Chinese Journal of Physiology 60(6): 353-362, 2017
DOI: 10.4077/CJP.2017.BAG512

Involvement of Endothelial Nitric Oxide Synthase Activation in Midkine-Mediated Central Hypotensive Effects

Ching-Huang Lin[1, 2, 3, *], Jun-Yen Pan[4, 5, *], Jung-Jui Chang[6, *], Hsin-Hung Chen[7, 8], Pei-Wen Cheng[7, 8], and Ching-Jiunn Tseng[7, 9, 10]

[1]Department of Biomedical Sciences, National Sun Yat-sen University, Kaohsiung 80424
[2]Department of Neurology, Kaohsiung Veterans General Hospital, Kaohsiung 81362
[3]Department of Physical Therapy, Shu-Zen Junior College of Medicine and Management, Kaohsiung 82144
[4]Division of Cardiovascular Surgery, Department of Surgery, Kaohsiung Veterans General Hospital, Kaohsiung 81362
[5]Department of Nursing, Shu-Zen Junior College of Medicine and Management, Kaohsiung 82144
[6]Department of Surgery, Zuoying Branch of Kaohsiung Armed Forces General Hospital, Kaohsiung 81342
[7]Department of Medical Education and Research, Kaohsiung Veterans General Hospital, Kaohsiung 81362
[8]Yuh-Ing Junior College of Health Care & Management, Kaohsiung 80765
[9]Institute of Biomedical Sciences, National Sun Yat-sen University, Kaohsiung 80424
and
[10]Department of Pharmacology, National Defense Medical Center, Taipei 11490, Taiwan, Republic of China

Abstract

The growth factor midkine (MK) has been implicated in various biologic and pathologic events. It has been shown that the peripheral influence of MK on cardiovascular regulation is due to an influence on the renin-angiotensin system (RAS). The nucleus tractus solitarii (NTS) is the primary integrative center for cardiovascular control and other autonomic functions in the central nervous system. However, the signaling mechanisms involved in MK-mediated cardiovascular effects in the NTS remain unclear. In this study, we investigated whether the RAS and/or N-methyl-D-aspartate (NMDA) receptor-calmodulin-endothelial nitric oxide synthase (eNOS) signaling pathways were both involved in MK-mediated blood pressure (BP) regulation in the NTS of Wistar-Kyoto (WKY) rats. Intra-NTS microinjection and immunoblot analysis were used to evaluate the signal pathway. WKY rats were anesthetized with urethane. Unilateral microinjection of MK (600 fmol) into the NTS produced a dose-dependent decrease in BP and heart rate (HR). The depressor effects were observed before and after microinjection of the angiotensin-converting enzyme (ACE) inhibitor lisinopril (2.4 fmol), or the angiotensin receptor blockers (ARB) inhibitor valsartan (7.5 pmol). However, lisinopril and valsartan did not diminish the MK-mediated cardiovascular effects in the NTS. Microinjection of the NMDA receptor antagonist MK801 (1 nmol) or the NOS inhibitor N-nitro l-arginine methyl ester (L-NAME), (33 nmol), into the NTS attenuated the MK-induced hypotensive effects. Pretreatment with an eNOS inhibitor N5-iminoethyl-l-ornithine (L-NIO) (6 nmol) attenuated the MK-induced

Corresponding author: Ching-Jiunn Tseng, MD, PhD, Department of Medical Education and Research, Kaohsiung Veterans General Hospital, No. 386, Ta-Chung 1st Rd., Kaohsiung, 81362, Taiwan, ROC. Tel: +886-7-3422121 ext.1505, Fax: +886-7-3468056, E-mail: cjtseng@vghks.gov.tw
*Contributed equally to this work.
Received: February 27, 2017; Revised (Final Version): June 6, 2017; Accepted: July 5, 2017.
 ISSN : 0304-4920. http://www.cps.org.tw

hypotensive effects. In this study, the data showed that MK might play a role in central cardiovascular regulation in the NTS. These results suggest that MK decreased BP and HR in the NTS probably acting *via* the NMDA receptor-calmodulin-eNOS signaling pathway.

Key Words: central cardiovascular regulation, midkine, nitric oxide synthases, nucleus tractus solitarii

Introduction

Midkine (MK) is a heparin-binding growth factor or cytokine that promotes growth, survival, differentiation, migration and other activities of target cells (16, 24, 25). MK has been shown to have a cardiac protective effect against acute ischemia-reperfusion injury and infarction, and one of the mechanisms for this being its anti-apoptotic effect (13). Administration of MK-expressing adenovirus can also ameliorate cardiac infarction (28). Even though MK has been shown to have cardiac protective effects against acute ischemia-reperfusion injury and to inhibit cardiac remodeling, MK also promotes intimal hyperplasia and vascular stenosis (12). In addition, one of the first links between MK and the renin-angiotensin system (RAS) was made by studying the levels of the RAS components in aorta of the wild-type and MK-deficient mice (7). MK has been proven to play an important role in the regulation of blood pressure (BP) *via* the RAS (10). However, the possible role of MK in central cardiovascular regulation remains unclear.

The RAS plays a central role in the regulation of BP. Previously, it was reported that the expression of an angiotensin-converting enzyme (ACE) was significantly impaired in the aorta of MK-deficient mice compared to that of wild-type mice, implying that MK regulated the expression of ACE (7). However, MK-deficient mice exhibit normal BP under physiological conditions. Hobo *et al*. (10) reported the biological significances of the MK-ACE pathway under pathological conditions by employing 5/6 nephrectomy. Wild-type mice exhibited marked hypertension and severe renal damage, while MK-deficient mice showed almost normal BP and only mild renal obstruction. MK was upregulated in both the lung and kidney in response to oxidative stress, and induced ACE expression *via* the phosphorylation of protein kinase C (PKC) in vascular endothelial cells (10). Recently, it has been shown that the peripheral influence of MK on cardiovascular regulation is due to an influence on RAS (1). Our previous studies indicated that the extracellular signal-regulated kinases 1 and 2 (ERK1/2)-ribosomal protein S6 kinase (RSK)-neuronal nitric oxide synthase (nNOS) signaling pathway might play a significant role in angiotensin II–mediated central BP regulation in the nucleus tractus solitarii (NTS) (4). In addition, MK-deficient mice were resistant to hypertension and developed less glomerulosclerosis and proteinuria after administration of a NOS inhibitor in the setting of uninephrectomy (26). Hypertension observed in uninephrectomized wild-type mice after NOS inhibition was ameliorated by an anti-MK antibody. Our previous studies showed that, when microinjected into the NTS, a non-selective inhibitor of NOS increased arterial pressure and renal sympathetic nerve activity (31). Moreover, administration of nicotine into the NTS demonstrated the involvement of N-methyl-D-aspartate (NMDA) receptor-calmodulin-endothelial nitric oxide synthase (eNOS)-NO signaling pathway in the cardiovascular effects of nicotine (2, 11).

