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Site-dependent inhibition of neuronal *c-jun* in the brainstem elicited by imidazoline I_1 receptor activation: Role in rilmenidine-evoked hypotension

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Abstract

Clonidine (a mixed α_2-adrenoceptor and imidazoline I_1 receptor agonist)-evoked hypotension was associated with dissimilar reductions in *c-jun* gene expression in the rostral ventrolateral medulla (RVLM) and the nucleus tractus solitarius (NTS) in normotensive rats. In the present study, we investigated the relative contribution of the α_2-adrenoceptor vs. the imidazoline I_1 receptor to the reduction in *c-jun* gene expression in these two brainstem areas. In conscious spontaneously hypertensive rats (SHRs), equihypotensive doses of three centrally acting hypotensive drugs with different selectivity for the two receptors were administered intracisternally (4 μl) to limit their actions to the brain. As a control, a similar hypotensive response was elicited by i.v. hydralazine. Clonidine (0.5 μg), or α-methylnorepinephrine (α-MNE, 4 μg), a highly selective α_2-adrenoceptor agonist, similarly reduced *c-jun* mRNA expression in the NTS and rostral ventrolateral medulla. In contrast, a similar hypotensive response (−37±3.5 mm Hg) caused by the selective imidazoline I_1 receptor agonist rilmenidine (25 μg) was associated with reduction in *c-jun* mRNA expression in the rostral ventrolateral medulla, but not in the NTS. Further, intra-rostral ventrolateral medulla rilmenidine (40 nmol) reduced c-Jun protein expression in rostral ventrolateral medulla and blood pressure and both responses were antagonized by selective imidazoline I_1 receptor (efaroxan, 4 nmol), but not α_2-adrenoceptor (SK&F 86466, 10 nmol) blockade. These results suggest: (1) the *c-jun* containing neurons in the brainstem are involved in the centrally mediated hypotension elicited by centrally acting antihypertensive agents, and (2) the α_2-adrenoceptor modulates *c-jun* gene expression in the NTS and rostral ventrolateral medulla implicated in centrally mediated hypotension, and (3) the imidazoline I_1 receptor mediated inhibition of *c-jun* gene expression in the rostral ventrolateral medulla, but not in the NTS, contributes to the centrally mediated hypotension by the second generation drugs.

Keywords: Rilmenidine; Clonidine; α-Methylnorepinephrine; Rostral ventrolateral medulla; Nucleus tractus solitarii; SHRs (spontaneously hypertensive rats); c-Jun

1. Introduction

Clonidine, a centrally acting antihypertensive agent, decreases blood pressure via activation of both of α_2-adrenoceptor and imidazoline I_1 receptors (Bousquet et al., 1984, 2003; Tank et al., 2004b). Newer drugs with higher selectivity for the imidazoline I_1 receptor lower blood pressure without the unwanted side-effects normally associated with the α_2-adrenergic agents (Head et al., 2001). The mechanisms of the anti-hypertensive actions of drugs like clonidine have been thoroughly investigated (Head et al., 1997). However, the relative roles of the α_2-adrenoceptor versus the imidazoline I_1 receptor in the antihypertensive actions of various agents remain controversial (Szabo, 2002).

The immediate early genes *c-fos* and *c-jun* are transiently expressed in neurons after a variety of physiological and pharmacological stimuli and are considered useful markers of neuronal activity at a variety of sites (Pertovaara et al.,

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1993; Minson et al., 1996b; El-Mas and Abdel-Rahman, 2001) including those involved in the central control of arterial pressure (El-Mas and Abdel-Rahman, 2000, 2001). Systemic clonidine suppresses hypotension-induced Fos expression in the NTS and rostral ventrolateral medulla in the rabbit (Li and Dampney, 1995) and rats (El-Mas and Abdel-Rahman, 2000). Further, we demonstrated, for the first time, that clonidine reduced basal *c-jun* gene expression in the NTS and rostral ventrolateral medulla of normotensive rats (El-Mas and Abdel-Rahman, 2000). The changes in immediate early genes, and their protein products (c-Fos and c-Jun) not only reflect neuronal activity but also serve a functional role in modulating neuronal signaling including sympathetic neurons in the rostral ventrolateral medulla (Minson et al., 1996b; Chan and Sawchenko, 1998; Dampney and Horiuchi, 2003).

