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Pressor response to pulsatile compression of the rostral ventrolateral medulla mediated by nitric oxide and c-*fos* expression

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1 It has been reported that neurovascular compression of the rostral ventrolateral medulla might be causally related to essential hypertension. Recently, we found that pulsatile compression of the rostral ventrolateral medulla increases sympathetic nerve activity and elevates arterial pressure *via* activation of glutamate receptors in rats. We also found that increases in sympathetic and cardiovascular activities by microinjection of L-glutamate into the rostral ventrolateral medulla are mediated by c-*fos* expression-related substance(s) following activation of the nitric oxide-cyclic GMP pathway.

2 Herein, we investigated whether responses to pulsatile compression are mediated by local activation of the nitric oxide-cyclic GMP pathway and/or c-*fos* expression-related substance(s) in rats.

3 Increases in arterial pressure (15 ± 1 mmHg), heart rate (9 ± 1 b.p.m.), and sympathetic nerve activity (% change: $8.5\pm1.1\%$) induced by pulsatile compression were partially but significantly inhibited after local microinjection of a nitric oxide synthase inhibitor, L-N^G-nitroarginine methyl ester (8 ± 2 mmHg, 1 ± 1 b.p.m., $4.0\pm1.3\%$; $P<0.05$ vs compression without pretreatment) or 7-nitroindazole (7 ± 2 mmHg, 2 ± 1 b.p.m., $4.0\pm1.5\%$; $P<0.05$), or a soluble guanylate cyclase inhibitor, methylene blue (9 ± 1 mmHg, 4 ± 1 b.p.m., $4.1\pm1.4\%$; $P<0.05$). In addition, increases in arterial pressure, heart rate, and sympathetic nerve activity by pulsatile compression were significantly reduced 6 h after microinjection of antisense oligodeoxynucleotide to c-*fos* mRNA (2 ± 2 mmHg, 2 ± 1 b.p.m., $1.0\pm1.0\%$; $P<0.05$ vs sense oligodeoxynucleotide).

4 These results suggest that increases in sympathetic and cardiovascular activities induced by pulsatile compression of the rostral ventrolateral medulla are mediated, at least in part, by local activation of the nitric oxide-cyclic GMP pathway and c-*fos* expression-related substance(s) in rats. *British Journal of Pharmacology* (2000) **129,** 859–864

Keywords: Rostral ventrolateral medulla; compression; sympathetic nerve activity; nitric oxide; cyclic GMP; c-*fos*

Abbreviations: AP, arterial pressure; D-NAME, D-N^G-nitroarginine methyl ester; HR, heart rate; L-NAME, L-N^G-nitroarginine methyl ester; 7-NI, 7-nitroindazole; NO, nitric oxide; ODN, oligodeoxynucleotide; RVLM, rostral ventrolateral medulla; SNA, sympathetic nerve activity; SNP, sodium nitroprusside

Introduction

The rostral ventrolateral medulla (RVLM) contains neurons that are the major tonic source of supraspinal sympathoexcitatory outflow, and thus this area is considered to be an important center for the regulation of sympathetic and cardiovascular activities (Dampney *et al*., 1982). Several clinical studies have indicated that neurovascular compression of the RVLM might be causally related to essential hypertension (Jannetta *et al*., 1985; Morimoto *et al*., 1997a; Naraghi *et al*., 1994). We have reported that, in rats, pulsatile compression of the RVLM increases arterial pressure (AP), heart rate (HR) and sympathetic nerve activity (SNA) (Morimoto *et al*., 1997b) *via* activation of glutamate receptors in RVLM neurons (Morimoto *et al*., 1999). However, the intracellular mechanism of the pressor response to compression remains to be elucidated.