The NTS is located in the dorsal medulla of the brain stem, which is the primary integrating center for cardiovascular regulation and other autonomic functions of the central nervous system. Our previous studies demonstrated that several neuromodulators, including adenosine (30), angiotensin II (4), angiotensin III (29), carbon monoxide (19), insulin (14), renin (5), neuropeptide Y (3), nicotine (2) and nitric oxide (20) are involved in cardiovascular control in the NTS. In this study, we investigated whether microinjection of MK into the NTS might regulate BP, and we determined which form of NOS could be activated by MK administration. In addition, we investigated which receptors and downstream signaling pathways were involved in MK-induced effects in the NTS. Our results suggest that a possible MK-calmodulin-eNOS signaling pathway is involved in the modulation of cardiovascular control by interaction with NMDA receptor in the NTS.

Materials and Methods

Animals

Male Wistar-Kyoto (WKY) rats weighing 250-300 g were obtained from National Laboratory Animal Center and housed in the animal room of Kaohsiung Veterans General Hospital (Kaohsiung, Taiwan, ROC). The rats were kept in individual cages in a room in which lighting was controlled (12 h on/12 h off), and the temperature was maintained at 23°C to 24°C. The rats were in stabilization to acclimatize to the

housing conditions for 1 week. The rats were given normal rat chow (Purina, St. Louis, MO, USA) and tap water *ad libitum*. All animal research protocols were approved by the Research Animal Facility Committee of Kaohsiung Veterans General Hospital.

Intra-NTS Microinjection

Rats were anesthetized with urethane (1.0 g/kg intraperitoneal injection (IP) and 0.3 g/kg intravenous injection (IV) if necessary). The preparation of animals for intra-NTS microinjection and the methods used to localize the NTS have been previously described (31). Briefly, a polyethylene cannula was inserted into the femoral vein for fluid supplementation, and BP was measured *via* a femoral-artery cannula by pressure transducer and polygraph (Gould, Cleveland, OH, USA). Heart rate (HR) was monitored by a tachograph preamplifier (Gould). To verify that the needle tip of the glass electrode was exactly in the NTS, L-glutamate (0.154 nmol/60 nl) was microinjected to test induction of a characteristically abrupt decrease in BP (BP ≧ 35 mmHg) and HR (HR ≧ 50 bpm) if the needle tips were to be located precisely in the NTS.

To investigate whether NMDA(R)-NOS signaling participated in the depressor effect of MK in the NTS, the electrode was filled with one of the following drugs: ACE inhibitor (lisinopril, 2.4 fmol), angiotensin receptor blockers (ARB) inhibitor (valsartan, 7.5 pmol), NMDA receptor inhibitor (MK801, 1 nmol), calmodulin inhibitor [N-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide; W7, 0.1 nmol], non-selective NOS inhibitor (non-selective NOS inhibitor, L-NAME, 33 nmol), selective nNOS-specific inhibitor (vinyl-L-NIO, 600 pmol), and selective eNOS-specific inhibitor (L-NIO, 6 nmol). Each of the drugs was dissolved in reduced-serum medium (Opti-MEM I; Invitrogen, Carlsbad, CA, USA). The injection volume in the NTS was 60 nl and the injection was made within 10 seconds so as to provide an effective compromise between excessive spread of the drug and coverage of the area being examined; injections were limited to 6-8 times in a rat.

Immunoblotting Analysis

The NTS of WKY rats was removed after injection of MK or vehicle. Total protein was prepared by homogenizing NTS in a lysis buffer containing 20 mM Imidazole-HCl (pH 6.8), 100 mM KCl, 2 mM MgCl, 20 mM ethylene glycol tetraacetic acid (EGTA) (pH 7.0), 300 mM sucrose, 1 mM NaF, 1 mM Na-vanadate, 1 mM Na molybdate, 0.2% Triton X-100, and a proteinase inhibitor cocktail for 1 h at 4°C. Protein extracts (20 μg/sample assessed by BCA protein assay, Pierce Chemical Co., Rockford, IL, USA) were subjected to 6-7.5% sodium dodecyl sulfate (SDS)-Tris glycerin gel electrophoresis and transferred to a polyvinylidene difluoride membrane (PVDF) (GE Healthcare, Buckingamshire, UK). The membrane was blocked with 5% non-fat milk in tris-buffered saline (TBS)/Tween 20 buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20, pH 7.4) and incubated with an appropriate anti-eNOS antibody (BD Biosciences, San Jose, CA, USA) or a mouse anti-β-actin antibody (1:10,000; Millipore, Bedford, MA, USA), which was diluted in phosphate buffered saline Tween-20 (PBST) with 5% bovine serum albumin and incubated at 4°C overnight. Horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibody was used as the secondary antibody (1:5,000) at room temperature for 1 h. Development was incubated for 1 min with enhanced chemiluminescence (ECL)-Plus detected kit (GE Healthcare) and exposure to ECL sensitive films (Super RX; Fuji Photo Film Co., Tokyo, Japan). The films were scanned by a photo scanner (4490; Epson, Long Beach, CA, USA) and analyzed with the NIH Image densitometry analysis software (National Institutes of Health, Bethesda, MD, USA).

Statistical Analysis

A paired *t*-test (before and after pre-treatments), unpaired *t*-test (for control and study group comparisons), or repeated-measures analysis of variance (ANOVA) followed by the Dunnett test was applied to compare group differences. Differences with a *P* value of less than 0.05 were considered significant. All data were expressed as means ± standard error of the mean (SEM).