To our knowledge, no study has evaluated the role of α_2-adrenoceptor vs. the imidazoline I_1 receptor in the suppression of immediate early genes expression in the brainstem caused by centrally acting antihypertensive drugs. Furthermore, in the reported studies, the drugs were administered systemically (Li and Dampney, 1995; El-Mas and Abdel-Rahman, 2000), which raises the possibility that peripheral effects may have contributed to the reported changes in immediate early genes. Therefore, we investigated in conscious SHRs the contribution of central imidazoline I_1 receptor vs. the α_2-adrenoceptor to the suppression of the immediate early gene *c-jun* in two brainstem areas (rostral ventrolateral medulla and NTS) known to be involved in blood pressure control by injecting intracisternally selective I_1 (rilmenidine) or α_2 (α-methylnorepinephrine) receptor agonist as well as the mixed I_1/α_2 receptor agonist clonidine. As a control, we investigated the impact of a comparable peripherally mediated hypotensive response, elicited by hydralazine, on brainstem *c-jun* gene expression. Because the rostral ventrolateral medulla contains functional α_2-adrenoceptors (Allen and Guyenet, 1993; Nicholas et al., 1993) and rilmenidine exhibits weak α_2-adrenoceptor agonist activity (Mao et al., 2003), we felt it important to directly microinject rilmenidine into the rostral ventrolateral medulla of conscious SHRs in the absence and presence of α_2 or imidazoline I_1 receptor blockade to: (i) bolster the conclusions obtained with the selective imidazoline I_1 receptor agonist, which was administered intracisternally in the first set of experiments and (ii) obtain direct evidence that the I_1, and not the α_2, receptor in the rostral ventrolateral medulla mediates the correlated reductions in c-Jun and blood pressure independent of projections from other brain areas. Therefore, we investigated in conscious SHRs the effect of intra- rostral ventrolateral medulla rilmenidine (40 nmol) on mean arterial pressure and c-Jun expression in the rostral ventrolateral medulla in the presence and absence of selective imidazoline (efaroxan, 4 nmol) or α_2-adrenergic (SK&F 86466, 10 nmol) receptor blockade.

2. Materials and methods

2.1. Animals

Male spontaneously hypertensive rats (SHRs) were used in the present study. The rats were obtained from Charles River (Raleigh, NC) at their 12th–13th week of age (250–300 g). Experiments were performed in strict accordance with institutional animal care and use guidelines.

2.2. Intracisternal (i.c.) or intra-cranial cannulation

Five days before the experiment, a stainless steel guide cannula (23 G; Small Parts, Miami, FL, USA) was implanted so that its tip sits in the cisterna magna or 2 mm above the rostral ventrolateral medulla under pentobarbital anesthesia (50 mg/kg, i.p.) by using a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). The total length of the i.c. guide cannula was 16 mm, of which 6 mm was passed between the occipital bone and the cerebellum so that its tip protruded into the cisterna magna. For intra- rostral ventrolateral medulla microinjections, a stainless steel guide cannula (23 G; Small Parts, Miami, FL, USA) was implanted as described in our previous study (Mao and Abdel-Rahman, 1998). The tip of guide cannula was positioned 2 mm above the rostral ventrolateral medulla (posterior −2.8 mm, lateral ±2.0 mm, dorsoventral −0.5 mm). The cannula was secured in place with small metal screws and dental acrylic cement (Durelon; Thompson Dental Supply, Raleigh, NC, USA), as described in our previous studies (El-Mas and Abdel-Rahman, 1999a,b). Each rat received a subcutaneous injection of buprenorphine (Buprenex; 30 μg/kg) to control pain and an intramuscular injection of 50 000 U/kg of penicillin G and penicillin G procaine in an aqueous suspension (Durapen).

2.3. Intravascular cannulation

Two days before the experiment, the rats were anesthetized with pentobarbital (50 mg/kg i.p.). Polyethylene-50 catheters filled with heparinized saline (100 U/ml) were introduced via the left femoral artery and vein to the abdominal aorta and inferior vena cava for measurements of blood pressure and intravenous injection of drugs, respectively. The catheters were tunneled subcutaneously and exteriorized at the back of the neck between the scapulae. Incisions were closed with surgical clips and swabbed with povidone-iodine solution (Wang and Abdel-Rahman, 2002). Each rat received postoperative care as described above.

