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Activation of Vestibular Neurons Projecting to Autonomic Brain Stem Nuclei Following Acute Hypotension in Rats

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Extracellular regulated protein kinase1/2 (pERK1/2) is one of the major regulatory factors for transcription of the c-fos oncogene in neurons. The purpose of this study was to evaluate the expression of phosphorylated ERK1/2 within the vestibular nuclei (VN) of rats following acute arterial hypotension. Following the acute arterial hypotension induced by rapid hemorrhage, a significant number of pERK1/2-immunoreactive neurons appeared bilaterally in the caudal aspect of the medial and inferior VN. No labeling of pERK1/2 was observed in the lateral VN. The peak expression of pERK1/2 in these nuclei occurred within 5 min after hemorrhage. However, in bilaterally labyrinthectomized rats, the appearance of pERK1/2-immunoreactive neurons was eliminated in the VN. Western blot confirmed the effect of bilateral labyrinthectomy on pERK1/2 protein expression in the medial vestibular nucleus 5 min after hemorrhage. These results suggest that, following acute hypotension, afferent signals from the peripheral vestibular receptors are required for activation of ERK 1/2 in the VN.

Key Words: Vestibular nucleus, Hypotension, pERK 1/2, Labyrinthectomy

INTRODUCTION

Some of the cardiovascular disorders cause vestibular symptoms, such as vertigo, spontaneous nystagmus, and postural instability (Andrews et al, 1988; Ohashi et al, 1990; Kikuchi et al, 1993). The changes of vestibular functions occur as blood flow in vertebrobasilar artery is reduced (Matsunaga et al, 1979; Yamamoto et al, 1985; Nario et al, 1997). Our previous electrophysiological study showed that acute arterial hypotension results in alteration of resting neuronal firing rate in the medial vestibular nucleus (MVN). Moreover, acute arterial hypotension also induces the expression of c-Fos protein, a marker of depolarization in a neuron, in the vestibular nuclei (VN) and ablation of vestibular end-organs markedly suppresses the expression of c-Fos protein that is induced by hypotension in the VN (Kim et al, 2003).

Extracellular signal-regulated protein kinase 1/2 (ERK 1/2), a member of the family of mitogen-activated protein kinases, is involved in a complex intracellular signaling cascade to controls various neurobiological effects in the nervous system (Seger and Krebs, 1995; Grewal et al, 1999). ERK1/2 is activated via phosphorylation catalysed by the MAPK/ERK kinase by stimuli such as membrane depolarization (Rosen et al, 1994), glutamate (Vanhoutte et al, 1999), and electrical stimulation (Sgambato et al, 1998). The phosphorylated form of ERK1/2 protein (pERK 1/2) is crucial for controlling transcription of immediate early-genes, such as c-fos (Xia et al, 1996; Sgambato et al, 1998; Vanhoutte et al, 1999).

Therefore, it has been suggested that activation of ERK occurs in the VN following acute hypotension. The purpose of this study was to evaluate the spatiotemporal changes in pERK1/2 expression in the VN following acute hypotension and the effects of labyrinthectomy on the expression of pERK1/2 hypotension induced.

METHODS

Experimental animals

Sprague-Dawley rats, weighing 250-300 g, were divided into three groups: a sham operation group (n=6) a control group (CON) with intact labyrinth (n=21) and a bilateral labyrinthectomy (BL) group (n=9). The procedures used were approved by the Institutional Ethical Committee on Experimental Use of Animals.

Labyrinthectomy and hemorrhage

Chemical BL and the recording of blood pressure (BP) were performed as previously described in detail (Kim et al, 2003). Briefly, under chloral hydrate anesthesia (300 mg/kg, i.p.), 100 μL of sodium arsanilate (100 mg/mL) were intratympanically injected into the bilateral middle ear. A catheter was inserted into each of the femoral arteries of

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ABBREVIATIONS: VN, vestibular nuclei; ERK1/2, extracellular signal-regulated protein kinase 1/2; BP, blood pressure; BL, bilateral labyrinthectomy.