Results

MK Modulates Central Cardiovascular Effect in the NTS

We initially investigated the effects of MK on the central cardiovascular system of WKY rats by microinjection of MK into the NTS. Unilateral microinjection of increasing doses of MK (66.7 to 1800 fmol) into the NTS produced dose-dependent depressor and bradycardic effects (Fig. 1A). Microinjection of MK at a dose of 66.7 fmol was sufficient to evoke hypotension and bradycardia (-6 ± 1 mmHg and -15 ± 5 bpm) in the WKY rats (Fig. 1B). The dose of 1800 fmol seemed to have reached the maximum effects (-75 ± 6 mmHg and -173 ± 22 bpm). Immunoblotting analysis also showed that microinjection of MK (600 fmol) increased the protein

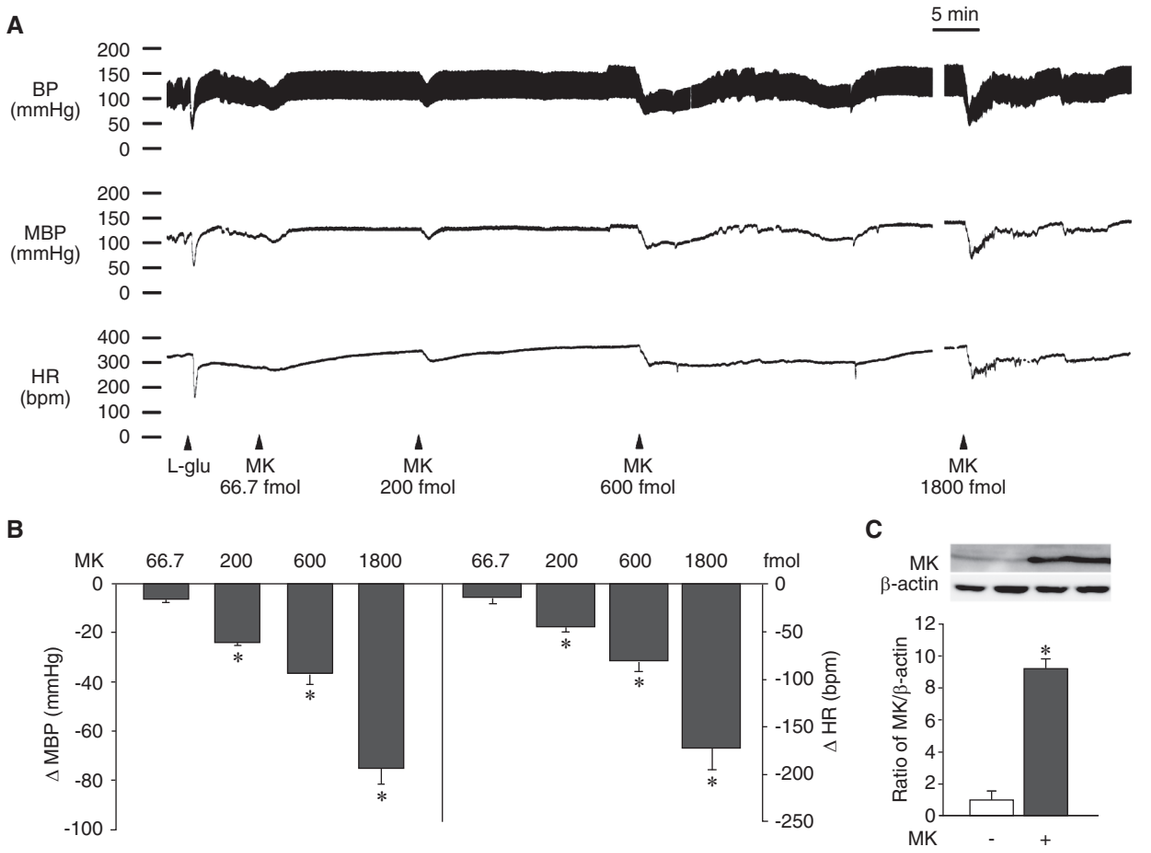

Fig. 1. Effects of different doses of MK microinjected into NTS on cardiovascular effects in rats. (A) Representative tracings demonstrating the cardiovascular effects of different doses (66.7~1800 fmol) of MK in the anesthetized rats. (B) Effects of microinjected MK on MBP and HR into unilateral NTS. (C) MK microinjection showed MK protein expression with and without 600 fmol MK in the NTS. (*$P < 0.05$ *vs* vehicle group).

levels in the NTS (Fig. 1C); therefore, 600 fmol of MK was chosen in subsequent studies to investigate the cardiovascular effects of MK in the NTS of WKY rats.

The RAS Is Not Involved in MK-Mediated Hypotensive Effects in the NTS

To determine whether MK can activate ACE to modulate the central cardiovascular effect through angiotensin II type I receptors (AT1R) in the NTS, the rats were pretreated with lisinopril, an ACE inhibitor, and valsartan, a selective ARB antagonist, and the cardiovascular responses induced by MK microinjection into the NTS were observed. Pretreatment with lisinopril (2.4 fmol) did not change the central cardiovascular effect (Fig. 2A). In addition, pretreatment with the AT1 receptor antagonist valsartan (7.5 pmol) also did not diminish the depressor effect of MK in the NTS (Fig. 2, B and C).

NMDA Receptor Is Involved in MK-Modulated Depressor Effects in the NTS

To examine the role of the NMDA receptor in cardiovascular responses to MK in the NTS, the rats were pretreated with MK801, a non-competitive NMDA receptor antagonist, and the cardiovascular responses induced by microinjection of MK into the NTS were observed. Ten min after MK801 (1 nmol) administration, hypotensive and bradycardic responses to the same dose of MK were significantly attenuated (-40 ± 4 versus -8 ± 2 mmHg and -56 ± 9 versus -26 ± 5 bpm; Fig. 3, A and B). The hypotensive and bradycardic effects of MK recovered after 90 min. These results indicate that the NMDA receptor plays a role in the downstream signaling of MK in the NTS. To determine whether Ca^{2+}/calmodulin is involved in the BP modulatory effects of the NMDA receptor in the NTS, we investigated the effects of a calmodulin inhibitor W7 (0.1 nmol) on the depressor effects

