Hypertension-Linked Decrease in the Expression of Brain γ-Adducin

Hong Yang,* Sharon C. Francis,* Kathleen Sellers, Mia DeBarros, Chengwen Sun, Colin Sumners, Carlos M. Ferrario, Michael J. Katovich, André F. Muro, Mohan K. Raizada

Abstract—Gene profiling data coupled with adducin polymorphism studies led us to hypothesize that decreased expression of this cytosolic protein in the brain could be a key event in the central control of hypertension. Thus, our objectives in the present study were to (1) determine which adducin subunit gene demonstrates altered expression in the hypothalamus and brainstern (two cardioregulatory-relevant brain areas) in two genetic strains of hypertensive rats and (2) analyze the role of adducins in neurotransmission at the cellular level. All three adducin subunits (α, β, and γ) were present in the hypothalamus and brainstem of Wistar Kyoto (WKY) and spontaneously hypertensive (SH) rats. However, only the γ-adducin subunit expression was 40% to 60% lower in the SH rat compared with WKY rat. A similar decrease in γ-adducin expression was observed in the hypothalamus and brainstem of the renin transgenic rat compared with its normotensive control. Losartan treatment of the SH rat failed to normalize γ-adducin gene expression. A hypertension-linked decrease of γ-adducin was confirmed by demonstrating a decrease in γ-adducin expression in hypothalamic/brainstem neuronal cultures from prehypertensive SH rats. Neuronal firing rate was evaluated to analyze the role of this protein in neurotransmission. Perfusion of a γ-adducin–specific antibody caused a 2-fold increase in the neuronal firing rate, an effect similar to that observed with angiotensin II. Finally, we observed that preincubation of neuronal cultures for 8 hours with 100 nmol/L angiotensin II caused a 60% decrease in endogenous γ-adducin and was associated with a 2-fold increase in basal firing rate. These observations support our hypothesis that a decrease in γ-adducin expression in cardioregulatory-relevant brain areas is linked to hypertension possibly by regulating the release of neurotransmitters. (Circ Res. 2002;91:633-639.)

Key Words: hypothalamus/brainstem ■ gene profiling ■ neurons ■ γ-adducin ■ hypertension

The central nervous system (CNS) plays a key role in the control of cardiovascular functions. Anatomical and physiological evidence have established that specific hypothalamic and brainstem nuclei participate in the control of sympathetic nerve activity (SNA), vasopressin release, and baroreceptor reflexes, which are all aspects involved in normal cardiovascular functions.1,2 The significance of these pathways in central control of cardiovascular functions is further underscored by the evidence that an increase in SNA and secretion of neurotransmitters/neurohormones is associated with the development and establishment of hypertension.3

In spite of this evidence, little is known about the cellular and molecular basis of hypertension-linked dysregulation in the CNS. In an attempt to elucidate the molecular basis of this dysregulation, we hypothesized that an inherent change in the expression profiling of gene(s) in cardiovascular-relevant brain nuclei is responsible for the development of hypertension. In this regard, our present studies have indicated that the expression profiles of 112 genes are significantly altered in the hypothalamus/brainstem of the spontaneously hypertensive (SH) rat compared with the normotensive Wistar Kyoto (WKY) rat with the use of Affymetrix microarray technology.4 One such gene whose expression we chose to investigate in the study was adducin. Adducin is a heterodimeric cytosolic protein that consists of α, β, and γ subunits. It interacts with multiple structural and functional proteins to regulate cytoskeletal-mediated functions such as intracellular protein trafficking, Ca2+ mobilization, and phosphorylation/dephosphorylation of certain protein kinases.5

Our rationale for selecting adducin as a candidate gene for its possible linkage to hypertension was as follows: (1) adducins are associated with the regulation of intracellular Ca2+ and kinases that are potentially important in neurotrans-
mitter release and thus relevant in the regulation of SNA; (2) aducin polymorphism was demonstrated in the Milan hypertensive rat6 (this polymorphism accounts for up to 50% of the total blood pressure (BP) difference between the hypertensive and normotensive strains6); (3) /H9252-adducin deficient mice express higher BP compared with their wild-type controls 7; (4) a high degree of linkage of the /H9251-adducin locus to hypertension in different populations of humans has been demonstrated 8– 9; and (5) the affinity of a hypertensive adducin variant to the Na
/H11001,K
/H11001-ATPase pump is greater than that of normotensives. 10 This is relevant because Na
/H11001,K
/H11001-ATPase is involved in hypertension and adducin-Na
/H11001,K
/H11001-ATPase interaction regulates its activity.11 Collectively, these studies suggest that the adducin gene is ideally poised for its role in the regulation of neurotransmitter activity and thus could be crucial in the control of SNA and hypertension. Thus, the objective of the present investigation was 2-fold: (1) determine which, if any subunit of adducin gene expression correlates with the hypertensive state in genetic models of hypertension, and (2) evaluate on the possible mechanism of involvement of adducin in neurotransmission and hypertension.