Since the discovery of endothelium-derived relaxing factors (Furchgott *et al*., 1980), considerable attention has been directed toward understanding its roles. In 1987, nitric oxide (NO) was proposed to be an endothelium-derived relaxing factor (Ignarro *et al*., 1987; Palmer *et al*., 1987). Since then, it has been reported that NO is synthesized not only in the vascular endothelium but also in the peripheral and central nervous systems (Bredt *et al*., 1990; Bult *et al*., 1990; Togashi *et al*., 1992). Intracisternal injection of a NO synthase inhibitor increases AP and SNA (Togashi *et al*., 1992), indicating that endogenous NO tonically affects sympathetic outflow *via* a direct action in the brain. It is also possible that NO plays a functional role in the RVLM since neurons that express NO synthase are found in this area (Hirooka *et al*., 1996; Iadecola *et al*., 1993).

The immediate early gene c-*fos* is expressed transiently in neurons after a variety of physiological and pharmacological stimuli in the central nervous system (Bullitt, 1990; Dragunow *et al*., 1989), including the RVLM (Minson *et al*., 1994; 1995). Transcription of c-*fos* occurs within 5 min after neuronal activation (Greenberg *et al*., 1985) and the protein product Fos enters cell nuclei and functions as a transcriptional regulator in cooperation with Jun through activator-protein-1 regulatory elements in neurons (Lee *et al*., 1988). In addition, basal and stimulated expression of c-*fos* is important in the central control of AP in the RVLM (Suzuki *et al*., 1994). We have also reported that increases in AP, HR, and SNA induced by local microinjection of L-glutamate are mediated by c-*fos* expression-related substance(s) following activation of the NO-cyclic

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GMP pathway (Sasaki *et al*., 1997). Therefore, in the present study, we investigated whether the responses to pulsatile compression of the RVLM are mediated by local activation of the NO-cyclic GMP pathway and/or c-*fos* expression-related substance(s) in rats.

Methods

Surgical procedures

All experiments were carried out using male Wistar rats (Charles River Breeding Laboratories, Kanagawa, Japan) weighing between 300 and 400 g. Animal care and procedures were approved by the Experimental Animal Care Committee of the Kyoto Prefectural University of Medicine. The rats were anaesthetized with urethane (1 g kg^{-1}, i.p.) and the anaesthetic was supplemented (10–30 mg 100 g^{-1}, i.p.) as indicated by the presence of corneal reflex and/or cardiovascular responses to surgical procedures. The rats were mounted on a stereotaxic apparatus (David Kopf Instrument, Tujunga, CA, U.S.A.) in the supine position. The lower trachea was cannulated, and the rats were artificially ventilated at a rate of 60 breaths min^{-1} with a respirator (Ealing Co., Ltd, U.K.) and were paralyzed with decamethonium bromide (0.2 mg 100 g^{-1}, i.v.). Catheters were inserted into the right femoral artery to record AP and HR, and into the right femoral vein for drug injection. The splanchnic nerve was placed over a bipolar stainless steel electrode, and spike potentials were amplified and counted as described in detail elsewhere (Sasaki *et al*., 1990). The ventral surface of the medulla oblongata was exposed and the RVLM was identified by a pressor response of more than 25 mmHg mean AP after microinjection of monosodium L-glutamate (2 nmol). Microinjections were made over a 30 s period with a computer-controlled pneumatic pump using glass micropipettes with tip diameters of 50 μm.

Experimental procedures

Pulsatile compression was applied to the unilateral RVLM as described previously (Morimoto *et al*., 1997b). In brief, a rubber membrane was attached to an end of a polyurethane cannula with an outer diameter of 1.5 mm, and the pneumatic pump was connected to the opposite end. By pumping air triggered by electrocardiographic monitoring, the membrane pulsated and the pressure wave inside the cannula mimicked an intraarterial pressure wave. Using a stereotaxic apparatus, the cannula was pressed 1 mm dorsal to the ventral surface of the RVLM. AP, HR, and splanchnic SNA induced by unilateral pulsatile compression of the RVLM were monitored for 5 min.