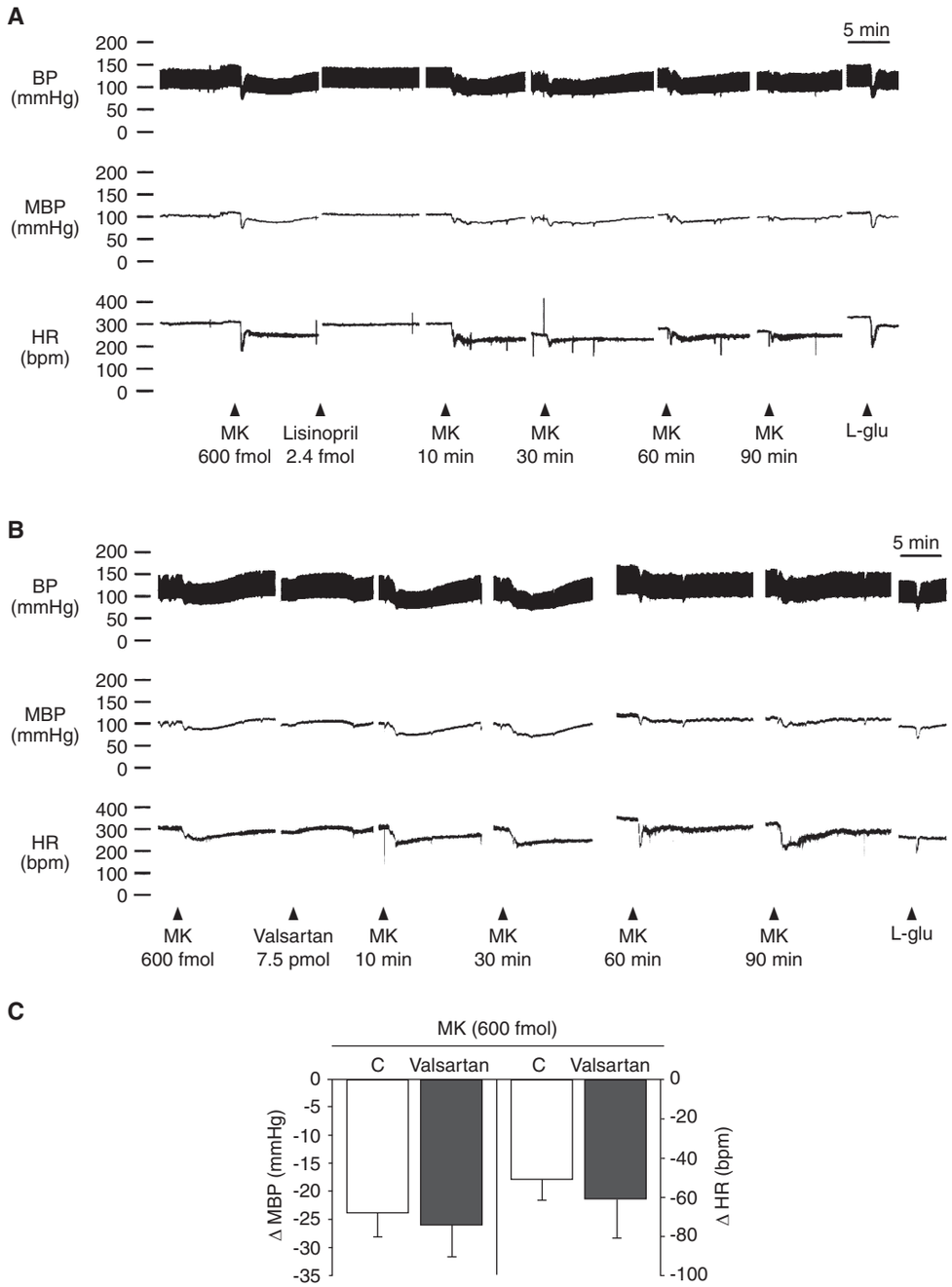

Fig. 2. Cardiovascular effects of MK in the NTS before and after administration of the ACE inhibitor, lisinopril and the ARB inhibitor, valsartan. A-B. Cardiovascular effects of MK (600 fmol) in unilateral NTS before and 10 min after pretreatment with (A) lisinopril (2.4 fmol) and (B) valsartan (7.5 pmol) in anesthetized rats. MK and lisinopril were injected at the indicated time points. BP, MBP, and HR recordings were made at a paper speed of 3 mm/min. The horizontal bar represents recording during 5-min intervals. (C) Effects of pretreatment with valsartan on MBP and HR after microinjection of MK into unilateral NTS. n = 3. MBP, mean blood pressure; L-glu, L-glutamate.

induced by the NMDA receptor in the NTS. The results showed that the BP response to MK was attenuated by prior microinjection of W7 in the NTS of the WKY rats (-35 ± 3 versus -21 ± 5 mmHg and -41 ± 9 versus -21 ± 4 bpm; Fig. 3, C and D). These results indicate that Ca^{2+} signaling is involved in the