Materials and Methods

Animals
Twelve-week-old Wistar Kyoto rats (WKY) and SH rats were obtained from Charles River Farm (Wilmington, Mass). Blood pressures for WKY rats averaged 117/5 mm Hg and those of the SH rat, 166/2 mm Hg. Twelve-week-old renin-transgenic rats (TGR mRen2/27, 220/7 mm Hg) and age-matched Sprague-Dawley (SD) rats (124/11 mm Hg) were obtained from the breeding colony at the Wake Forest University, Winston-Salem, NC. Animals were housed at 25±2°C on a 12:12-hour light-dark cycle, fed rat chow (Harlan Teklad), and were provided water ad libitum. All animal protocols were approved by the University Animal Care and Use Committee.

### Altered Expression of Certain Genes in the Hypothalamus-Brainstem of the SH vs WKY Rat Brain

<table>
<thead>
<tr>
<th>Description (Accession No.)</th>
<th>Increase (↑) or Decrease (↓):</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP citrate-lyase (J05210)</td>
<td>↓</td>
</tr>
<tr>
<td>Bone morphogenetic protein4 (Z22607)</td>
<td>↓</td>
</tr>
<tr>
<td>Carnitine octanoyltransferase (U26033)</td>
<td>↓</td>
</tr>
<tr>
<td>Calpastatin (X56729)</td>
<td>↑</td>
</tr>
<tr>
<td>Cell adhesion-like molecule (M88709)</td>
<td>↑</td>
</tr>
<tr>
<td>Cyclooxygenase 1 (U03388)</td>
<td>↑</td>
</tr>
<tr>
<td>Cytochrome oxidase subunit VIIa (U75927)</td>
<td>↓</td>
</tr>
<tr>
<td>Cytochrome oxidase subunit IV (X54081)</td>
<td>↑</td>
</tr>
<tr>
<td>Cytosolic epoxide hydrolase (X65083)</td>
<td>↑</td>
</tr>
<tr>
<td>GABA A receptor α-4 (L08493)</td>
<td>↑</td>
</tr>
<tr>
<td>GABA B receptor 1c (AB016160)</td>
<td>↑</td>
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<tr>
<td>γ glutamylcysteine synthetase light chain (S65555)</td>
<td>↓</td>
</tr>
<tr>
<td>Heart shock protein 70 (L16764/275029)</td>
<td>↑</td>
</tr>
<tr>
<td>GAS-5 (U77829)</td>
<td>↓</td>
</tr>
<tr>
<td>α-fibrinogen (M35601)</td>
<td>↓</td>
</tr>
<tr>
<td>Kidney urea transporter (AF031642)</td>
<td>↑</td>
</tr>
<tr>
<td>Sodium/glucose cotransporter (D16101)</td>
<td>↑</td>
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<tr>
<td>Sodium/potassium ATPase α1 (M28648)</td>
<td>↓</td>
</tr>
<tr>
<td>Krox20 (U78102)</td>
<td>↑</td>
</tr>
<tr>
<td>Epsilon 1 globin gene (X56326)</td>
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<tr>
<td>Proline-rich proteoglycan (L17318)</td>
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<tr>
<td>SNAP25a (U56261)</td>
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<tr>
<td>Synaptic vesicle protein (L05435)</td>
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<tr>
<td>PPK kinase p35 (D64045)</td>
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<tr>
<td>Phospholipase Cβ3 (M99567)</td>
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<tr>
<td>SPARC (U75929)</td>
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<tr>
<td>Fructose 6-P, 2-kinase: fructose 2,6-bisphosphatase (SK7900)</td>
<td>↓</td>
</tr>
<tr>
<td>Galanin receptor (I05209)</td>
<td>↓</td>
</tr>
<tr>
<td>PTH-like peptide (M34112)</td>
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Total RNA from the hypothalamus/brainstem areas of SH and WKY rats was used for expression profiling with the use of Affymetrix GeneChip Arrays, Rat Genome Chip U34A, according to the company’s recommended protocol. Some of the genes with a statistically significant (P<0.05) expression are listed above.
Neuronal Cells in Primary Culture