all experimental animals after anesthesia. The BP was recorded from the unilateral femoral artery using a pressure transducer, physiograph, and digital recording system (CED Ltd., Cambridge, UK). On the contralateral femoral artery, the catheter was connected to a 5-mL heparinized syringe mounted to a peristaltic pump (WPI, Sarasota, FL, USA), which was partly modified to withdraw the syringe also. Baseline measurements of BP were obtained during a 2-h period following the surgery. The peristaltic pump retracted the syringe at a constant rate of 200 mL/h, removing about 4 mL of arterial blood from the femoral artery, where the BP dropped rapidly within 1 min to about 50% of the baseline value. Rectal temperature was monitored and body temperature was maintained at 37± 1°C during the experiment. Most of the experiments on acute hypotension in the BL group were carried out 2 weeks after labyrinthectomy.

Immunohistochemistry

Animals were sacrificed at 5 min (n=6), 30 min (n=6), and 1 h (n=6) (CON), or 5 min (BL) after the induction of hypotension. Immunohistochemistry and image analysis were performed as previously described in detail (Kim et al, 2003). Briefly, under deep anesthesia, rat brain was perfused and fixed with 4% paraformaldehyde phosphate-buffered saline solution. The sucrose-embedded brainstem was sectioned at a thickness of 40 μm on a cryostat. After nonspecific binding sites were blocked with normal goat serum, primary anti-rabbit polyclonal pERK1/2 antibody (1 : 1000)(Cell Signaling Technology, Beverly, MA, USA) was applied overnight at 4°C. The following day, tissue sections were incubated with the secondary antibody and then with the avidin/biotin complex at room temperature. The bound complex was visualized by incubating the tissue with 0.05% DAB and 0.003% H_2O_2. For quantification, p-ERK1/2-positive neurons in the VN were counted using an image analysis system (Media Cybernetics, Inc., Silver Spring, MD, USA) at four different levels (rostrally to caudally)(Paxinos and Watson, 1986). Three rats received the same surgical and immunohistochemical procedures with an exception of hemorrhage being replaced with a sham operation.

Western blot

For Western blotting, the rats were killed by decapitation at specified times under deep anesthesia. The brain was rapidly isolated and mounted on a rat brain matrix in ice-cold artificial cerbrospinal fluid. The brain stem was cut, and tissue containing the medial and inferior vestibular nuclei was selectively isolated from a brain stem slice using a tissue puncher (Fine Science Tools Inc., CA, USA) under a surgical microscope. Tissues were homogenized in 1 : 10 (w/v) ice-cold homogenization buffer consisting of 20 mM Tris-HCl (pH 8.0), 1% NP-40, 150 mM NaCl, 0.5 mM dithiothreitol, 2 mM sodium orthovanadate, 1 mM EDTA, 0.1% beta-mercaptoethanol, and a mixture of proteinase inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 1 μg/ml pepstatin A). The homogenates were centrifuged at 15,000 g for 30 min at 4°C. The supernatant was collected, frozen, and kept at −80°C. The protein content was determined the method of Bradford (Bradford, 1976). Proteins were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, according to the method of Laemmli (Laemmli, 1970). These equal amounts of protein (20 μg in all assays) were loaded in each lane with loading buffer containing 0.125 M Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 10% mercaptoethanol, and 0.002% bromphenol blue. The samples were heated at 95°C for 5 min before gel loading. The protein separated by gel electrophoresis was transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon; Millipore, Bedford, MA, USA) using an electrophoretic transfer system (Semi-Dry Transfer System; Biocraft, Fair Lawn, NJ, USA) at 12 V for 1 hr. The blotting buffer used contained 48 mM Tris, 39 mM glycine, 1.3 mM SDS, and 20% methanol (pH 8.3). The membrane filters were then washed with Tris-buffered saline solution (pH 7.4) and 0.05% Tween 20 (TTBS), and then blocked in TTBS containing 5% skim milk for 60 min. Next, the filter was probed overnight at 4°C with anti-ERK1/2 antibody (polyclonal, 1 : 10,000 dilution) or anti-diphosphorylated ERK1/2 (monoclonal, 1 : 5,000 dilution) diluted in TTBS containing 3% bovine serum albumin. Then, the membrane filters were incubated for 1 h with a secondary antibody conjugated with horseradish peroxidase diluted in TTBS containing 3% bovine serum albumin. Several washes with TTBS 20 were performed between each step. The Western blots were developed using SuperSignal West Pico Chemiluminescent Substrate from Pierce (Rockford, IL, USA) and exposed to Amersham ECL films. A computer- assisted analysis of the bands was performed with the Bio-Rad GS700 system (Bio-Rad, CA, USA), and the data were processed with the Bio-Rad Molecular Analyst image program using a personal computer. Repeated scans were taken for film non-linearity corrections. Negative controls were carried out without the first antibodies (anti-ERK1/2 antibody or anti-diphosphorylated ERK1/2 antibody), but including all the other procedures. The ratios between pERK1 and ERK1 and pERK2 and ERK2 were determined mainly by absolute values of pERK1 and pERK2.