2.4. Cardiovascular measurement

When the experiment started, the arterial catheter was connected to a Gould-Statham (Oxnard, CA) pressure transducer. Blood pressure was recorded on a Grass poly-

graph (model 7D, Grass Instrument Co., Quincy, MA); heart rate was computed from blood pressure waveforms by a Grass tachograph and recorded on another channel of the polygraph.

2.5. In situ hybridization

Fifty minutes after injection, the rats were anesthetized, decapitated, and the brains were removed and placed in dry ice cold 2-methylbutane. Coronal sections (14 μm) were cut in a cryostat and thawed onto gelatin/chrom alum-coated slides. Four sections included the middle NTS or rostral ventrolateral medulla (centered 13.24, and 12.30 mm, respectively, caudal to bregma) were collected on each slide. In situ hybridization was performed as described in our previous study (El-Mas and Abdel-Rahman, 2001). Oligodeoxynucleotide probes of 48 base pairs complementary to rat *c-jun* (Oncogene Science, Manhasset, NY, USA) were used. This probe and its comparison with 'sense' probe were evaluated in our previous studies (El-Mas and Abdel-Rahman, 2000, 2001). All oligodeoxynucleotide probes were labeled at the 3′end using alpha-[^{35}S]deoxyadenosine triphosphate (1250 Ci/mmol, New England Nuclear, Boston, MA, USA) and terminal deoxynucleotidyl transferase (Boehringer Mannheim, Indianapolis, IN, USA) to a specific activity of $7-12\times10^5$ cpm/μl. The sections were incubated with 25 μl/section containing 1×10^6 cpm labeled probe and hybridized overnight at 37 °C in a humid environment. Following hybridization, the slides were washed, dehydrated and apposed, along with [^{14}C] standards (American Radiolabeled Chemicals, St. Louis, MO, USA), to Kodak X-ray film for 2–3 weeks.

2.6. Immunohistochemical analysis

Fifty minutes after microinjection into the rostral ventrolateral medulla, the rats were anesthetized and perfuse with 4% buffered-paraformaldehyde solution. The brain tissues were removed and post-fixed in 4% paraformaldehyde/Phosphate-buffered saline (PBS) solution for 4 h at 4 °C. Then the tissues were transferred to 20% sucrose/PBS solution and incubated for 48 h at 4 °C. The sucrose-infiltrated tissues were frozen and sectioned. Cryostat sections (14 μm in thickness) were immunostained with anti-c-Jun polyclonal antibody (Santa Cruz Biotechnology) using a modification of the avidin–biotin-complex method (ABC). ABC kit (Vector Laboratories, Inc. Burlingame, CA) was used to perform Immunohistochemistry (Wang and Abdel-Rahman, 2002). Control sections that were only incubated with the primary or secondary antibody showed no positive staining (data not shown).

2.7. Quantitative assessment of neuronal c-Jun

Neuronal profile counts were performed via light microscopy. Six sections, which included the rostral ventrolateral medulla were collected from each rat, were used for quantification of c-Jun protein expression. An identical region (field = 0.125 mm^2) of the rostral ventrolateral medulla was examined in brain sections from treated and control rats. Within the field in each section a number denoting the total number of neuronal profiles exhibiting immunoreactivity for c-Jun was obtained. Positive profiles were defined as cells with dark granular staining indicative of a 3, 3′-Diaminobenzidine (DAB) reaction product. The average per-field count of positive neuronal profiles was then determined and subsequently converted into the number of profiles per unit area (mm^2) for each rat (Marcus et al., 1998).

2.8. Experimental protocols

2.8.1. Effect of α_2- vs. I_1 activation on c-jun gene expression in the NTS and rostral ventrolateral medulla

In the first set of experiments, we examined the relative contribution of α_2-adrenoceptor and imidazoline I_1 receptor to the reduction of *c-jun* mRNA expression in brainstem elicited by centrally acting drugs and determined whether the *c-jun* mRNA response was dependent on the neuronal pool; the NTS vs. the rostral ventrolateral medulla. Five groups of SHRs ($n=8$ each) were used in this study. The rats in a particular group received intracisternal artificial cerebrospinal fluid (ACSF) (4 μl), clonidine (0.5 μg), α-methylnorepinephrine (4 μg) or rilmenidine (25 μg). The rats in the 5th group received the peripherally acting hypotensive hydralazine (0.4 mg/kg) and served as control. To facilitate data interpretation, doses of the hypotensive agents were chosen, based on reported studies including ours, to produce comparable reductions in blood pressure.

