Neuregulin-1/ErbB signaling in rostral ventrolateral medulla is involved in blood pressure regulation as an antihypertensive system

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Neuregulin-1/ErbB signaling in rostral ventrolateral medulla is involved in blood pressure regulation as an antihypertensive system

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Objectives Neuregulin-1 (NRG-1), located in the central nervous system (CNS), plays

an important role in synaptic function, neurite outgrowth, and survival of

neurons and glia acting on the ErbB receptor family. However, the functional role of

NRG-1/ErbB signaling in the CNS and blood pressure regulation is unknown,

particularly in the rostral ventrolateral medulla (RVLM), a major vasomotor center.

Thus, we investigated whether NRG-1/ErbB signaling in the RVLM is involved in

blood pressure regulation.

Methods and results Microinjection of NRG-1 into the RVLM decreased arterial blood

pressure, heart rate (HR), and renal sympathetic nerve activity (RSNA) in Wistar rats. In

contrast, microinjection of an ErbB2 or ErbB4 inhibitor into the RVLM increased

arterial pressure, HR, and RSNA. ErbB2 expression levels in the brainstem were

significantly lower in spontaneously hypertensive rats (SHRs) than in Wistar–

Kyoto (WKY) rats. Depressor responses to NRG-1 and pressor responses to the ErbB2

inhibitor were significantly smaller in SHRs than in WKY rats ($P < 0.05$). Furthermore,

the inhibition of ErbB2 expression in the RVLM by RNA interference significantly

increased arterial pressure, HR, and urinary norepinephrine excretion in conscious

WKY rats ($P < 0.01$).

Conclusion Our findings indicate that the NRG-1/ErbB signaling in the RVLM has

depressor and sympathoinhibitory effects. Reduced NRG/ErbB2 signaling

in the RVLM may contribute to the neural mechanisms of hypertension.

Keywords: blood pressure, ErbB, hypertension, neuregulin-1, rostral ventrolateral

medulla, sympathetic nervous system

Abbreviations: CNS, central nervous system; GABA, g-aminobutyric acid; HR, heart

rate; MAP, mean arterial pressure; NRG-1, neuregulin-1; RSNA, renal sympathetic

nerve activity; RVLM, rostral ventrolateral medulla; SHR, spontaneous hypertensive

rat; siRNA, small-interference RNA; uNE, urinary norepinephrine excretion; WKY,

Wistar–Kyoto

Introduction

Neuregulin-1 (NRG-1) is a member of the epidermal growth factor family, which is

involved in cell–cell communication [1,2]. NRG-1 is expressed in the nervous

system, heart, and other organ systems [1–3]. NRG-1 binds to the extracellular domain

of the tyrosine kinase ErbB3 or ErbB4, which often leads to the formation of

ErbB heterodimers with ErbB2. This activates intra-cellular signaling pathways leading

to synaptic function, neurite outgrowth, and survival of neurons and glia [3–5].

It is also known that NRG-1 and ErbB receptors are widely distributed in the central nervous system (CNS), including the brainstem [6–10]. Several studies have demonstrated the involvement of NRG-1/ErbB signaling in the regulation of neurotransmitters, such as L -glutamate and g-aminobutyric acid (GABA) [11,12]. In addition, ErbB2 is known to be involved in the development of glial cells and their transformation into astrocytes [13]. However, little is known regarding the role of the NRG-1/ErbB signaling pathway in the brain-stem and the action of this pathway on blood pressure regulation. With regard to CNS blood pressure regulation, the rostral ventrolateral medulla (RVLM) of the brainstem is one of the most important regions involved in central cardio-vascular regulation [14–17]. RVLM neurons project toward sympathetic preganglionic neurons in the intermediolateral cell column of the spinal cord and provide an essential excitatory drive, thereby maintaining sympathetic vasomotor tone [14]. In addition, the RVLM neurons regulate sympathetic vasomotor tone by inte-grating the inputs from baroreceptors, chemoreceptors, and higher autonomic nuclei [18]. Accumulating evidence suggests that increased RVLM activity leads to chronic sympathetic hyperactivity in many forms of hypertension [17]. For example, in spontaneously hypertensive rats (SHRs), excitation of the RVLM vasomotor neurons is increased by L -glutamate-mediated excitation and decreased