Statistical anaylsis

All data are presented as means±S.D. The statistical significance of differences was assessed using Kruskal-Wallis non-parametric test and Mann-Whitney U test. Values of $p<0.05$ were considered significant.

RESULTS

Before the induction of hemorrhagic hypotension, baseline mean arterial pressure was 98.3±4.3 mmHg (n=6) for the CON group. Hemorrhage caused the mean arterial pressure to fall rapidly within the first few seconds with a maximum decrease of 47.3±5.3 mmHg (~50%) occurring at 1min (Fig. 1).

The sham operation had a negligible effect on the expression of pERK1/2-like immunoreactivity (LI) in neurons in all regions of the VN. In the control group, acute hemorrhage gave rise to significant expression of pERK1/2-LI in neurons in the bilateral medial vestibular nucleus (MVN), inferior vestibular nuclei (IVN), with pERK1/2-LI being most prominent in the caudal MVN (Fig. 2, 3), On the other hand, no pERK1/2 immunoreactivity was detected in the superior and lateral VN 5 min after the hemorrhage. The expression of pERK1/2-LI in the MVN and IVN peaked at 5 min after the hemorrhage and then decreased rapidly at

30 min, finally disappearing at 90 h after the hemorrhage (Fig. 2, 4).

In contrast, complete suppression of pERK1/2 protein expression, induced by acute hemorrhage, was seen in the VN of the BL group ($p<0.01$)(Fig. 3). Therefore, there was statistical difference in the number of pERK1/2-LI neurons of the VN between the control group and BL group 5 after the hemorrhage ($p<0.01$)(Fig. 4).

Western blot analyses of phospho-ERK 1/2 were performed in whole cell lysate from the medial vestibular nuclei. In the sham operation group, ERK1 and pERK1 were visible as 44-kDa bands, and ERK2 and pERK2 as 42-kDa bands (Fig. 5). The phosphorylated ERK1/2 band was very weak, as compared to that of the ERK1/2 proteins. Five minutes after the hemorrhage, marked increases in pERK1 and pERK2 were observed in the medial vestibular nuclei of the control group. The relative density of pERK1/2 was significantly increased in the vestibular nuclei of sham operation ($p<0.05$). There was a marked reduction in the relative density of pERK1/2 in vestibular nuclei of the BL group 5 min after the hemorrhage. In the same experiment, non-phosphorylated ERK1 and ERK2 remained relatively constant. The average increases in the ratios of pERK1 to ERK1 and pERK2 to ERK2 (pERK/ERK ratios) after UL are shown in Fig. 6. The result of the Western blot to examine the expression of pERK1/2 protein was consistent with the immunohistochemical findings.

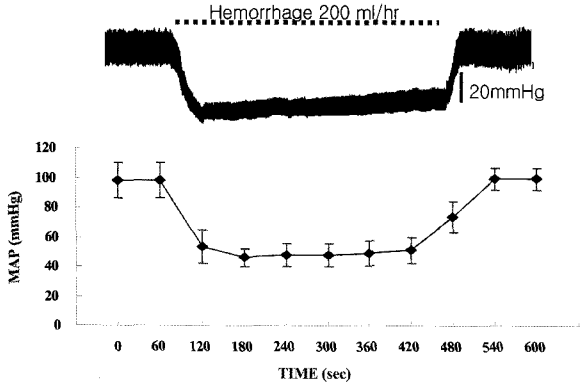

Fig. 1. Representative recording of arterial pressure (A) and line histograph showing change of the mean arterial pressure (MAP) (B) by acute hemorrhage with amount of 4 ml at 200 ml per hour from the femoral artery in control group. Values are mean±S.D. Number of rats was 12.