2.8.2. Effect of selective I_1 vs. α_2 blockade on reductions in c-Jun expression in the rostral ventrolateral medulla and blood pressure elicited by rilmenidine

In this experiment, direct evidence was sought to identify the receptor subtype in the rostral ventrolateral medulla implicated in the reduction of neuronal c-Jun expression and the associated hypotension. The experiment focused on the responses elicited by rilmenidine microinjection into the rostral ventrolateral medulla because intracisternal rilmenidine had no effect on neuronal *c-jun* in the NTS in experiment 1. Four groups of SHRs were used in this experiment; 5–6 rats in each group successfully completed the experiment. The rats in a particular group received intra-rostral ventrolateral medulla rilmenidine (40 nmol) 15 min after intra-rostral ventrolateral medulla: (i) ACSF (40 nl), (ii) the selective imidazoline I_1 receptor antagonist efaroxan (4 nmol); or (iii) the selective α_2-adrenoceptor antagonist SK&F 86466 (10 nmol). The doses of the antagonists adequately blocked the respective receptor in reported studies (Haxhiu et al., 1994). The rats in the 4th group received ACSF as the second microinjection and served as control.

2.9. Data analysis

Values are presented as mean±S.E.M. Quantification of the mRNA hybridization signal on X-ray films was performed using the NIH Image software (Version 1.60) as described in our previous study (El-Mas and Abdel-Rahman, 2001). The [^{14}C] standards were measured, plotted against known dpm/mg signals, and converted to [^{35}S] equivalents to generate a calibration curve. Non-uniform illumination was corrected for by saving a 'blank field'. Quantitative changes were expressed as integrated density: the product of area times mean density, which relates the area over which the hybridization signal is present to the mean density of the signal. ANOVA followed by Newman-Keuls post hoc analysis was used for multiple comparisons, and the level of significance was set at $P<0.05$.

3. Results

3.1. Baseline data

Table 1 shows the baseline mean arterial pressure and heart rate values obtained before drug treatment in conscious unrestrained SHRs. As shown in Table 1, the baseline values of mean arterial pressure and heart rate were similar in the all groups of SHRs, which received ACSF, clonidine (0.5 μg, i.c.), rilmenidine (25 μg, i.c.), α-methylnorepinephrine (4 μg, i.c.) or hydralazine (0.4 mg/kg, i.v.). Table 2 shows the similar baseline mean arterial pressure and heart rate values obtained before intra- rostral ventrolateral medulla ACSF (40 nl) or antagonist (efaroxan, 4 nmol or SKF 86466, 10 nmol), which was followed by intra-rostral ventrolateral medulla rilmenidine (40 nmol) in conscious unrestrained SHRs.

3.2. Hemodynamic effects of clonidine, rilmenidine, α-methylnorepinephrine or hydralazine

Fig. 1 depicts the time-course of blood pressure and heart rate responses elicited by intracisternal clonidine, rilmenidine, or α-methylnorepinephrine, compared with ACSF, in conscious unrestrained SHRs. Mean arterial pressure was similarly and significantly reduced after clonidine, rilmenidine or α-methylnorepinephrine (Fig. 1). Hydralazine (i.v.) also significantly decreased mean arterial pressure (Fig. 1). The maximal hypotensive response elicited by i.c clonidine (−32.3±4.2 mm Hg), rilmenidine (−37±3.5 mm Hg), or α-methylnorepinephrine (−36.4±4.7 mm Hg) or by i.v. hydralazine (−26.6±4.9 mm Hg) observed at 15 min was similar. The heart rate was significantly reduced by clonidine, rilmenidine, or α-methylnorepinephrine, but was significantly increased after hydralazine administration (Fig. 1). The blood pressure and heart rate responses were still

Table 1
Baseline values of mean arterial pressure (MAP, mm Hg), heart rate (HR, beats/min) of SHRs obtained before antihypertensive drug or artificial cerebrospinal fluid (ACSF) treatment

Group	*n*	MAP	HR
ACSF (4 μl, i.c.)	8	162.6±1.4	383±11
Clonidine (0.5 μg, i.c.)	8	158.4±4.7	389±8
Rilmenidine (25 μg, i.c.)	8	158.8±4.5	386±10
α-MNE (4 μg, i.c.)	8	161.5±5.9	391±7
Hydralazine (0.4 mg/kg, i.v.)	8	159.9±5.5	392±9

α-MNE: α-methylnorepinephrine.