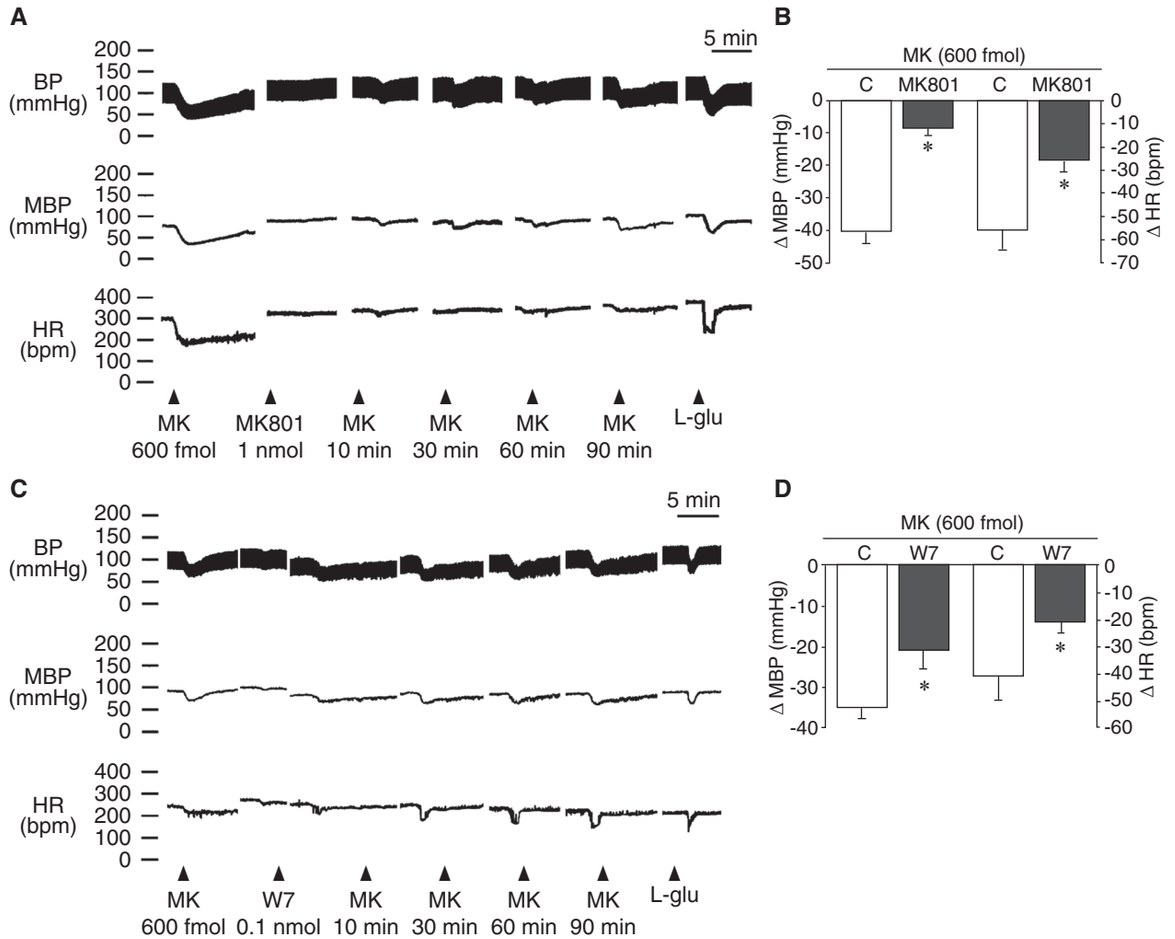

Fig. 3. Cardiovascular effects of MK in the NTS before and after administration of the non-competitive NMDA receptor antagonist, MK801. (A) Cardiovascular effects of microinjection of MK (600 fmol) into unilateral NTS before and 10 min after pretreatment with MK801 (1 nmol) in anesthetized rats. (B) Effects of pretreatment with MK801 on MBP and HR after microinjection of MK into unilateral NTS. (C) Cardiovascular effects of microinjection of MK (600 fmol) into unilateral NTS before and 10 min after pretreatment with W7 (0.1 nmol) in anesthetized rats. (D) Effects of pretreatment with W7 on MBP and HR after microinjection of MK into unilateral NTS. *$P < 0.05$ *vs* vehicle group, n = 4-6.

downstream of stimulation by the NMDA receptor in mediating MK-induced depressor effects.

MK Induces Systemic Vasodepressor Effect by eNOS in the NTS

We further investigated whether NOS contributes to depressor effects of MK in the NTS of WKY rats. Pretreatment with the non-selective NOS inhibitor, L-NAME (33 nmol), attenuated the depressor effect of MK (Fig. 4A). The depressor and bradycardic responses to MK in the NTS were attenuated after L-NAME treatment (-29 ± 4 versus -12 ± 5 mmHg and -66 ± 19 versus -27 ± 17 bpm; Fig. 4, A and B). The depressor effect of MK in the NTS recovered gradually 30 min after L-NAME treatment (Fig. 4B). These results indicate that MK may induce the NOS in the NTS. In addition, the immunoblotting analysis showed that pretreatment with L-NAME could attenuate MK-induced eNOS phosphorylation in the NTS (Fig. 4C).

Previous results showed that depressor responses to MK-mediate NMDA receptor are achieved through activation of NOS in the NTS. To identify which constitutive NOS contribute(s) to these depressor effects of MK in the NTS, the effects of a selective eNOS inhibitor, L-NIO, on the depressor response by MK-mediated NMDA receptor in the NTS were investigated. Ten min after pretreatment with L-NIO (6 nmol), MK significantly attenuated depressor and

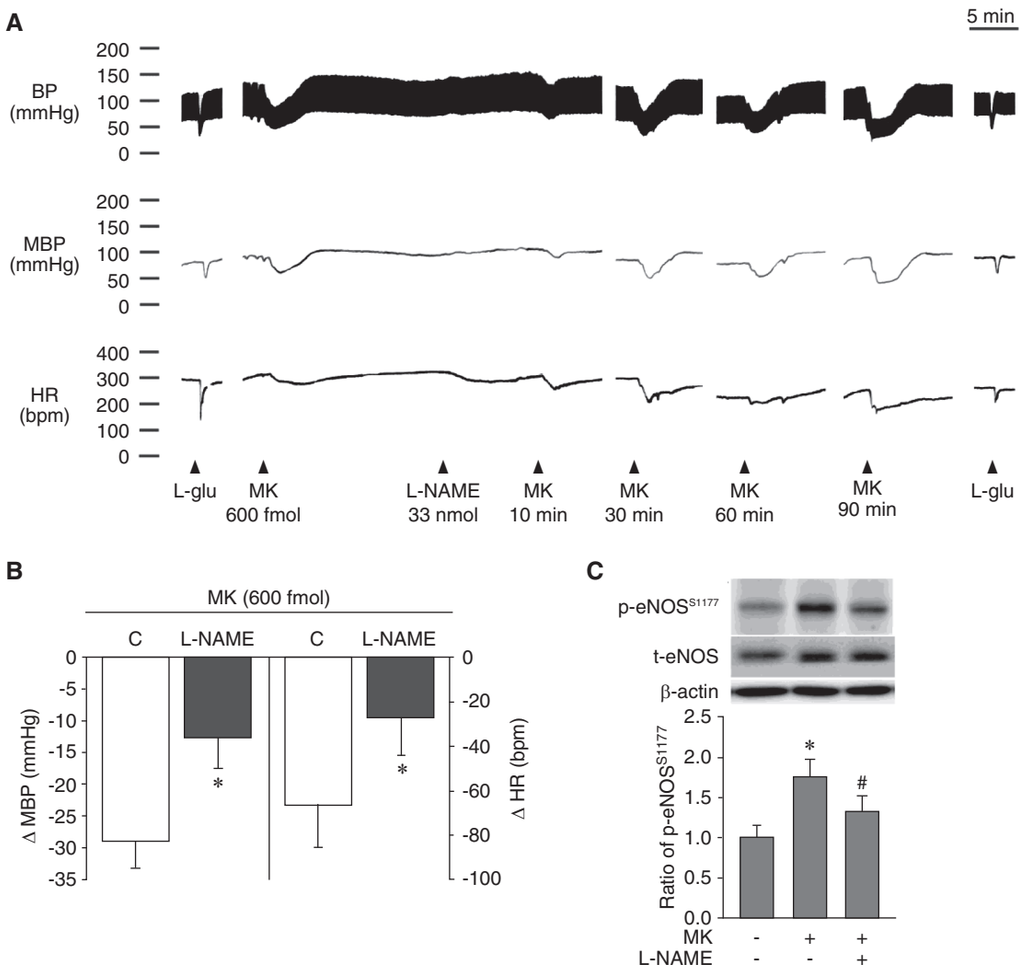

Fig. 4. The NO system participates in the modulation of BP mediated by MK. (A) Cardiovascular effects of microinjection of MK (600 fmol) into unilateral NTS before and 10 min after pretreatment with L-NAME (33 nmol). (B) Effects of pretreatment with L-NAME on MBP and HR after microinjection of MK into unilateral NTS. (C) Immunoblotting revealed that phosphorylated eNOS^{S1177} was increased in the NTS that received MK. Phosphorylation of eNOS was blocked by pretreatment with L-NAME 10 min before MK microinjection. *$P < 0.05$ *vs* vehicle group, $^{\#}P < 0.05$ *vs* MK group, n = 4-6. MBP, mean blood pressure; L-glu, L-glutamate; p-eNOS, phospho-eNOS (S1177); t-eNOS, total eNOS.

bradycardic responses (-37 ± 4 versus -21 ± 3 mmHg and -51 ± 9 versus -21 ± 4 bpm; Fig. 5, A and B). In contrast, pretreatment with vinyl-L-NIO (600 pmol), a nNOS-specific inhibitor, did not diminish the MK-mediated pressor (-30 ± 4 versus -27 ± 6 mmHg) and bradycardic effects (-41 ± 9 versus -46 ± 5 bpm; Fig. 5, C and D).

