keeping with the findings in humans, demonstrating the association of β-ADD polymorphism (in interaction with α-ADD and other genes) with the thickness of femoral artery but not of carotid artery [Struijker-Boudier 2002, personal communication]. The widespread downregulation of β4-Add in MHS may be the result of a parallel but independent biological mechanism with respect to that of β-Add. In summary, the β-Add transcripts may differ between MNS and MHS at the prehypertensive stage or may change with blood pressure increment in MHS. Since the active protein works into the cell only as a dimer composed by different subunits, it is likely that these differences in β transcripts also affect the heterodimer assembly. As a consequence, an alteration of the protein specific cell function may occur, as suggested by the targeted disruption of β-Add gene in mice which led to the concomitant modification of α and γ protein levels [28,33] and blood pressure [34]. Further studies will be required to elucidate the biological meanings of these findings.

References

[1] G. Bianchi et al., The Milan hypertensive strain of rats, in: J.D. Swales (Ed.), Textbook of Hypertension, Blackwell Scientific Publications, Oxford, UK, 1994, pp. 457–460.

[2] P.A. Kuhlman, C.A. Hughes, V. Bennett, V.M. Fowler, A new function for adducin, calcium/calmodulin regulated capping of the barbed ends of actin filaments, J. Biol. Chem. 271 (1996) 7986–7991.

[3] Y. Matsuoka, X. Li, V. Bennett, Adducin: structure, function and regulation, Cell. Mol. Life 57 (2000) 884–895.

[4] G. Bianchi, G. Tripodi, G. Casari, S. Salardi, B.R. Barber, R. Garcia, P. Leoni, L. Torielli, D. Cusi, M. Ferrandi, L.A. Pinna, F.E. Baralle, P. Ferrari, Two point mutations within the adducin genes are involved in blood pressure variation, Proc. Natl. Acad. Sci. 91 (1994) 3999–4003.

[5] L. Zagato, R. Modica, M. Florio, L. Torielli, M.T. Bihoreau, G. Bianchi, G. Tripodi, Genetic mapping of blood pressure quantitative trait loci in Milan hypertensive rats, Hypertension 36 (2000) 734–737.

[6] G. Tripodi, F. Valtorta, L. Torielli, E. Chieregatti, S. Salardi, L. Trusolino, A. Menegon, P. Ferrari, P.C. Marchisio, G. Bianchi, Hypertension-associated point mutation in the adducin α and β subunits affect cytoskeleton and ion transport, J. Clin. Invest. 97 (1996) 2815–2822.

[7] M. Ferrandi, S. Salardi, G. Tripodi, P. Barassi, R. Rivera, P. Manunta, R. Goldshleger, P. Ferrari, G. Bianchi, S.J.D. Karlish, Evidence for an interaction between adducin and Na^+–K^+–ATPase: relation to genetic hypertension, Am. J. Physiol. 277 (1999) H1338–H1349.

[8] M. Ferrandi, G. Tripodi, I. Molinari, P. Barassi, E. Minotti, C. Camisasca, R. Modica, G. Bianchi, P. Ferrari, Protective effect of α-adducin gene from Milan hypertensive rats on age-dependent progression of proteinuria in Milan normotensive rats, J. Mol. Med. 79 (2001) B4 (abstract).

[9] D. Cusi, C. Barlassina, T. Azzani, G. Casari, L. Citterio, M. Devoto, N. Glorioso, C. Lanzani, P. Manunta, M. Righetti, R. Rivera, P. Stella, C. Troffa, L. Zagato, G. Bianchi, α-Adducin polymorphism in primary hypertension: linkage and association study; relationship to salt sensitivity, Lancet 349 (1997) 1353–1357.

[10] N. Glorioso, F. Filigheddu, D. Cusi, C. Troffa, M. Conti, M. Natalizio, G. Argiolas, C. Barlassina, G. Bianchi, α-Adducin 460Trp allele is associated with erythrocyte Na transport rate in primary hypertension, Hypertension 39 (2002) 357–362.

[11] A. Ciechanowicz, K. Widecka, R. Drozd, G. Adler, L. Cyrylowski, S. Czekalski, Lack of association between Gly460Trp polymorphism of α-adducin gene and salt sensitivity blood pressure in Polish hypertensives, Kidney Blood Press. Res. 24 (2001) 201–206.

[12] N.J. Schork, A. Chakravarti, B. Thiel, M. Fornage, H.J. Jacob, R. Cai, C.N. Rotimi, R.S. Cooper, A.B. Weder, Lack of association between a biallelic polymorphism in the adducin gene and blood pressure in whites and African Americans, Am. J. Hypertens. 13 (2000) 396–398.

[13] J.G. Wang, J.A. Staessen, C. Barlassina, R. Fagard, T. Kuznetsova, H.A. Struijker-Boudier, L. Zagato, L. Citterio, E. Messaggio, G. Bianchi, Associations between hypertension and variation in the α and β-adducin genes in a white population, Kidney Int. 62 (2002) 2152–5159.

[14] A.C. Morrison, M.S. Bray, A.R. Folsom, E. Boerwinkle, ADD1 460W allele associated with cardiovascular disease in hypertensive individuals, Hypertension 39 (2002) 1053–1057.

[15] M. Winnicki, V.K. Somers, V. Accurso, M. Hoffmann, R. Pawlowski, G. Frigo, P. Visentin, P. Palatino, On behalf of the HARVEST study group. α-Adducin Gly460Trp polymorphism, left ventricular mass and plasma renin activity, J. Hypertens. 20 (2002) 1771–1777.