Effects of NO synthase inhibitor on the responses to pulsatile compression

Mean AP, HR and SNA were monitored for 30 min after microinjection of a non-specific NO synthase inhibitor, L-N^G-nitroarginine methyl ester (L-NAME, 100 nmol), the inactive isomer of L-NAME, D-N^G-nitroarginine methyl ester (D-NAME, 100 nmol), or a specific neuronal NO synthase inhibitor, 7-nitroindazole (Moore *et al*., 1993) (7-NI, 10 nmol) either unilaterally or bilaterally into the RVLM. Maximum changes in mean AP, HR, and SNA induced by unilateral pulsatile compression of the RVLM were compared 1 min after microinjection of L-NAME (100 nmol), D-NAME (100 nmol), or 7-NI (10 nmol) ipsilaterally.

Effects of a soluble guanylate cyclase inhibitor on the responses to pulsatile compression

Mean AP, HR, and SNA were monitored for 30 min after microinjection of an inhibitor of soluble guanylate cyclase, methylene blue (10 nmol), either unilaterally or bilaterally into the RVLM. Maximum changes in mean AP, HR, and SNA induced by unilateral pulsatile compression of the RVLM were compared 10 min after microinjection of methylene blue (10 nmol) ipsilaterally.

In the unilateral experiments from above, an identical experiment was performed in the same rat on the opposite side after full recovery from the first experiment and a waiting period of at least 30 min. In the bilateral experiments, only one experiment was performed in each rat.

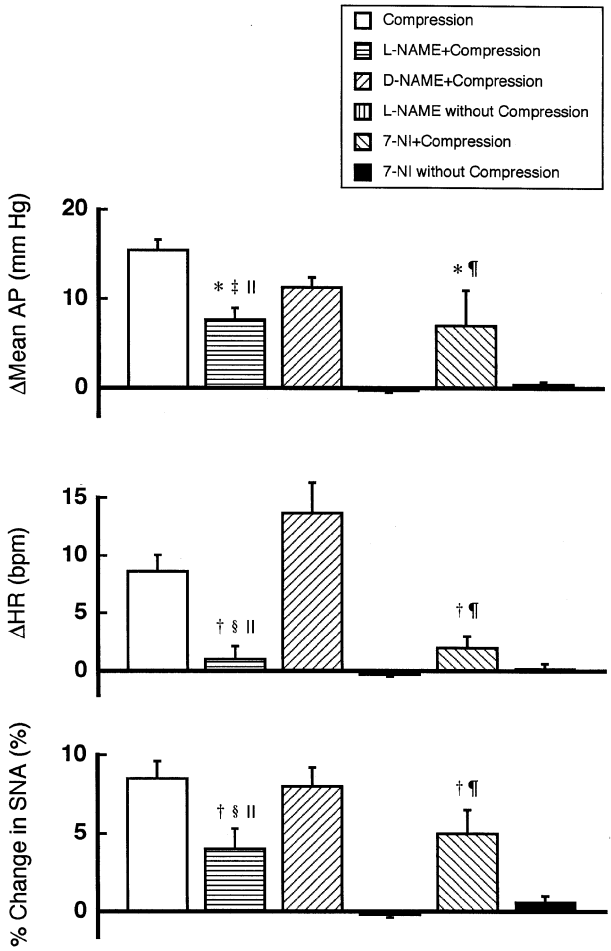

Figure 1 Maximum changes in mean arterial pressure, heart rate, and sympathetic nerve activity induced by unilateral pulsatile compression of the rostral ventrolateral medulla or by unilateral compression 1 min after ipsilateral microinjection of L-N^G-nitroarginine methyl ester, D-N^G-nitroarginine methyl ester, or 7-nitroindazole into the rostral ventrolateral medulla. Maximum changes by microinjection of L-N^G-nitroarginine methyl ester or 7-nitroindazole unilaterally into the rostral ventrolateral medulla without compression are also shown as controls. $n=6$ for all groups, $^*P<0.05$ and $^\dagger P<0.01$ compared to compression without pretreatment and $^\ddagger P<0.05$ and $^\S P<0.01$ compared to compression after microinjection of D-N^G-nitroarginine methyl ester. $^{||}P<0.01$ compared to microinjection of L-N^G-nitroarginine methyl ester and $^\P P<0.01$ compared to 7-nitroindazole without compression. L-NAME, L-N^G-nitroarginine methyl ester; D-NAME, D-N^G-nitroarginine methyl ester; 7-NI, 7-nitroindazole; AP, arterial pressure; HR, heart rate; SNA, sympathetic nerve activity. Bars represent mean ± s.e.mean.