Table 2
Baseline values of mean arterial pressure (MAP, mm Hg), heart rate (HR, beats/min) obtained before artificial cerebrospinal fluid (ACSF, 40 nl) or antagonist microinjected into rostral ventrolateral medulla (RVLM)

Group	*n*	MAP	HR
ACSF+ACSF	5	173.5±9.5	375±15
ACSF+Rilmenidine	6	167.3±6.8	365±14
Efaroxan (4 nmol)+Rilmenidine	6	163.8±4.5	368±15
SK&F 86466 (10 nmol)+Rilmenidine	6	176.6±7.2	366±16

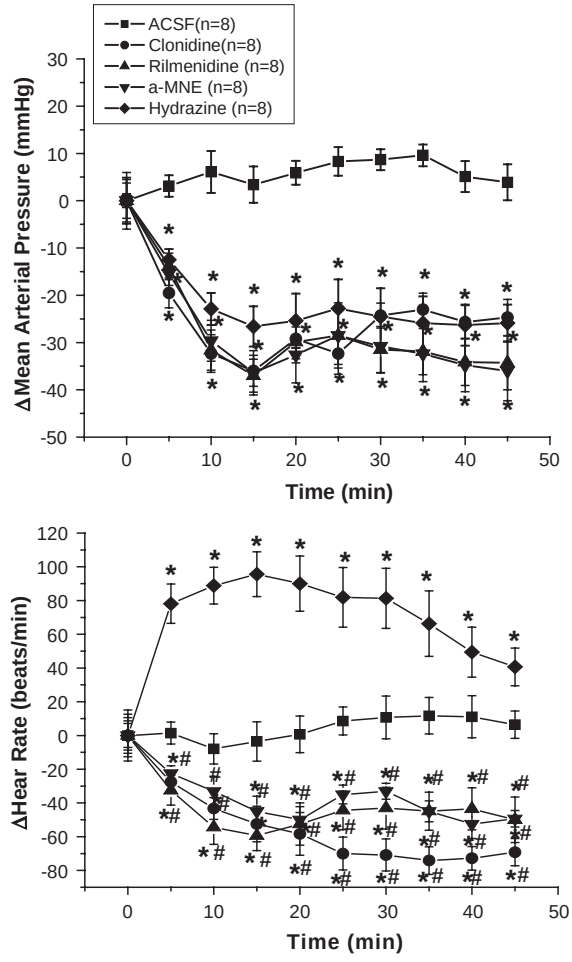

Fig. 1. The time-course changes in mean arterial pressure and heart rate elicited by the intra-cisternal injection of antihypertensive agents or ACSF in conscious SHRs. Values are mean±S.E.M. ($n=8$ in each group). *$P<0.05$ and #$P<0.05$ compared with control artificial cerebrospinal fluid (ACSF) and hydralazine values, respectively.

evident when the brains were harvested for the measurement of brainstem *c-jun* gene expression.

3.3. Effect of central α_2-adrenergic and/or imidazoline I_1 receptor activation on c-jun mRNA expression in brainstem

In this experiment we evaluated the effect of activating the imidazoline I_1 receptor and/or α_2-adrenoceptor on *c-jun* gene expression in two areas of the brainstem, the NTS and the rostral ventrolateral medulla. Compared with control group (ACSF), intracisternal clonidine significantly ($P<0.05$) reduced the *c-jun* mRNA expression in the NTS by 24±3.7% and in the rostral ventrolateral medulla by 27.7±4.3% (Fig. 2). *c-jun* mRNA expression was also significantly reduced by the α_2-adrenergic agonist α-methylnorepinephrine in the NTS by 36.5±6.7% and rostral ventrolateral medulla by 28.5±5.7% (Fig. 2). The selective imidazoline I_1 receptor agonist rilmenidine significantly reduced *c-jun* mRNA expression in the rostral ventrolateral medulla by 37.5±5.4%, but not in the NTS (Fig. 2). On the other hand, the peripheral antihypertensive drug hydralazine significantly increased *c-jun* mRNA expression in the NTS and rostral ventrolateral medulla (Fig. 2).