GABA-mediated inhibition [16,17]. Therefore, the aim of the present study was to determine whether NRG-1/ErbB signaling in the RVLM contributes to blood pressure regulation through the sympathetic nervous system, and, if so, whether alteration of this signaling in the RVLM occurs in SHRs, leading to the activation of the sympathetic nervous system associated with neurogenic hypertensive mechanisms. We investigated the effect of NRG-1 and the ErbB receptor blockers injected into the RVLM on arterial blood pressure, heart rate (HR), and sympathetic nervous system activity. Expression levels of NRG-1 and ErbB receptors were also evaluated. These effects were examined in SHRs to elucidate the role of this signaling in hypertension. Finally, we further examined the effects of this signaling in conscious rats using small-interference RNA (siRNA) techniques.

Methods

This study was reviewed and approved by the Committee on Ethics of Animal Experiments, Kyushu University Graduate School of Medical Sciences, and it was performed according to the Guidelines for Animal Experiments of Kyushu University. Microinjection studies Animals were anesthetized and measured for arterial pressure, HR and renal sympathetic nerve activity (RSNA). For details of general surgical

preparation, see online Data Supplemental Digital Content 1, http://links. lww.com/HJH/A108. Microinjections into the RVLM were made according to the following protocols: unilateral microinjection of recombinant NRG-1b (NRG-1b: 0.025, 0.25, and 2.5 pmol in 50 nl injections; Ray Biotech, Norcross, Georgia, USA) in Wistar rats and NRG-1b (2.5 pmol in 50 nl injections) was microinjected in Wistar–Kyoto (WKY) rats and SHRs; bilateral micro- injections of the ErbB2 antagonists AG825 (0.5 pmol in 50 nl injections; Santa Cruz Biotechnology, Santa Cruz, California, USA) in Wistar rats, WKY rats, and SHRs; bilateral microinjections of the ErbB4 antagonists AG1478 (0.5 pmol in 50 nl injections; Santa Cruz Biotechnology) in Wistar rats, WKY rats, and SHRs; unilateral microinjection of NRG-1b (2.5 pmol in 50 nl injections) 10 min after unilateral injection of both AG825 (0.5 pmol in 50 nl injections) and AG1478 (0.5 pmol in 50 nl injections) in Wistar rats; unilateral microinjection of NRG-1b (2.5 pmol in 50 nl injections) 5 min after unilateral injection of the GABA-A receptor antagonist bicuculline (100 pmol in 50 nl injections; Santa Cruz) in Wistar rats; and unilateral microinjection of L -glutamate (1.0 nmol in 50 nl injections; Nakarai Tesque, Fukuoka, Japan) 10 min after unilateral injection of NRG-1b (2.5 pmol in 50 nl injections) in Wistar rats. Arterial pressure, HR, and RSNA are monitored in the first (2.5 pmol NRG-1b), second, and third protocol. Arterial pressure and HR are

monitored in the first (0.025 and 0.25 pmol NRG-1b) and the fourth to sixth protocol.

Dosages of reagents were determined as follows. The dose of NRG-1b was determined

on the basis of previous studies [19,20]. Additionally, we confirmed the dose response in

the present study. Doses of AG825 and AG1478 were determined on the basis of

previous studies and half maximal inhibitory concentration (IC 50) values [21–25].

Furthermore, to exclude the peripheral effects of the agents, we performed intravenous

administration of the agents (the same amount of the agents used in microinjection

studies in 0.2 ml isotonic saline). However, there were no effects on mean arterial

pressure (MAP) and HR [DMAP: NRG-1 (2.5 pmol), 0.9 1.8 mmHg; AG825 (1.0

pmol), 0.6 1.8 mmHg; AG1478 (1.0 pmol), 0.3 $\pm$ 1.1 mmHg; and DHR: NRG-1,

0.6 $\pm$ 2.3 beats/min; AG825, 0.9 $\pm$ 2.8 beats/min; AG1478, 1.0$\pm$ 0.9 beats/min (n

= 3 for each)]. Doses of bicuculline and L –glutamate were determined on the basis of

previous studies [26–28].