These results collectively indicate that MK, acting *via* eNOS but not nNOS, might play a role in NMDA receptor-mediated depressor and bradycardic responses in the NTS. Therefore, eNOS signaling served as the downstream molecules of MK-mediated cardiovascular responses in the NTS.

Discussion

In the study, we have provided evidence that microinjection of MK into the NTS induced depressor and bradycardic effects in normotensive WKY rats (Fig. 1). It was reported previously that the growth factor MK is a novel regulator of the RAS (7, 10). MK is a novel promoter of hypertension associated with chronic kidney disease, and upregulates the RAS and mediates the kidney-lung interaction. These results suggest a possible link between MK and ACE (14). Indeed, the presence of exogenous MK protein in primary cultured human lung microvascular endothelial cells significantly enhances ACE expres-

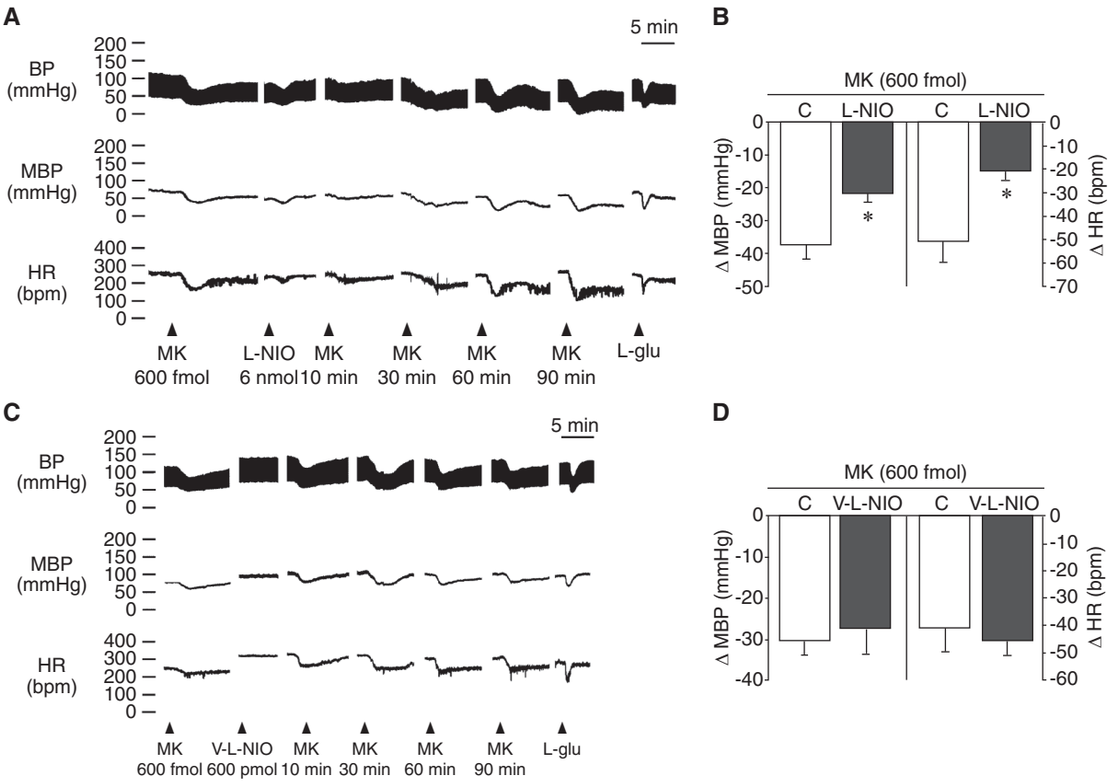

Fig. 5. The depressor effects of MK in the NTS before and after administration of selective nitric oxide synthase inhibitor. (A) The depressor effects of microinjection of MK (600 fmol) into unilateral NTS before and 10 min after pretreatment with L-NIO (6 nmol). (B) Effects of pretreatment with L-NIO on MBP and HR after microinjection of MK into unilateral NTS. (C) The depressor effects of microinjection of MK into unilateral NTS before and 10 min after pretreatment with vinyl-L-NIO (600 pmol). (D) Effects of pretreatment with vinyl-L-NIO on MBP and HR after microinjection of MK into unilateral NTS. *$P < 0.05$ *vs* vehicle group, n = 4-6. V-L-NIO, vinyl-L-NIO; MBP, mean blood pressure; L-glu, L-glutamate.

sion (10). In the periphery, MK can mediate RAS system on the kidney-lung interaction to regulate BP (10). In the central nervous system, NTS is one of the important organs of major central cardiovascular control. Therefore, we wanted to understand the mechanisms by which MK regulated central BP in the NTS. However, MK did not induce the ACE pathway on central cardiovascular regulation in the NTS of WKY rats (Fig. 2). Our study suggests that a possible MK-calmodulin-eNOS signaling pathway is involved in the modulation of cardiovascular control by interaction with NMDA receptor in the NTS.

Protein-tyrosine phosphatase-zeta (PTPζ), a chondroitin sulfate proteoglycan, is an established component of the MK receptor (21). Analyses of MK-binding proteins from embryonic brains have revealed that low-density lipoprotein receptor-related protein (LRP) (22), $\alpha_4\beta_1$-integrin and $\alpha_6\beta_1$-integrin (23) also serve as MK receptors. MK, which acts as a dimer to bind to components of the receptor including the glycosaminoglycan chains, and promotes the formation of the receptor complex. When an MK dimer binds to an integrin and the chondroitin sulfate chain of PTPζ, the cytoplasmic phosphatase domain of PTPζ becomes closer to the cytoplasmic domain of the integrin, resulting in an increase in the tyrosine phosphorylation of key signaling molecules (33). However, our study demonstrated that MK might operate through the NMDA receptor-mediated central cardiovascular regulation in the NTS of the WKY rats (Fig. 3). This may be a novel finding for MK-mediated cardiovascular effects.