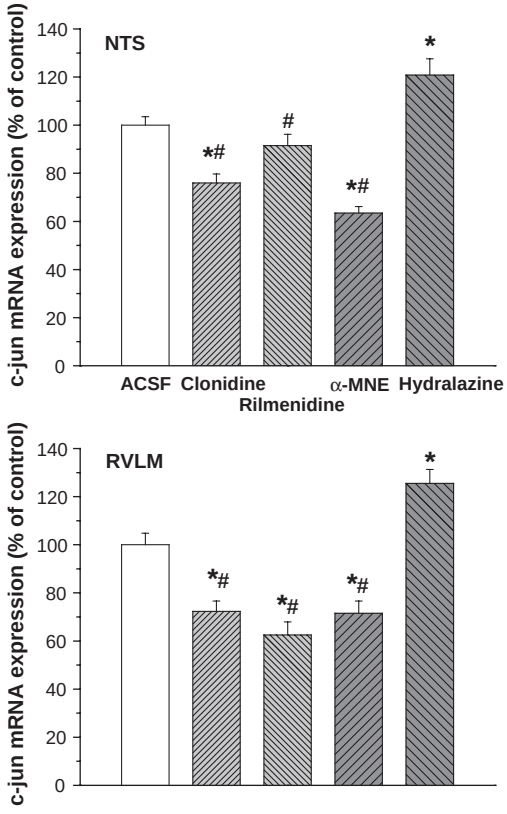

Fig. 2. Effects of intracisternal injection of antihypertensive agents or artificial cerebrospinal fluid (ACSF) on *c-jun* mRNA expression in the NTS (top panel) and rostral ventrolateral medulla (RVLM, bottom panel) of conscious SHRs. Compared with ACSF, clonidine and α-methylnorepinephrine significantly reduced *c-jun* mRNA expression in the NTS and RVLM. Rilmenidine reduced *c-jun* mRNA expression only in the RVLM while hydralazine increased *c-jun* mRNA expression in the NTS and RVLM. Values are mean±S.E.M. ($n=8$ in each group). *$P<0.05$ and #$P<0.05$ compared with control (ACSF) and hydralazine values, respectively.

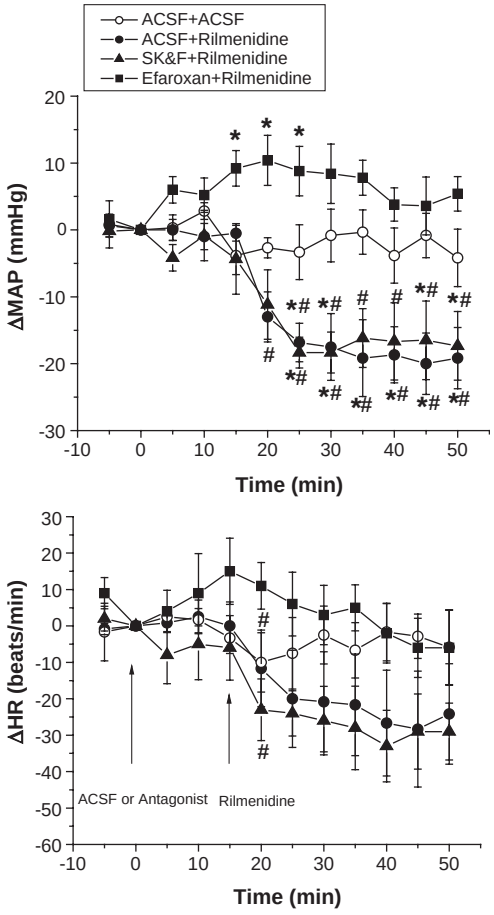

Fig. 3. The time-course changes in mean arterial pressure and heart rate elicited by the microinjection of rilmenidine or artificial cerebrospinal fluid (ACSF) into the rostral ventrolateral medulla (RVLM) of conscious SHRs. ACSF or antagonist (efaroxan or SK&F 86466) was microinjected 15 min before rilmenidine or ACSF microinjection into the RVLM. Values are mean±S.E.M. ($n=5$ or 6 in each group). *$P<0.05$ compared with control (ACSF+ACSF group) values; #$P<0.05$ compared with values of (efaroxan+rilmenidine) group.