Neuronal cells from the hypothalamus/brainstem areas of 1-day old WKY and SH rats dissociated by trypsin/pancreatin and plated in Poly-1-Lysine precoated tissue culture dishes at a density of 3×10^6 cells/35-mm diameter dish as described previously. These cultures consisted of 85% to 90% neurons and 10% to 15% astrocytic cells.

Real-Time RT-PCR for Adducins and GAPDH

Oligonucleotide primers and TaqMan probes for specific rat adducin subunits were designed from the GenBank databases using Primer Express (Applied Biosystems, Inc.).

α-Adducin

The primers and probes for α-adducin were as follows: forward primer, 5′-CAGCGGCGGCTCACCT-3′; reverse primer, 5′-GACAAATGCACTTGCGACATCT-3′; probe, 5′-CAGTGAGGATCGTGCTG-3′ with 5′-6-FAM label.

β-Adducin

The primers and probes for β-adducin were as follows: forward primer, 5′-AAGACCCTGCCTCGTACACT-3′; reverse primer, 5′-CTTGCGCGCACAGTTCC-3′; probe, 5′-CCGACGCACTGACATTGGG-3′ with 5′-FAM label.

γ-Adducin

The primers and probes for γ-adducin were as follows: forward primer, 5′-ACGGATCTGCTAGCGTTACAG-3′; reverse primer, 5′-TGAGAAAGACCGTGAAAGGA-3′; probe, 5′-AGATGCAGGACTACATCATGCGCA-3′ with 5′-FAM label.

Western Blots for Adducins

Tissues were homogenized in the lysis buffer (50 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 10% glycerol, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 2 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, and 2 μg/mL leupeptin). The lysates were centrifuged; proteins were then separated by SDS-PAGE, transferred to nitrocellulose membrane, and used for Western blotting with adducin subunit-specific antibodies. Characterization and specificity of adducin antibodies have been established. Adducin subunit protein bands were quantitated by normalizing to endogenous tubulin protein.

Electrophysiological Recording

Spontaneous action potentials were recorded with the use of whole-cell patch clamp procedure in the current-clamp mode as described previously. Neurons were bathed in a solution containing (in mmol/L) 140 NaCl, 5.4 KCl, 2.0 CaCl₂, 2.0 MgCl₂, 0.3 NaH₂PO₄, 10 HEPES, and 10 dextrose, pH 7.4. Patch electrodes had resistances of 3 to 4 MΩ when filled with an internal pipette solution containing (in mmol/L) 140 KCl, 2.0 MgCl₂, 4 ATP, 0.1 GTP, 10 HEPES, and 10 dextrose, pH 7.2. Angiotensin II (Ang II; 100 nmol/L) was added into the perfusion solution, and γ-adducin antibody was applied into the perfusion solution, and γ-adducin antibody was applied intracellularly through the recording pipette.

Experimental Groups and Statistical Analysis

Four animals per group and 5 culture dishes per data point were used for mRNA and protein measurements. Each experiment was repeated at least 3 times unless stated otherwise. Images from the autoradiographs were captured with the GS700 densitometer (Bio-Rad Laboratories). The immunoreactive bands were quantitated using Quantity One analysis software (Bio-Rad Laboratories) and corrected for equal sample loading by normalizing with a standard protein. Data are presented as mean±SE. Comparison between the control and experimental groups were made using Student t test with Statistica Software. Eight cells have been used for electrophysiological recording in each group.

Results

Expression of Adducins in WKY and SH Rat Brains

Affymetrix GeneChip array was used to compare the expression profile of SH rat brain with its WKY rat control. The Table shows a list of genes whose expression was significantly altered in the SH rat. In this study, we decided to compare the expression of γ-adducin between these two strains of rat in detail.