Several neuromodulators modulate central BP effects *via* different signaling pathways in the NTS. For example, our previous study showed that the ERK-eNOS signaling pathway existed and played an important role in the modulation of cardiovascular responses to adenosine in the NTS (9). In addition, we also demonstrated that Ang II might modulate central BP effects *via* reactive oxygen species (ROS) to downregulate ERK1/2, RSK and nNOS (4). These results indicate that different neuromodulators

might modulate central BP effects *via* different signaling pathways in the NTS. We propose here that the neuromodulator NO plays a major role in hypotensive effects induced in the NTS (31). In addition, it has been reported that MK mediates BP through NOS in the chronic kidney disease (CKD) (26). In the present study, MK-induced depressor effects in the NTS were reduced by prior administration of the NOS inhibitor L-NAME (Fig. 4). However, MK-mediated BP responses in the NTS were attenuated by L-NIO but not by vinyl-L-NIO (Fig. 5). This suggests that eNOS might be one of the downstream targets of MK involved in NO production, which in turn modulates BP in the NTS of the WKY rats.

Integrin ligands enhance NMDA receptor currents and this effect is dependent on Src activity (18). The extracellular matrix (ECM)–integrin interactions can regulate spine remodeling through NMDAR/CaMKII-mediated actin reorganization (27). In addition, for native PTP as well as native protein-tyrosine kinase (PTK), regulation of the NMDA receptors is associated with the channel complex (32). It is generally thought that synaptic NMDA receptors are retained at the synapse by an attachment to postsynaptic density protein 95 (PSD-95) through a PSD-95/Dlg1/ZO-1 (PDZ) interaction with the NR2 subunits (8). PSD-95, in turn, binds to nNOS, a Ca^{2+}-activated form of NOS, through a PDZ-domain interaction in which the NOS amino terminal PDZ domain binds to a PDZ domain of PSD-95 (6). Therefore, PSD-95 may concentrate nNOS near the NMDA receptor at postsynaptic sites in neurons thereby connecting NMDA receptors to specific signal transduction pathways (17). However, our study demonstrated that MK operated *via* eNOS but not nNOS on the NMDA receptor-mediated depressor and bradycardic responses in the NTS (Fig. 5). This evidence confirms that cardiovascular modulation by MK in the NTS is predominantly through eNOS activation and not nNOS.

In conclusion, this is the first study to demonstrate the mechanism by which MK regulates depressor effects in the central cardiovascular effects in the NTS. Our study suggests that MK might modulate BP *via* the centrally located NMDA receptor, to activate eNOS. Our findings suggest new insights into the central nervous system regulation of BP, and may be of help for further development of therapy against cardiovascular disease.

Acknowledgements

This study was supported by grants from the Ministry of Science and Technology (MOST104-2320-B-075B-003-MY3), National Science Council (NSC102-2320-B-075B-002), Kaohsiung Veterans General Hospital (VGHKS106-016, VGHKS106-149, VGHKS106-G01-3, VGHKS106-G01, VGHKS106-G01-2) (to Dr. J.-Y. P., C.-J. T., Y.-T. L., and C.-C. L.) and Zouying Branch of Kaohsiung Armed Forces General Hospital (ZBH 106-09) to Dr. J.-J. Chang.

Conflict of Interests

The authors declare that there are no conflicts of interests.

References

1. Badila, E., Daraban, A.M., Tintea, E., Bartos, D., Alexandru, N. and Georgescu, A. Midkine proteins in cardio-vascular disease. Where do we come from and where are we heading to? *Eur. J. Pharmacol*. 762: 464-471, 2015.
2. Cheng, P.W., Lu, P.J., Chen, S.R., Ho, W.Y., Cheng, W.H., Hong, L.Z., Yeh, T.C., Sun, G.C., Wang, L.L., Hsiao, M. and Tseng, C.J. Central nicotinic acetylcholine receptor involved in Ca^{2+}-calmodulin-endothelial nitric oxide synthase pathway modulated hypotensive effects. *Br. J. Pharmacol*. 163: 1203-1213, 2011.
3. Cheng, P.W., Wu, A.T., Lu, P.J., Yang, Y.C., Ho, W.Y., Lin, H.C., Hsiao, M. and Tseng, C.J. Central hypotensive effects of neuropeptide Y are modulated by endothelial nitric oxide synthase after activation by ribosomal protein S6 kinase. *Br. J. Pharmacol*. 167: 1148-1160, 2012.
4. Cheng, W.H., Lu, P.J., Ho, W.Y., Tung, C.S., Cheng, P.W., Hsiao, M. and Tseng, C.J. Angiotensin II inhibits neuronal nitric oxide synthase activation through the ERK1/2-RSK signaling pathway to modulate central control of PTP. *Circ. Res*. 106: 788-795, 2010.
5. Cheng, W.H., Lu, P.J., Hsiao, M., Hsiao, C.H., Ho, W.Y., Cheng, P.W., Lin, C.T., Hong, L.Z. and Tseng, C.J. Renin activates PI3K-Akt-eNOS signalling through the angiotensin AT_1 and Mas receptors to modulate central blood pressure control in the nucleus tractus solitarii. *Br. J. Pharmacol*. 166: 2024-2035, 2012.
6. Christopherson, K.S., Hillier, B.J., Lim, W. A. and Bredt, D.S. PSD-95 assembles a ternary complex with the N-methyl-D-aspartic acid receptor and a bivalent neuronal NO synthase PDZ domain. *J. Biol. Chem*. 274: 27467-27473, 1999.
7. Ezquerra, L., Herradon, G., Nguyen, T., Silos-Santiago, I. and Deuel, T.F. Midkine, a newly discovered regulator of the renin-angiotensin pathway in mouse aorta: significance of the pleiotrophin/midkine developmental gene family in angiotensin II signaling. Biochem. *Biophys. Res. Commun*. 333: 636-643, 2005.
8. Gardoni, F. and Di Luca, M. New targets for pharmacological intervention in the glutamatergic synapse. *Eur. J. Pharmacol*. 545: 2-10, 2006.
9. Ho, W.Y., Lu, P.J., Hsiao, M., Hwang, H.R., Tseng, Y.C., Yen, M.H. and Tseng, C.J. Adenosine modulates cardiovascular functions through activation of extracellular signal-regulated kinases 1 and 2 and endothelial nitric oxide synthase in the nucleus tractus solitarii of rats. *Circulation* 117: 773-780, 2008.
10. Hobo, A., Yuzawa, Y., Kosugi, T., Kato, N., Asai, N., Sato, W., Maruyama, S., Ito, Y., Kobori, H., Ikematsu, S., Nishiyama, A., Matsuo, S. and Kadomatsu, K. The growth factor midkine regulates the renin-angiotensin system in mice. *J. Clin. Invest*. 119: 1616-1625, 2009.
11. Hong, L.Z., Cheng, P.W., Cheng, W.H., Chen, S.R., Wang, L.L. and Tseng, C.J. Involvement of NMDA receptors in nicotine-mediated central control of hypotensive effects. *Chin. J. Physiol*. 55: 337-345, 2012.
12. Horiba, M., Kadomatsu, K., Nakamura, E., Muramatsu, H., Ikematsu, S., Sakuma, S., Hayashi, K., Yuzawa, Y., Matsuo, S., Kuzuya, M., Kaname, T., Hirai, M., Saito, H. and Muramatsu,