3.4. Effect of local selective I_1 or α_2 adrenergic receptor blockade on the reductions in blood pressure and neuronal c-Jun elicited by intra-rostral ventrolateral medulla rilmenidine

As shown in Fig. 3, intra-rostral ventrolateral medulla rilmenidine (40 nmol) in conscious SHRs elicited significant hypotensive and bradycardic responses. Imidazoline I_1 receptor blockade with intra-rostral ventrolateral medulla efaroxan (4 nmol in 80 nl) caused slight increases in blood pressure and heart rate (Fig. 3). Imidazoline I_1 receptor blockade (efaroxan), virtually abolished the hypotensive and bradycardic responses elicited by intra-rostral ventrolateral medulla rilmenidine (Fig. 3). However, α_2-adrenergic

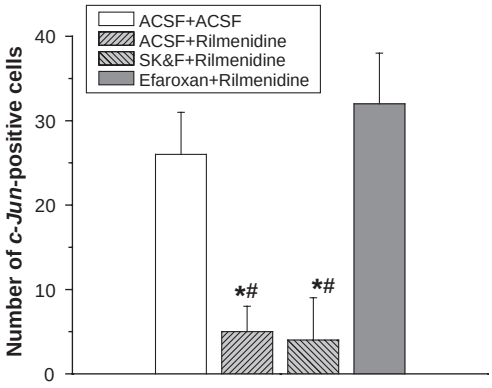

Fig. 4. Effect of rilmenidine microinjected into the rostral ventrolateral medulla (RVLM) of conscious SHRs on c-Jun expression in the RVLM. Counts are expressed as average number of positive neurons per mm^2 within the RVLM of control or treated rats. Compared with artificial cerebrospinal fluid (ACSF), rilmenidine produced significant reductions in the mean (±S.E.M.) number of c-Jun-positive cells in RVLM. This rilmenidine-induced reduction in the RVLM c-Jun protein expression was virtually abolished by pretreatment with intra-RVLM efaroxan but not SK&F 86466. *$P<0.05$ compared to control values. Values are mean± S.E.M. ($n=6$ in each group).

blockade in the rostral ventrolateral medulla (SKF 86466; 10 nmol/80 nl) had no effect on hemodynamic responses elicited by intra-rostral ventrolateral medulla rilmenidine (Fig. 3). Similarly, the significant reduction in neuronal c-Jun elicited by intra-rostral ventrolateral medulla rilmenidine was virtually abolished by prior I_1 (efaroxan) but not α_2-adrenergic (SKF 86466) receptor blockade in the rostral ventrolateral medulla (Fig. 4). Representative images depicting c-Jun-positive cells in the rostral ventrolateral medulla of SHRs treated with intra-rostral ventrolateral medulla rilmenidine in the presence or absence of imidazoline I_1 receptor or α_2-adrenoceptor blockade in the rostral ventrolateral medulla are shown in Fig. 5.

4. Discussion

Neuronal *c-jun* and/or *c-fos* mRNA are useful markers of neuronal activity (Morgan and Curran, 1989) and more importantly their corresponding proteins seem to serve a functional role in neuronal signaling (Dampney and Horiuchi, 2003; Dampney et al., 2003). In the few studies that demonstrated reduction of immediate early genes in brainstem by centrally acting drugs (Li and Dampney, 1995; El-Mas and Abdel-Rahman, 2000), the receptor type (I_1 or α_2) and the targeted neuronal pool could not be ascertained because non-selective agonists were administered systemically. In the present study, selective agonists were administered intracisternally or directly into the rostral ventrolateral medulla in conscious SHRs in doses that produced comparable hypotensive responses. The most important findings of the present study are: (1) clonidine, a mixed imidazoline I_1 receptor and α_2-adrenergic agonist, or the highly selective "pure" α_2-adrenoceptor agonist α-methylnorepinephrine reduced *c-jun* mRNA expression in the NTS and rostral ventrolateral medulla; (2) in contrast, the selective imidazoline I_1 receptor agonist rilmenidine reduced *c-jun* mRNA expression in the rostral ventrolateral medulla, but not in the NTS; (3) the peripherally acting antihypertensive drug hydralazine significantly increased *c-jun* mRNA expression in NTS and rostral ventrolateral medulla; and (4) intra-rostral ventrolateral medulla rilmenidine reduced neuronal c-Jun protein expression and the pretreatment with the imidazoline I_1 receptor (efaroxan), but not the α_2 (SKF 86466) antagonist prevented the neuronal and hemodynamic responses elicited by subsequent intra-rostral ventrolateral medulla rilmenidine. These findings suggest that neuronal immediate early gene expression in cardiovascular controlling areas of the brainstem is modulated by centrally acting drugs in a receptor- and site-dependent manner.