Western blot analysis

Western blots were made according to the following protocols: first, expressions of

NRG-1 and ErbB receptors (ErbB2, ErbB3, and ErbB4) in the brainstem of 4-week-

old and 12-week-old WKY rats and SHRs were confirmed. Rabbit IgG monoclonal

antibodies against NRG-1 (1 : 1000), ErbB2 (1 : 1000), ErbB3 (1 : 1000), and ErbB4 (1 : 1000) were used as the primary antibody. Additionally, expression of ErbB2 in the cerebral cortex or hypothalamus of 12-week-old WKY rats and SHRs was confirmed. Second, in a study of chronic inhibition of ErbB2 in the RVLM by siRNA, western blot was performed to confirm the effects of RNA interference on the ErbB2 receptor using RVLM tissues of WKY rats. At 0, 1, 5, and 14 days after administration of ErbB2 or control siRNA (siErbB2 or siControl), rats were anesthetized using sodium pentobarbital and perfused transcardially with PBS. RVLM tissues defined according to a rat brain atlas were obtained as previously described [29]. Anti-ErbB2 antibody (1 : 1000) was used. For details see online Data Supplemental Digital Content 1, http://links. lww.com/HJH/A108.

In-vivo small-interference RNA technique

The siErbB2 sequence was determined as described in a previous study [30]. Specific siErbB2 (AAGUGUGUGUACCGGCACAGACA) and scrambled siRNA (siControl: AGCCUAACUGAACGCGUAGGA), as a control, were purchased from Koken, Tokyo, Japan. Furthermore, we used the in-vivo siRNA delivery kit AteloGene (Koken) for stable local delivery of the siRNA into the RVLM. SiErbB2 or siControl

was administered into the RVLM, and MAP and HR were monitored using radiotelemetry system for 14 days. For details see online Data Supplemental Digital Content 1, http://links.lww.com/HJH/A108.

Measurement of urinary norepinephrine excretion

We measured urinary norepinephrine excretion (uNE) concentration before and at 1, 7, and 14 days after the start of the administration of siRNA and calculated uNE as described previously [26,31]. Statistical analysis All values are expressed as the mean SEM. Differences were considered significant when the P value was less than 0.05. For details see online Data Supplemental Digital Content 1, http://links.lww.com/HJH/A108.

Results

Baseline mean arterial pressure and heart rate of microinjection study.

The baseline MAP and HR in microinjection protocols of NRG-1b, AG825, and AG1478 are described in Supplemental Digital Content 2, Online Table 1, http://links.lww.com/HJH/A109.

Effects of neuregulin-1 injection into the rostral ventrolateral medulla on arterial blood pressure, heart rate, and renal sympathetic nerve activity

Microinjection of NRG-1b (2.5 pmol) into the RVLM unilaterally significantly

decreased MAP, HR, and RSNA [DMAP, 22.3 $\pm$ 1.8 mmHg; DHR, 36.1 $\pm$ 5.5 beats/min; DRSNA %baseline, 36.9 $\pm$ 6.2%; P < 0.01, n =5; Fig. 1a]. These changes occurred slowly and peaked approximately 10–15 min after injection. The depressor and bradycardic responses induced by NRG-1b occurred in a dose-dependent manner (Fig. 1b).

Effects of inhibition of ErbB2 and ErbB4 receptors in the rostral ventrolateral medulla on mean arterial pressure, heart rate, and renal sympathetic nerve activity

MAP, HR, and RSNA values increased significantly after bilateral injection of the ErbB2 receptor blocker, AG825 (1.0 pmol), into the RVLM (ΔMAP, +17.6 $\pm$ 2.3 mmHg; ΔHR, +16.5 $\pm$ 1.1 beats/min; ΔRSNA %baseline, +29.6 $\pm$4.1%; P < 0.01, n = 5; Fig. 2a, c). These variables also increased after injection of the ErbB4 inhibitor, AG1478 (1.0 pmol) (ΔMAP, 11.0 $\pm$ 1.6 mmHg; ΔHR, 12.9$\pm$ 1.9 beats/min; ΔRSNA %baseline, þ18.3 $\pm$ 3.8%; P < 0.01, n = 5; Fig. 2b, c). These changes occurred slowly and peaked approximately 10–15 min after microinjection of the blockers. After microinjection of both AG825 (0.5 pmol) and AG1478 (0.5 pmol), the depressor response to NRG-1b (2.5 pmol) injected into the RVLM was nearly abolished (ΔMAP, 22.3$\pm$ 1.8 vs. 2.0$\pm$ 0.7 mmHg; ΔHR, 36.1 $\pm$ 5.5 vs.