DISCUSSION

The present results indicated that transient but marked expression of pERK1/2 protein occurs in the bilateral VN, especially in the caudal MVN and IVN, in response to acute hypotension in anesthetized rats. The present experment also demonstrated that the pERK1/2 protein, induced by acute hypotension, was completely inhibited by labyrin-

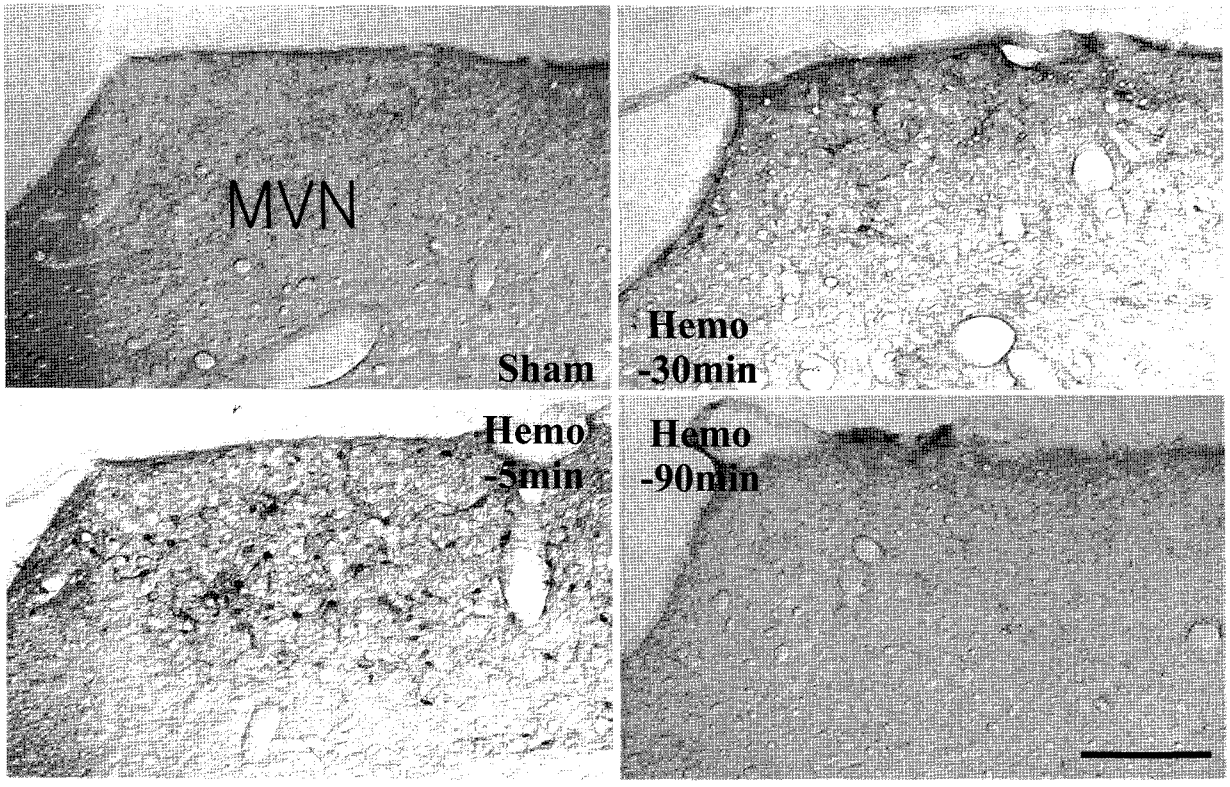

Fig. 2. Photomicrographs depicting the expression of pERK1/2 immunoreactive neurons in the medial vestibular nucleus (MVN) of sham operation and of control group at 5, 30, and 90 min after acute arterial hypotension. Solid bar, 200 μm.