[16] E.J. Balkestein, J.A. Staessen, J. Wang, J.J. van der Heijden-Spek, L.M. Van Bortel, C. Barlassina, G. Bianchi, E. Brand, S.M. Herrmann, H.A. Struijker-Boudier, Carotid and femoral artery stiffness in relation to three candidate genes in a white population, Hypertension 38 (2002) 1190–1197.

[17] E.J. Balkestein, J.G. Wang, H.A. Struijker-Boudier, C. Barlassina, G. Bianchi, W.H. Birkenhager, E. Brand, E. Den Hond, R.

Fagard, S.M. Herrmann, L.M. Van Bortel, J.A. Staessen, Carotid and femoral intima-media thickness in relation to three candidate genes in a Caucasian population, J. Hypertens. 20 (2002) 1551–1561.

[18] J.G. Wang, J.A. Staessen, L. Tizzoni, E. Brand, W.H. Birkenhagen, R. Fagard, S.M. Herrmann, G. Bianchi, Renal function in relation to three candidate genes, Am. J. Kidney Dis. 38 (2001) 1158–1168.

[19] J. Nicod, B.M. Frey, F.J. Frey, P. Ferrari, Role of the α-adducin genotype on renal disease progression, Kidney Int. 61 (2002) 1270–1275.

[20] B.M. Psaty, N.L. Smith, S.R. Heckbert, H.L. Vos, R.N. Lemaitre, A.P. Reiner, D.S. Siscovick, J. Bis, T. Lumely, W.T. Longstreth, F.R. Rosendaal, Diuretic therapy, the α-adducin gene variant and the risk of myocardial infarction or stroke in person with treated hypertension, JAMA 287 (2002) 1680–1689.

[21] B. Lin, J. Nasir, H. McDonald, R. Graham, J.M. Rommens, Y.P. Goldberg, M.R. Hayden, Genomic organization of the human α-adducin gene and its alternatively spliced isoforms, Genomics 25 (1995) 93–99.

[22] D.M. Gilligan, L. Lozovatsky, A. Silberfein, Organization of the human β-adducin gene (ADD2), Genomics 43 (1997) 141–148.

[23] L. Citterio, T. Azzani, S. Duga, G. Bianchi, Genomic organization of the human γ adducin gene, Biochem. Biophys. Res. Commun. 266 (1999) 110–114.

[24] G. Tripodi, G. Casari, S. Tisminetsky, G. Bianchi, G. Devescovi, A. Muro, R. Tuteja, F.E. Baralle, Characterization and chromosomal localisation of the rat α- and β-adducin-encoding genes, Gene 166 (1995) 307–311.

[25] G. Tripodi, C. Szpirer, C. Reina, J. Szpirer, G. Bianchi, Polymorphism of γ-adducin gene in genetic hypertension and mapping of the gene to rat chromosome 1q55, Biochem. Biophys. Res. Commun. 237 (1997) 685–689.

[26] S.P. Suriyapperuma, L. Lozovatsky, S.L. Ciciotte, L.L. Peters, D.M. Gilligan, The mouse adducin gene family: alternative splicing and chromosomal localization, Mamm. Genome 11 (1999) 16–23.

[27] J.H. Sinard, G.W. Stewart, P.R. Stabach, A.C. Argent, D.M. Gilligan, J.S. Morrow, Utilization of an 86 bp exon generates a novel adducin isoform (β4) laking the MARCKS homology domain, Biochem. Biophys. Acta 1396 (1998) 57–66.

[28] D.M. Gilligan, L. Lozovatsky, B. Gwynn, C. Brugnara, N. Mohandas, L.L. Peters, Targeted distruption of the β adducin gene (Add2) causes red blood cell spherocytosis in mice, Proc. Natl. Acad. Sci. USA 96 (1999) 10717–10722.

[29] R. Joshi, D.M. Gilligan, E. Otto, T. McLaughlin, V. Bennett, Primary structure and domain organization of human α and β adducin, J. Cell Biol. 115 (1991) 665–675.

[30] L. Dong, C. Chapline, B. Mousseau, L. Fowler, K. Ramsay, J.L. Steven, S. Jaken, 35H, a sequence isolated as a protein kinase C binding protein, is a novel member of the adducin family, J. Biol. Chem. 270 (1995) 25534–25540.

[31] C.A. Hughes, V. Bennett, Adducin: a physical model with implications for function in assembly of spectrin-actin complexes, J. Biol. Chem. 270 (1995) 18990–18996.

[32] H. Yang, S.C. Francis, K. Sellers, M. DeBarros, C. Sun, C. Sumners, C.M. Ferrario, M.J. Katovich, A.F. Muro, M.K. Raizada, Hypertension-linked decrease in the expression of brain γ-adducin, Cir. Res. 91 (2002) 633–639.

[33] A.F. Muro, M.L. Marro, S. Gajovic, F. Porro, L. Luzzatto, F.E. Baralle, Mild spherocytic hereditary elliptocytosis and altered levels of α and γ adducins in β-adducin-deficient mice, Blood 95 (2000) 3978–3985.

[34] M.L. Marro, O.U. Scremin, M.C. Jordan, L. Huynh, F. Porro, K.P. Roos, S. Gajovic, F.E. Baralle, A.F. Muro, Hypertension in β-adducin deficient mice, Hypertension 36 (2000) 449–453.