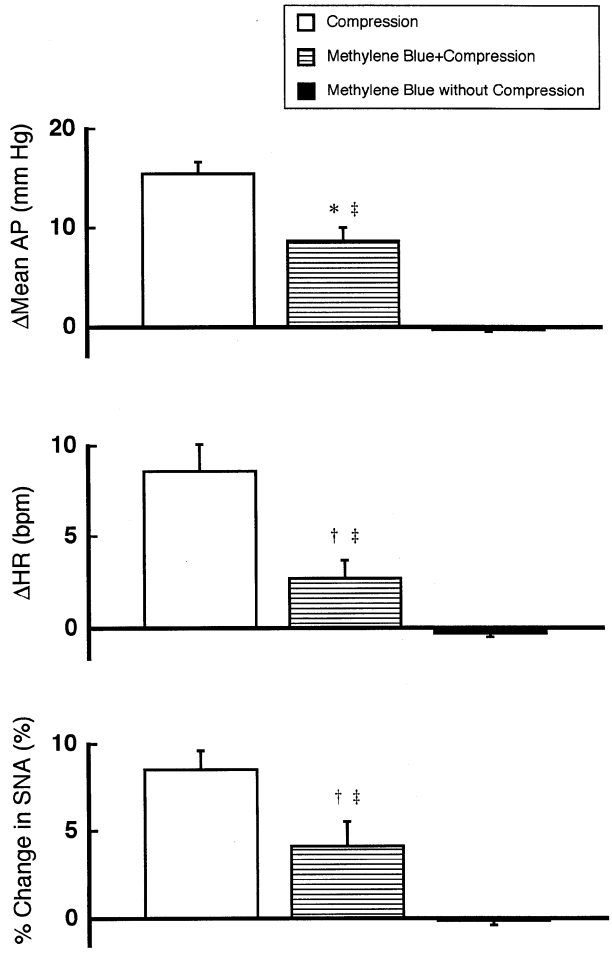

Figure 2 Maximum changes in mean arterial pressure, heart rate, and sympathetic nerve activity induced by unilateral pulsatile compression of the rostral ventrolateral medulla or by unilateral compression 10 min after ipsilateral microinjection of methylene blue into the rostral ventrolateral medulla. Maximum changes by microinjection of methylene blue unilaterally into the rostral ventrolateral medulla without compression are also shown as controls. $n=6$ for both groups, $^{*}P<0.05$ and $\dagger P<0.01$ compared to compression without pretreatment. $\ddagger P<0.01$ compared to microinjection of methylene blue without compression. AP, arterial pressure; HR, heart rate; SNA, sympathetic nerve activity. Bars represent mean$\pm$s.e.mean.

*Effects of antisense oligodeoxynucleotide (ODN) to c-*fos *mRNA on the responses to pulsatile compression*

Mean AP and HR were monitored for 6 h after microinjection of c-*fos* antisense or sense ODN either unilaterally or bilaterally into the RVLM. SNA was not monitored in this experiment because long-term (6 h) measurements of SNA are not possible due to nerve damage. In a second series of experiments, maximum changes in mean AP, HR, and SNA induced by unilateral pulsatile compression of the RVLM were compared 15 min, 2 h or 6 h after microinjection of antisense (5′-GAA-CAT-CAT-GGT-CGT-3′) or sense ODN (5′-ACG-ACC-ATG-ATG-TTC-3′) to c-*fos* mRNA ipsilaterally and at the other time points, the electrode was displaced from the splanchnic nerve to prevent nerve damage. In this series of experiments, a single experiment was done in each rat.