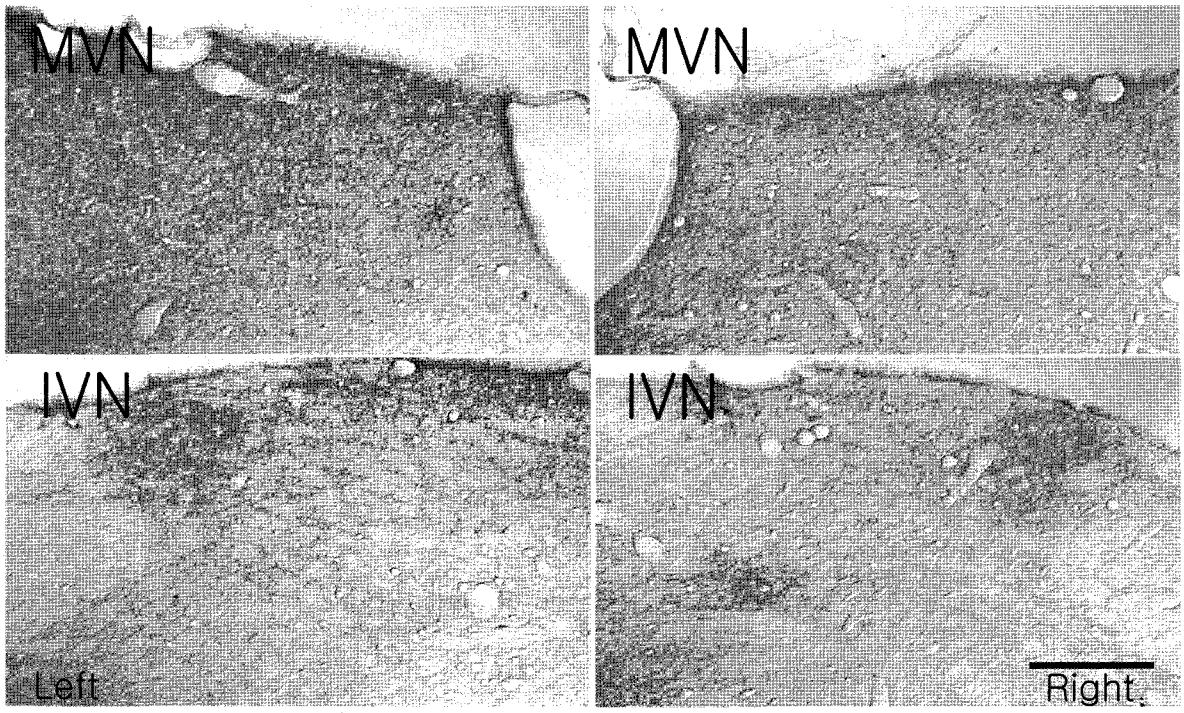

Fig. 3. Photomicrographs depicting the expression of pERK1/2 immunoreactive neurons in the medial (MVN) and inferior (IVN) vestibular nucleus of unilateral labyrinthectomized rats 5 min after acute arterial hypotension. Solid bar, 200 μm.

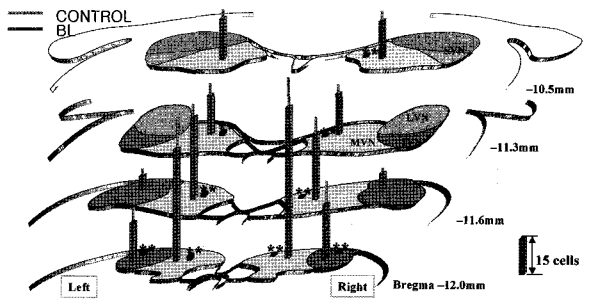

Fig. 4. Bar histograph showing number of pERK1/2 immunoreactive neurons in the vestibular nuclei of CON and BL groups at 4 different levels 5 min after acute hemorrhage. Values are mean±S.D. Number of rats in each group was 6. CON, control; BL, bilateral labyrinthectomy; SVN, superior vestibular nucleus; LVN, lateral vestibular nucleus; MVN, medial vestibular nucleus; IVN, inferior vestibular nucleus. *Denotes significant difference between control and BL (**$p<0.01$).