2.5± 0.7 beats/min; P < 0.01, n = 5 for each; Fig. 2d). Furthermore, to demonstrate

that the effects of the reagents in the RVLM are site specific, microinjections

of NRG-1b and AG825 were performed 1.0-mm dorsal apart from the RVLM in Wistar

rats. The changes in MAP, HR, and RSNA were not significant (Supplemental

Digital Content 2, Online Table 2, http://links.lww.com/HJH/A109).

Effect of blockade of γ -aminobutyric acid-A receptors in the rostral ventrolateral

medulla on the depressor response to neuregulin-1

The baseline MAP before NRG-1b injection increased after the injection of the

GABA-A receptor antagonist bicuculline (100 pmol) (MAP increased from 118.3

3.8 to 146.3± 10.7 mmHg, whereas HR decreased from 337.8± 13.2 to 332.3 ±17.6

beats/min). The depressor response to NRG-1b (2.5 pmol) into the RVLM was sig-

nificantly attenuated after bicuculline injection (Δ MAP, 22.9 ± 2.9 vs. 4.6± 2.0

mmHg; Δ HR, 38.1 ± 5.5 vs. 11.9 ± 2.3 beats/min; P < 0.01, n = 5 for each).

Effect of neuregulin-1 in the rostral ventrolateral medulla on the pressor response

to L -glutamate

The baseline MAP before L -glutamate injectiondecreased after NRG-1b injection (2.5

pmol) from 126.0 ± 5.2 to 109.9 ± 4.3 mmHg. The pressor response to L-glutamate

(1.0 nmol) into the RVLM was significantly attenuated after NRG-1b injection (Δ MAP,

23.4 $\pm$ 1.0 vs. 10.8 $\pm$ 1.6 mmHg; P < 0.01, n =5 for each).

ErbB receptors expression levels in the brainstem in Wistar–Kyoto and spontaneously hypertensive rats

NRG-1 and ErbB receptors (ErbB2, ErbB3, and ErbB4) are expressed in the brainstem in both the 12-week-old in SHRs and WKY rats. However, only ErbB2 expression levels were significantly lower in SHRs than in WKY rats (P < 0.05, n =5 for each; Fig. 3a). We further examined NRG-1 and ErbB receptor expression levels in the brainstem of 4-week-old prehypertensive SHRs and age-matched WKY rats. ErbB2 expression levels were significantly lower in 4-week-old SHRs than in WKY rats (n = 5 for each; Fig. 3a). NRG-1 and other ErbB receptor expression levels, however, were similar between SHRs and WKY rats for both ages (n = 5 for each; Fig. 3b). On the contrary, in cerebral cortex and hypothalamus, ErbB2 expression levels were similar between 12-week old SHRs and WKY rats (Fig. 3c).

Acute effects of neuregulin-1 or ErbB inhibitors on mean arterial pressure in Wistar–Kyoto and spontaneously hypertensive rats

The magnitudes of decreases in MAP, HR, and RSNA evoked by the unilateral injection of NRG-1b (2.5 pmol) into the RVLM were significantly smaller in SHRs than in WKY rats (Δ MAP, 23.3 $\pm$ 2.5 vs. 12.5 $\pm$ 1.7 mmHg, P < 0.01; Δ HR, 29.9 $\pm$ 4.0