T. Neointima formation in a restenosis model is suppressed in midkine-deficient mice. *J. Clin. Invest*. 105: 489-495, 2000.
13. Horiba, M., Kadomatsu, K., Yasui, K., Lee, J.K., Takenaka, H., Sumida, A., Kamiya, K., Chen, S., Sakuma, S., Muramatsu, T. and Kodama, I. Midkine plays a protective role against cardiac ischemia/reperfusion injury through a reduction of apoptotic reaction. *Circulation* 114: 1713-1720, 2006.
14. Huang, H.N., Lu, P.J., Lo, W.C., Lin, C.H., Hsiao, M. and Tseng, C.J. *In situ* Akt phosphorylation in the nucleus tractus solitarii is involved in central control of blood pressure and heart rate. *Circulation* 110: 2476-2483, 2004.
15. Kadomatsu, K. Midkine regulation of the renin-angiotensin system. *Curr. Hypertens. Rep*. 12: 74-79, 2010.
16. Kadomatsu, K. and Muramatsu, T. Midkine and pleiotrophin in neural development and cancer. *Cancer Lett*. 204: 127-143, 2004.
17. Kennedy, M.B. Signal-processing machines at the postsynaptic density. *Science* 290: 750-754, 2000.
18. Lin, B., Arai, A.C., Lynch, G. and Gall, C.M. Integrins regulate NMDA receptor-mediated synaptic currents. *J. Neurophysiol*. 89: 2874-2878, 2003.
19. Lin, C.H., Lo, W.C., Hsiao, M. and Tseng, C.J. Interaction of carbon monoxide and adenosine in the nucleus tractus solitarii of rats. *Hypertension* 42: 380-385, 2003.
20. Lo, W.J., Liu, H.W., Lin, H.C., Ger, L.P., Tung, C.S. and Tseng, C.J. Modulatory effects of nitric oxide on baroreflex activation in the brainstem nuclei of rats. *Chinese J. Physiol*. 39: 57-62, 1996.
21. Maeda, N., Ichihara-Tanaka, K., Kimura, T., Kadomatsu, K., Muramatsu, T. and Noda, M. A receptor-like protein-tyrosine phosphatase PTPzeta/RPTPbeta binds a heparin-binding growth factor midkine. Involvement of arginine 78 of midkine in the high affinity binding to PTPzeta. *J. Biol. Chem*. 274: 12474-12479, 1999.
22. Muramatsu, H., Zou, K., Sakaguchi, N., Ikematsu, S., Sakuma, S. and Muramatsu, T. LDL receptor-related protein as a component of the midkine receptor. *Biochem. Biophys. Res. Commun*. 270: 936-941, 2000.
23. Muramatsu, H., Zou, P., Suzuki, H., Oda, Y., Chen, G.Y., Sakaguchi, N., Sakuma, S., Maeda, N., Noda, M., Takada, Y. and Muramatsu, T. Alpha4beta1- and alpha6beta1-integrins are functional receptors for midkine, a heparin-binding growth factor. *J. Cell Sci*. 117: 5405-5415, 2004.
24. Muramatsu, T. Midkine and pleiotrophin: two related proteins involved in development, survival, inflammation and tumorigenesis. *J. Biochem*. 132: 359-371, 2002.
25. Muramatsu, T. Midkine, a heparin-binding cytokine with multiple roles in development, repair and diseases. *Proc. Jpn. Acad. Ser. B. Phys. Biol. Sci*. 86: 410-425, 2010.
26. Sato, Y., Sato, W., Maruyama, S., Wilcox, C.S., Falck, J.R., Masuda, T., Kosugi, T., Kojima, H., Maeda, K., Furuhashi, K., Ando, M., Imai, E., Matsuo, S. and Kadomatsu, K. Midkine regulates BP through cytochrome P450-derived eicosanoids. *J. Am. Soc. Nephrol*. 26: 1806-1815, 2015.
27. Shi, Y. and Ethell, I.M. Integrins control dendritic spine plasticity in hippocampal neurons through NMDA receptor and Ca^{2+}/calmodulin- dependent protein kinase II-mediated actin reorganization. *J. Neurosci*. 26: 1813-1822, 2006.
28. Sumida, A., Horiba, M., Ishiguro, H., Takenaka, H., Ueda, N., Ooboshi, H., Opthof, T., Kadomatsu, K. and Kodama, I. Midkine gene transfer after myocardial infarction in rats prevents remodelling and ameliorates cardiac dysfunction. *Cardiovasc. Res*. 86: 113-121, 2010.
29. Tseng, C.J., Chou, L.L., Ger, L.P. and Tung, C.S. Cardiovascular effects of angiotensin III in brainstem nuclei of normotensive and hypertensive rats. *J. Pharmacol. Exp. Ther*. 268: 558-564, 1994.
30. Tseng, C.J., Ger, L.P., Lin, H.C. and Tung, C.S. Attenuated cardiovascular response to adenosine in the brain stem nuclei of spontaneously hypertensive rats. *Hypertension* 25: 278-282, 1995.
31. Tseng, C.J., Liu, H.Y., Lin, H.C., Ger, L.P., Tung, C.S. and Yen, M.H. Cardiovascular effects of nitric oxide in the brain stem nuclei of rats. *Hypertension* 27: 36-42, 1996.
32. Wang, Y.T., Yu, X.M. and Salter, M.W. Ca^{2+}-independent reduction of N-methyl-D-aspartate channel activity by protein tyrosine phosphatase. *Proc. Natl. Acad. Sci. USA*. 93: 1721-1725, 1996.
33. Xu, C., Zhu, S., Wu, M., Han, W. and Yu, Y. Functional receptors and intracellular signal pathways of midkine (MK) and pleiotrophin (PTN). *Biol. Pharm. Bull*. 37: 511-520, 2014.