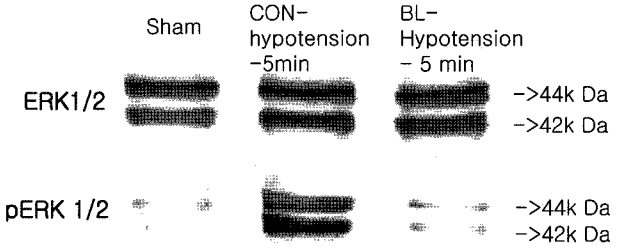

Fig. 5. Photograph showing a typical finding of Western blot for expression of ERK1/2, inactive form, and pERK1/2 proteins on a filter membrane from the whole cell lysate of the medial vestibular nucleus 5 min after acute hypotension.

thectomy. Acute hypotension significantly increases the firing rate of type I MVN neurons, which are excited by ipsilateral horizontal rotation (Park et al, 2001). Nevertheless, this excitatory response of type I MVN neurons is markedly suppressed by labyrinthectomy (Park et al, 2001). Glutamate receptors mediate or modulate transmission of excitatory afferent signals from the peripheral vestibular receptors to the central vestibular neurons (Guth et al, 1998; Smith, 2000). Moreover, the VN also receives excitatory synaptic inputs from the vestibular commissural fibers originating in the contralateral VN, where glutamate receptors are critical for this response (Cochran et al, 1987; Knopfel, 1987). An increase in the intracellular Ca^{++} level of the MVN neurons occurs following stimulation of the ipsilateral vestibular afferent nerve and commissural fibers through glutamate NMDA receptor (Takahashi et al, 1994). Glutamate stimulation, which increases the intracellular calcium level, also activated ERK in neurons (Xia et al, 1996; Vanhoutte et al, 1999). These observations, together with the results of the present study, suggest that the main reason for activation of ERK1/2 in response to acute hemorrhage in the VN is the excitation of glutamatergic afferent synapses between VN neurons and the peripheral

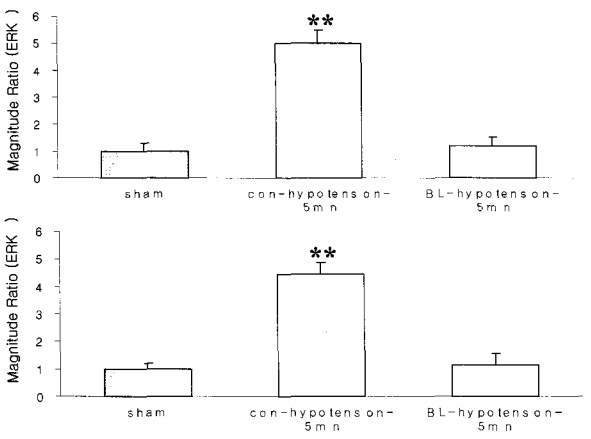

Fig. 6. Bar histograph showing densitometric results for pERK1/2 protein in the medial vestibular nucleus 5 min after acute hypotension. The ratios between pERK1 and ERK1 and pERK2 and ERK2 were determined mainly by the absolute values of pERK1 and pERK2. Number of rats in each group were 3. Values are mean ±S.D. *Denotes significant difference between Sham or BL-hypotension-5min and con-hypotension-5min (**$p < 0.01$).

vestibular receptors. Excitation of the contralateral VN through the commissural system may also involve, in part, hypotension-induced expression of pERK1/2 in the VN. It is not clear, however, how reduced blood flow causes the activation of vestibular afferent signals in the peripheral vestibular receptors. One possible explanation is that the reduction of blood flow generated an ischemic environment in the inner ear, increasing glutamate concentration (Haruta et al, 1998; Hyodo et al, 2001). In summary, these experiments demonstrate that a significant number of pERK1/2-immunoreactive neurons appeared bilaterally in the caudal aspect of the medial and inferior VN 5 min after acute hemorrhage. The appearance of pERK1/2-immunoreactive neurons was eliminated in the vestibular nuclei of bilaterally labyrinthectomized rats. These findings suggest that, following acute hypotension, afferent signals from the peripheral vestibular receptors are required for activation of ERK1/2 in the vestibular nuclei.

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