vs. 15.5 $\pm$ 1.6 beats/min, P < 0.05; Δ RSNA %baseline, 33.8 $\pm$4.2 vs. 17.2 $\pm$ 3.0%, P < 0.01; n =5 for each; Fig. 4a). The magnitudes of increases in MAP, HR, and RSNA evoked by the injection of AG825 (1.0 pmol) were significantly smaller in SHRs than in WKY rats (Δ MAP, þ16.7$\pm$ 1.4 vs. þ8.9 $\pm$ 1.0 mmHg, P < 0.05; Δ HR, þ20.5$\pm$ 4.2 vs. þ10.0 $\pm$ 1.2 beats/min, P < 0.05; Δ RSNA %baseline, þ31.8 $\pm$ 4.9 vs. þ17.8$\pm$ 2.3%, P < 0.01; n= 5 for each; Fig. 4b). In contrast, the magnitudes of increase in MAP, HR, and RSNA evoked by the injection of AG1478 (1.0 pmol) did not differ between SHRs and WKY rats (Δ MAP, +8.7 $\pm$ 0.9 vs. +9.9 $\pm$ 1.2 mmHg; Δ HR, +11.2 $\pm$1.8 vs. +10.9 $\pm$ 0.8 beats/min; Δ RSNA %baseline, +18.9$\pm$3.5 vs. +19.8 $\pm$ 3.7%; P =NS, n = 5; Fig. 4c).

Effects of local inhibition of ErbB2 in the rostral ventrolateral medulla caused by in-vivo small interference RNA on mean arterial pressure, heart rate, and urinary norepinephrine excretion in conscious

Wistar–Kyoto rats Inhibition of ErbB2 receptors in the RVLM using siRNA increased MAP and HR between days 1 and 5 after the siErbB2 treatment in WKY rats. In contrast, these variables did not change in the siControl-treated rats (P < 0.05, n = 5 for each; Fig. 5a). Twenty-four-hour uNE levels at day 1 and 7 were significantly greater in siErbB2 than that in siControl-treated rats (P < 0.01, n = 5 for each; Fig. 5b). The ErbB2

protein expression levels of RVLM in siErbB2-treated rats were successfully inhibited

between days 1 and 5 compared with day 0 (Fig. 5c).

Discussion

The findings of the present study are the first to suggest that NGR-1/ErbB signaling in

the RVLM reduces blood pressure through the inhibition of the sympathetic nervous

system activity. This suggestion is supported by the results of microinjection of NRG-1

or ErbB2 and ErbB4 antagonists into the RVLM, which demonstrated a decrease or

increase in blood pressure associated with changes in RSNA in acute anesthetized rats.

This is further supported by the experiments involving the inhibition of ErbB2 receptors

in the RVLM using siRNA for ErbB2 receptors in chronic conscious state. Furthermore,

our findings suggest that signaling in the RVLM is impaired in SHRs. This is based on

the results indicating that the depressor response to NRG-1 and pressor response to the

ErbB2 in the RVLM are attenuated in SHRs together with the reduced ErbB2

expression levels in the RVLM of SHRs. Therefore, signaling abnormalities in the

RVLM may contribute to, at least in part, the neurogenic mechanisms of hypertension.

NRG-1/ErbB signaling in the RVLM was found to have depressor effects with

sympathoinhibition. Microinjection of recombinant NRG-1b into the RVLM decreased

arterial pressure, HR, and RSNA in anesthetized normotensive rats. In contrast,

microinjection of the ErbB2 receptor antagonist as well as the ErbB4 receptor

antagonist into the RVLM increased arterial pressure, HR, and RSNA. These data

suggest that NRG-1/ErbB signaling in the RVLM is involved in regulating resting blood

pressure. The depressor response to NRG-1 was nearly completely blocked when both

ErbB2 and ErbB4 receptor blockers were administered. We cannot exclude the

possibility that the ErbB3 receptors might also be involved in the depressor response to

NRG-1 because NRG-1 stimulation could induce ErbB2/ErbB3 heterodimer or ErbB3

homodimer formation. We did not examine the effect of ErbB3 inhibition because

the ErbB3 receptor antagonist is commercially not available. However, the role of the

ErbB3 receptor in the NRG-1-induced hypotensive response in the RVLM is probably

not that strong based on the results obtained using ErbB2 and ErbB4 receptor

antagonists. It has been reported that only NRG-1, ErbB2, and ErbB4 are present

in synapse-rich regions [32]. It should be noted that it has also been reported that

peripheral NRG-1 affects cardiomyocytes, leading to negative inotropic effects [19].