Histological analysis for the microinjection sites

At the end of each experiment, 50 nl of Evans blue dye was microinjected to mark the injection site. The rats were then perfused transcardially with 100 ml of 0.9% w v^{-1} NaCl followed by 150 ml of 10% w v^{-1} phosphate-buffered formaldehyde. Serial 4 μm transverse sections of the medulla oblongata were stained with cresyl violet and subjected to light microscopic examination.

Drugs

ODNs were obtained from Sawady Co. Ltd. (Tokyo, Japan) and phosphorothioated in all positions. Antisense ODN to c-*fos* mRNA was complementary to bases -6 to $+9$ from the initiation codon of the rat c-*fos* mRNA. ODNs did not show any significant complementarity to any other gene sequence in the Gen Bank database. All other drugs were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.). Urethane and decamethonium bromide were dissolved in saline. For each microinjection, monosodium L-glutamate was dissolved in 100 nl of Ringer's solution and ODNs in 200 nl of saline. All other drugs were dissolved in 50 nl of Ringer's solution (mM) Na 130, K 4, Ca 6, Cl 109 and lactate 28 for each microinjection.

Statistical analysis

Values are expressed as mean$\pm$s.e.mean. Comparisons between the three groups were made by one factor ANOVA followed by Fisher's multiple range test. Group-to-group comparisons were made by a nonpaired Student's *t*-test. A *P* value <0.05 was considered statistically significant.

Results

Involvement of NO in the response to pulsatile compression

Microinjection of L-NAME (maximum Δmean AP, -0.3 ± 0.2 mmHg; maximum ΔHR, -0.3 ± 0.2 b.p.m.; maximum % change in SNA, -0.2 ± 0.2%; $n=6$), 7-NI (0.4 ± 0.3 mmHg, 0.2 ± 0.4 b.p.m., 0.6 ± 0.4%; $n=6$), or D-NAME (-1.0 ± 0.3 mmHg, 0.1 ± 0.2 b.p.m., 0.3 ± 0.8%; $n=6$) unilaterally into the RVLM did not significantly alter mean AP, HR, or SNA. Microinjection of L-NAME or 7-NI bilaterally into the RVLM significantly decreased mean AP (maximum Δmean AP: -12 ± 4, -8 ± 3 mmHg, respectively; $n=6$; $P<0.01$ for both groups) and SNA (maximum % change: -7.8 ± 2.7, -5.8 ± 2.8%; $P<0.01$ for both groups) and slightly decreased HR (maximum ΔHR: -4 ± 3, -2 ± 2 b.p.m.), whereas D-NAME failed to affect these parameters (0.9 ± 0.4 mmHg, 0.4 ± 0.4 b.p.m., 0.5 ± 0.6%; $n=6$). Unilateral pulsatile compression of the RVLM increased mean AP [from 92 ± 4 (just before start of compression) to 108 ± 8 (peak value) mmHg; $n=10$; $P<0.01$], HR [from 328 ± 14 (just before start of compression) to 338 ± 13 (peak value) b.p.m.; $P<0.01$], and SNA (maximum % change, 8.5 ± 1.1%; $P<0.01$; Figure 1). Increases in mean AP, HR, and SNA induced by compression were partially but significantly reduced after microinjection of L-NAME or 7-NI but not D-NAME ipsilaterally into the RVLM (Figure 1).

Involvement of cyclic GMP in the response to pulsatile compression

Microinjection of methylene blue unilaterally into the RVLM did not significantly alter mean AP, HR, or SNA (0.6 ± 0.3 mmHg, -0.4 ± 0.6 b.p.m., -0.9 ± 0.7%; $n=6$).