Messenger RNAs for all three adducin subunits (α, β, and γ) were present in both the hypothalamus and the brainstem of the WKY and SH rats (Figure 1). There was a 40 to 50% decrease in the mRNA levels of γ-adducin in both brain regions of the SH rat compared with the WKY rat (Figure 1). In contrast, mRNA levels for the α- and β-adducins did not change in either brain areas of the SH rat. The decrease in the γ-adducin mRNA was reflected in its protein levels (Figure 2A). Antibody to α-adducin recognized protein bands of 120 kDa and 90 kDa, whereas antibody to γ-adducin recognized protein bands of 120 kDa and 105 kDa (Figures 2A and 2B). In contrast, only one protein band of 120 kDa was detected by the β-adducin antibody (Figure 2C). This was consistent with previous data. A 56% and 60% decrease in γ-adducin protein levels were observed in the SH rat hypothalamus and brainstem, respectively, compared with WKY rat controls. Levels of α- and β-adducin proteins were comparable between SH rat and WKY rats (Figures 2B and 2C).

Is Decreased Expression of γ-Adducin Linked to Hypertension?

Four approaches have been taken to determine if the decrease in γ-adducin was linked to hypertension. First, SH rats were treated with losartan for 2 weeks. This resulted in a reduction in high BP (166±2 mm Hg control versus 140±2 mm Hg losartan treatment). No significant change in the brain pressure was observed in losartan treated WKY rats (120±5 mm Hg control versus 119±6 mm Hg losartan treated).

Figure 1. Messenger RNA levels of adducins in hypothalamus and brainstem of WKY and SH rats. Total RNA was isolated by TRIzol Reagent, samples were treated with DNase I, and 25 ng total RNA was subjected to quantitative real-time RT-PCR with α-, β-, and γ-adducin-specific primers. Data were normalized to endogenous GAPDH and presented as arbitrary unit. *Significantly different from SH rat vs WKY rat (P<0.01, n=3).
adducin levels were observed in the SH rat treated with losartan (data not shown). This indicated that decreased adducin in SH rat is not associated with high BP. A second approach utilized the Ren TGR as another model of genetic hypertension. Both the mRNA and protein levels of adducin were 50% to 60% lower in the hypothalamus and brainstem areas of TGR compared with SD normotensive control rats (Figure 3). Similar to the WKY and SH rats, we observed no significant changes in the mRNA or protein levels of either the α- or β-adducin subtype of TGR compared with SD rat (data not shown). Third, neuronal cells in coculture from hypothalamus/brainstem of prehypertensive SH rats were used to differentiate between genetic predisposition-induced versus high BP-induced change in adducin. Figure 4 shows that neuronal cultures express all three subunits of adducins similar to that seen in adult tissues. However, only γ-adducin expression was decreased in the SH rat compared with WKY rat. Fourth, the effect of Ang II on γ-adducin expression was studied in neuronal cultures. The rationale for this experiment was as follows: hyperactivity of the brain renin-angiotensin system plays a key role in the development and establishment of hypertension. In addition, Ang II increases norepinephrine (NE) transmission. Because Ang II exhibits an enhancement in NE modulatory activity in SH rat neurons, one could argue that it would decrease γ-adducin levels. Figure 5 shows that incubation of neuronal cultures of WKY rats with 100 nmol/L Ang II resulted in a 60% decrease in γ-adducin mRNA and protein levels within 8 hours. The decrease, although less pronounced, was also observed in the neurons from the SH rat (Figure 5).

**Role of γ-Adducin on Neuronal Activity**

We have established that an increase in neuronal activity is, in part, responsible for Ang II stimulation of NE transmission. Thus, it is likely that γ-adducin will regulate neuronal firing rate if this protein is involved in the regulation of NE neuromodulation. Superfusion of WKY rat neuronal cultures with Ang II produced a significant increase in neuronal firing...
rate compared with baseline values, consistent with our previous studies (Figures 6A and 2B). Similarly, intracellular application of γH9253-adducin antibodies resulted in a significant increase in the firing rate (Figure 6C). In a control experiment, intracellular application of normal goat serum or anti-rabbit IgG had no effect on firing rate. However, combined application of both γH9253-adducin antibodies and Ang II elicited no greater increase in neuronal activity, indicating that there is no facilitatory interaction between these agents (Figure 7D). Preincubation of neuronal cultures with Ang II (100 nmol/L) for 8 hours at 37°C results in a 60% depletion of endogenous γH9253-adducin levels (Figure 5). Under these pretreatment conditions, there was a 2-fold increase in neuronal firing rate (Figure 6E). Collectively, these observations suggest that the levels of γH9253-adducin are inversely related to neuronal activity. It has been proposed that the mechanism by which γH9253-adducin regulates neuronal activity and neurotransmitter release is by interacting with and regulating the activity of Na⁺,K⁺-ATPase. Therefore, this interaction was examined in neuronal cultures by a coimmunoprecipitation experiment. Figure 7 shows that γH9253-adducin coimmunoprecipitated with Na⁺,K⁺-ATPase from both WKY and SH rats neurons. There was a significant decrease in coimmunoprecipitate of these proteins in the SH rat compared with the WKY rat brain neurons.