Our findings are evoked by the agents of their central effects because the amount of the

agents used in the present study is very small and directly administered into the RVLM.

In fact, we did not find blood pressure and HR changes when the same amount of agents

was administered systemically in Wistar rats. It is possible that synaptic function alteration in the RVLM might be involved in the NRG-1-induced hypotensive response. The depressor response to NRG-1 injection into the RVLM was attenuated by the blockade of the GABA-A receptors. This supports the hypothesis that NRG-1 in the RVLM increases GABA, the major inhibitory neurotransmitter, and releases and/or augments GABA-A receptor activity. The pressor response to L -glutamate, the major excitatory neurotransmitter, was also attenuated prior to the injection of NRG-1 into the RVLM. This suggests that N-methyl- D -aspartic acid (NMDA) and/or non-NMDA response to L –glutamate is attenuated by NRG-1 stimulation. Because the major neurotransmitters involved in regulating the activity of RVLM neurons include glutamate and GABA [18], alteration of synaptic transmission induced by L -glutamate and GABA is important for regulating SNA [17]. NRG-1 and ErbB receptors are extensively distributed in the brain, including the medulla, and they exist in neurons, glia, and oligodendrocytes [3]. Also, it has been reported that glutamate and GABA receptors colocalizes with ErbB receptors in postsynaptic lesions [6–9]. Several studies have shown that the NRG-1/ErbB pathway is involved in the regulation of postsynaptic glutamate receptor function and presynaptic release of GABA, although these functions have not been determined for the RVLM [11,12,33,34]. For example, NRG-1

significantly enhances the depolarization-induced release of GABA in hippocampal

neurons [11] and inhibits NMDA receptor currents in prefrontal cortex neurons

[12,33,34]. Determining whether the actions are presynaptic vs. postsynaptic is difficult.

We did not address which cells were responsible for our observations. Further studies

are necessary to clarify the precise mechanisms involved. In addition to the blood

pressure-lowering effect of the NRG-1/ErbB signaling in the RVLM through

sympathoinhibition, we found that this signaling in the RVLM is impaired in SHRs

compared with that in WKY rats. Particularly, reduced ErbB2 receptor expression levels

in the RVLM of SHRs occurred during the prehypertensive age and persisted through

the established hypertensive age of SHRs. Although NRG-1 and ErbB receptors are

expressed in the brain, we did not observe reduced ErbB2 expression levels in other

areas of the brain in SHRs (cerebral cortex and hypothalamus). Importantly, the

depressor response to NRG-1 and the pressor response to the ErbB2 antagonist were

attenuated in SHRs compared with responses in WKY rats. However, the pressor

response to ErbB4 inhibitor did not differ between SHRs and WKY rats. Thus, we

suggest that the reduction of ErbB2 receptors in the RVLM might contribute to the

hypertensive state of SHRs. On the basis of these findings, we further investigated

whether a reduction in ErbB2 expression in the RVLM contributes to increased blood

pressure in the conscious state. We inhibited ErbB2 expression in the RVLM of WKY

rats using siRNAs. In this experiment, we used the AteloGene kit to deliver siErbB2

into the RVLM. AteloGene is a commercial kit used to locally administer siRNA into

tissues in vivo. It has no toxicity and forms a gel in the body [35]. Thus, siRNA is

maintained at the administration site [35]. Our findings indicate that reducing ErbB2

receptor expression levels in the RVLM increases blood pressure and HR and is

associated with sympathoexcitation. These findings also suggest a dysfunction in

NRG-1/ErbB signaling; reduction in ErbB2 levels in the RVLM might contribute to the

neural mechanisms of hypertension in SHRs. In conclusion, our findings indicate that

the NRG-1/ErbB signaling in the RVLM exerts antihypertensive effects by reducing

SNA in normotensive rats. Furthermore, impairment of NRG-1/ErbB signaling in the

RVLM due to reduced levels of endogenous ErbB2 is a possible neural mechanism of

hypertension in SHRs.

Conflicts of interest

There are no conflicts of interest.

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