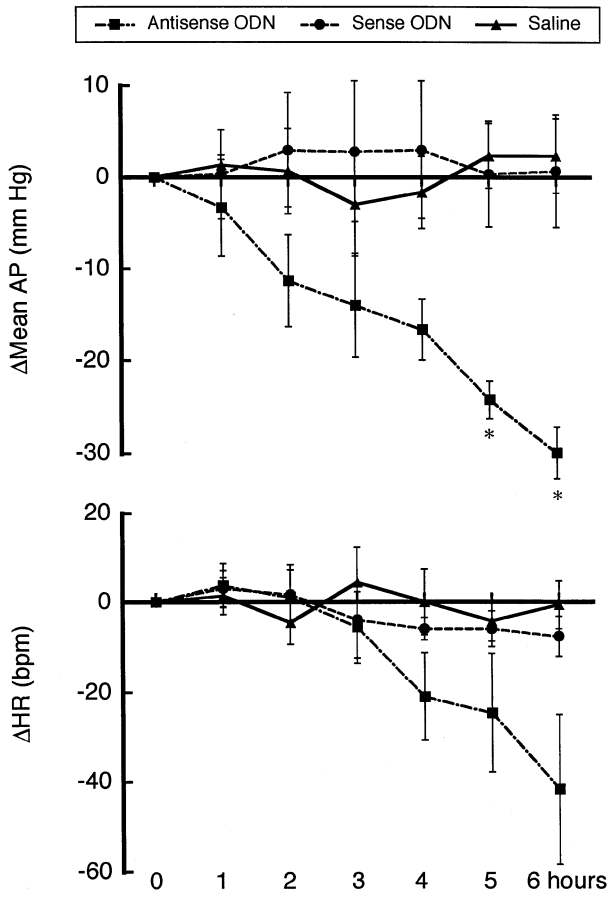

Figure 3 Changes in mean arterial pressure and heart rate after microinjection of antisense or sense oligodeoxynucleotide to c-*fos* mRNA or saline bilaterally into the rostral ventrolateral medulla. $n=6$ for all groups, $^{*}P<0.05$ compared to microinjection of sense oligodeoxynucleotide to c-*fos* mRNA or saline. AP, arterial pressure; HR, heart rate; ODN, oligodeoxynucleotide. Bars represent mean$\pm$s.e.mean.

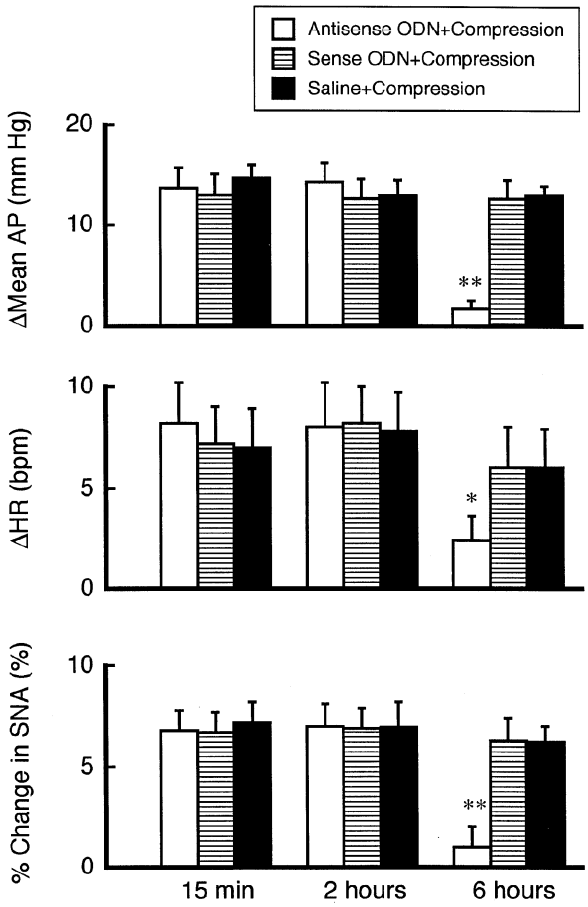

Figure 4 Maximum changes in mean arterial pressure, heart rate, and sympathetic nerve activity induced by unilateral pulsatile compression of the rostral ventrolateral medulla 15 min, 2 h, or 6 h after microinjection of antisense or sense oligodeoxynucleotide to c-*fos* mRNA or saline ipsilaterally into the rostral ventrolateral medulla. $n=6$ for all groups, $^{*}P<0.05$ and $^{**}P<0.01$ compared to microinjection of sense oligodeoxynucleotide to c-*fos* mRNA or saline. AP, arterial pressure; HR, heart rate; SNA, sympathetic nerve activity; ODN, oligodeoxynucleotide. Bars represent mean$\pm$s.e.mean.