**Discussion**

The observations of this study establish that a decrease in γH9253-adducin gene expression in the hypothalamic/brainstem areas of the brain is linked to hypertension. Thus, γH9253-adducin provides a novel target for us to explore its potential therapeutic value in the CNS-mediated hypertension.

Adducin gene polymorphism has long been proposed to be linked to hypertension in both animal models and in human hypertension. In fact, BP quantitative trait loci in the Milan hypertensive rat has been identified in chromosomal region that harbors hypertension-relevant genes such as Na⁺-H⁺ exchanger and α-adrenergic receptor. In spite of these studies, a unifying view as to the involvement of adducin gene in hypertension is lacking as a result of conflicting data. We believe that our study establishes that the pattern of expression of γH9253-adducin in the CNS may be linked to this pathophysiological state. Evidence in support of our view include the following: (1) decrease in γH9253-adducin gene expression was found in the hypothalamus and brainstem of both polygenetic (SH rat) and monogenetic (Ren-2 TGR) models of hypertension. Administration of lentiviral vector containing γH9253-adducin in the brain caused a modest decrease in BP in the SHR (preliminary data); (2) decreased γH9253-adducin expression was not high BP–dependent, but it is likely to be linked to the genetic aspects of hypertension, based on losartan experiments; (3) a decrease in γH9253-adducin expression was observed in neuronal cultures from prehypertensive SH rat, compared with WKY rat neuronal cells. Although our data indicate an association of hypertension and increased Ang II with decrease in brain γH9253-adducin, the mechanism of
this association remains unknown. It could not be due to the fact that the SH rat expresses multiple phenotype because the Ren-2 rat monogenetic model of hypertension exhibits a similar decrease. It could also not be related to the action of Ang II via AT2 receptor subtype because its effects on both $\gamma$-adducin and neuronal activity are completely inhibited by losartan and AT1 receptor antagonist. However, because adducin is a key regulatory protein, it could interact with $\gamma$-adducin polyclonal antibody (1:200 dilution) for 20 minutes in the absence (A and C) or presence (B, D, and E) of 100 nmol/L Ang II as described in the Materials and Methods. Data are from 8 neurons of each group. *Significantly different from untreated neurons ($P<0.01$).

Figure 7. Interaction of $\gamma$-adducin with Na$^+$.K$^+$-ATPase. Neuronal cultures from WKY and SH rats brains were established in 100-mm dishes for 14 days. Cells were collected and lysed. Cell lysates were incubated without or with $\gamma$-adducin antibody or goat normal IgG overnight at 4°C. Immunoprecipitates were collected by protein A/G PLUS-agarose and immunoblot was performed with Na$^+$.K$^+$-ATPase $\alpha_2$-subunit antibody. Top, representative autoradiograph; Bottom, mean data from two experiments.

Figure 6. Effect of $\gamma$-adducin antibody on Ang II–induced chro-notropic actions in WKY rat brain neurons. Neuronal cells were perfused intracellularly with $\gamma$-adducin polyclonal antibody (1:200 dilution) for 20 minutes (C and D). Neuronal firing rates were recorded for 2 minutes in the absence (A and C) or presence (B, D, and E) of 100 nmol/L Ang II as described in the Materials and Methods. Data are from 8 neurons of each group. *Significantly different from untreated neurons ($P<0.01$).
In conclusion, our findings indicate that γ-adducin is an important protein in the regulation of neuronal activity and neurotransmitter release. Thus, its decrease could be associated with an increase in SNA in hypertension. This proposal is being tested.

Acknowledgments
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References
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