Microinjection of methylene blue bilaterally into the RVLM significantly decreased mean AP (maximum Δmean AP: -11 ± 4 mmHg; $n=6$; $P<0.05$), HR (maximum ΔHR: -9 ± 5 b.p.m.; $P<0.05$), and SNA (maximum % change: $-8.7\pm4.5\%$; $P<0.05$). Increases in mean AP, HR, and SNA induced by compression were partially but significantly inhibited after microinjection of methylene blue ipsilaterally into the RVLM (Figure 2).

*Involvement of c-*fos *expression in the response to pulsatile compression*

Microinjection of c-*fos* antisense ODN unilaterally into the RVLM did not significantly alter mean AP or HR (1.1 ± 1.8 mmHg, 0.8 ± 1.4 b.p.m., $1.2\pm1.8\%$; $n=6$). Mean AP was significantly decreased 5 and 6 h after microinjection of c-*fos* antisense ODN bilaterally into the RVLM as compared with sense ODN or saline. HR was also slightly reduced after microinjection of antisense ODN, but the change failed to reach statistical significance (Figure 3). Increases in mean AP, HR, and SNA by pulsatile compression of the RVLM did not differ significantly 15 min or 2 h after microinjection of c-*fos* antisense or sense ODN, or saline. However, these increases were significantly reduced 6 h after microinjection of c-*fos* antisense ODN as compared with sense ODN or saline (Figure 4).

Histological analysis for the microinjection sites

In all rats, Evans blue was confirmed to be restricted to areas ventral to the nucleus ambiguus, caudal to the facial nucleus, and rostral to the rostral end of the lateral reticular nucleus, comparable to the RVLM as shown in our previous report (Morimoto *et al*., 1999).

Discussion

In the present study, we found that increases in sympathetic and cardiovascular activities by pulsatile compression of the RVLM are significantly inhibited after microinjection of L-NAME, 7-NI, methylene blue or antisense ODN to c-*fos* mRNA. These results suggest that the responses to pulsatile compression of the RVLM are mediated, at least in part, by local activation of the NO-cyclic GMP pathway and c-*fos* expression-related substance(s) in rats.

Involvement of NO-cyclic GMP pathway in the response to pulsatile compression

In the present study, increases in AP, HR, and SNA induced by compression were partially but significantly reduced after microinjection of L-NAME but not D-NAME (Figure 1).

Another NO synthase inhibitor, 7-NI, which is reputed to be more specific for neuronal NO synthase (Moore *et al*., 1993), exhibited similar results. Therefore, these results were likely to be due to neuronal NO synthase inhibition, but not due to non-specific effects or inhibition of other NO synthases. NO is a powerful activator of soluble guanylate cyclase, the cyclic GMP synthesizing enzyme (Miki *et al*., 1977). Increases in AP, HR, and SNA by compression were also partially but significantly inhibited after microinjection of methylene blue (Figure 2). Our data suggest that responses to compression are mediated, at least in part, by the NO-cyclic GMP pathway in the RVLM.

Methylene blue is not a selective inhibitor of NO-sensitive guanylate cyclase (Moro *et al*., 1996). It also inhibits prostacyclin production (Martin *et al*., 1989; Okamura *et al*., 1990) and generates superoxide anions (Marczin *et al*., 1992; Marshall *et al*., 1988; Wolin *et al*., 1990). However, we have found that the pressor response to compression is not affected by local pretreatment with a cyclo-oxygenase inhibitor, indomethacin or a free radical scavenger, superoxide dismutase (unpublished data, Morimoto *et al*., 1999). Therefore, although we have not tested the effects of more specific NO-sensitive guanylate cyclase inhibitors such as 1H-[1,2,4]oxadiazolo[4,3-a] quinoxalin-1-one (ODQ) (Garthwaite *et al*., 1995), we assume that these effects of methylene blue may be due to NO-sensitive guanylate cyclase inhibition.

NO synthase inhibition contracts arteries (Egashira *et al*., 1996; Toda *et al*., 1992) and reduces blood flow (Egashira *et al*., 1996; White *et al*., 1998). Therefore, microinjected L-NAME or 7-NI may contract arteries, reduce blood flow, and induce hypoxia in the RVLM. Hypoxia increases neuronal activity in the RVLM (Nolan *et al*., 1993). Thus, altered local blood flow induced by L-NAME or 7-NI would increase neuronal activity. Decreases in the cardiovascular response to compression after microinjection of L-NAME or 7-NI may not be the result of local blood flow reduction.

*Involvement of c-*fos *expression in the responses to pulsatile compression*

In the present study, increases in AP, HR, and SNA induced by pulsatile compression of the RVLM were significantly inhibited 6 h after microinjection of c-*fos* antisense ODN ipsilaterally into the RVLM (Figure 4). We propose that c-*fos* expression in RVLM neurons is important for the sympathoexcitatory and pressor responses to pulsatile compression. However, substance(s) produced *via* c-*fos* expression just after pulsatile compression probably do not mediate the rapid response to pulsatile compression. In the present study, a significant decrease in basal AP appeared 5 h after local microinjection of c-*fos* antisense ODN bilaterally into the RVLM (Figure 3). Furthermore, significant decreases in AP, HR, and SNA by pulsatile compression of the RVLM were observed 6 h but not 15 min or 2 h after microinjection of antisense ODN (Figure 4). Therefore, we assume that product(s) of Fos or activator-protein-1, that are synthesized under resting conditions may mediate the response to pulsatile compression of the RVLM. Chan *et al*. (1997) have reported enhancement of spontaneous baroreflex by c-*fos* antisense ODN treatment in the nucleus tractus solitarii, suggesting increased neuronal activity by c-*fos* antisense ODN. It is possible that c-*fos* antisense ODN treatment may inhibit synthesis of not only excitatory but also inhibitory substance(s). Further studies are required to determine what substance(s) related to c-*fos* expression mediate the response to pulsatile compression of the RVLM.

It needs to be discussed whether activation of substance(s) related to c-*fos* expression is mediated by NO-cyclic GMP pathway. Some investigators have reported that c-*fos* expression is induced by NO (Haby *et al*., 1994; Lin *et al*., 1999; Tassorelli *et al*., 1995) while others have failed to show such an effect (Lo *et al*., 1995; Takizawa *et al*., 1997). We have reported that the pressor response to compression is mediated by glutamate receptor activation in RVLM neurons (Morimoto *et al*., 1999). In addition, we have found that the pressor response induced by local microinjection of SNP or a cyclic GMP agonist, 8-bromo-cyclic GMP is inhibited significantly after microinjection of c-*fos* antisense ODN (Sasaki *et al*., 1997). Thus, we suggest that activation of substance(s) related to c-*fos* expression may be mediated by NO-cyclic GMP pathway in our model.

Cells expressing NO synthase are often distinct from those expressing c-*fos* in the central nervous systems (Chan *et al*., 1998; Maqbool *et al*., 1997). Furthermore, only a small fraction of NO synthase-containing neurons are sympathoexcitatory neurons in the RVLM (Iadecola *et al*., 1993). However, NO is considered to be diffusible and have a role in cell–cell signalling (Furchgott & Zawadzki, 1980; Ignarro *et al*., 1987; Palmer *et al*., 1987). Accordingly, we suspect that NO induced by pulsatile compression also had a cell–cell signalling role in our rat model.

In conclusion, we have found that increases in sympathetic and cardiovascular activities by pulsatile compression of the RVLM are significantly inhibited after microinjection of NO synthase inhibitors, a soluble guanylate cyclase inhibitor, or antisense ODN to c-*fos* mRNA. These results suggest that the responses to pulsatile compression of the RVLM are mediated, at least in part, by local activation of the NO-cyclic GMP pathway and c-*fos* expression-related substance(s) in rats.

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