A functional role for the immediate early genes and their corresponding proteins in the central control of arterial pressure is suggested (Minson et al., 1994, 1996a,b). However, the role of these immediate early genes and their respective proteins in the hemodynamic responses elicited by centrally acting antihypertensive agents has not been thoroughly investigated. Notably, many studies have demonstrated parallel changes in the two immediate early genes *c-fos* and/or *c-jun* in response to physiological or pharmacological interventions (El-Mas and Abdel-Rahman, 2000; Gerlach et al., 2002; Terashima

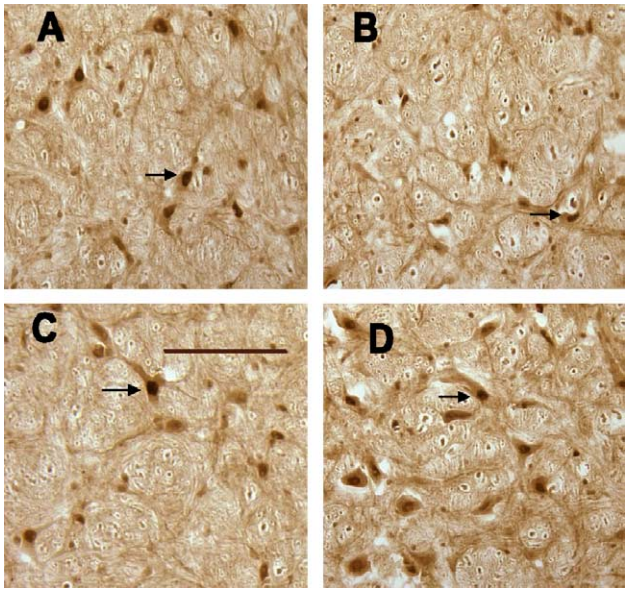

Fig. 5. Representative photomicrographs that illustrate the effects of microinjection of rilmenidine into the rostral ventrolateral medulla (RVLM) on the expression of c-Jun protein in the RVLM. (A) ACSF+ACSF; (B) ACSF+Rilmenidine; (C) Efaroxan+Rilmenidine; (D) SK&F 86466+Rilmenidine. Positive cells were defined as cells with dark granular staining indicative of a DAB reaction product (arrows). The solid line in panel C represents 10 μm.

et al., 2003). The present finding that intracisternal clonidine reduced *c-jun* mRNA expression in the brainstem (NTS and rostral ventrolateral medulla) of SHRs supports our earlier observation with systemic clonidine in normotensive rats (El-Mas and Abdel-Rahman, 2000). The present and the previous findings (El-Mas and Abdel-Rahman, 2000) suggest that the hypotensive action of clonidine may depend on inhibition of medullary neurons that express the *c-jun* gene. However, because clonidine is a mixed α_2-adrenoceptor/imidazoline I_1 receptor agonist, (Ernsberger et al., 1993), the type of the receptor implicated in the suppression of *c-jun* gene expression in reported studies (Tsujino et al., 1992; Li and Dampney, 1995; El-Mas and Abdel-Rahman, 2000) is not known. To address this issue, we used selective I_1 and α_2 receptor agonists and antagonists. Further, we utilized the SHRs and administered the drugs centrally for the following reasons. First, the hypotensive response elicited by centrally acting drugs is much more evident in conscious SHRs than in conscious normotensive rats (Head and de Jong, 1986). Second, we have recently shown that basal *c-jun* mRNA is significantly higher in the brainstem of the SHRs compared with normotensive rats (Wang and Abdel-Rahman, 2004). This higher basal *c-jun* mRNA level in the SHRs made it possible to quantify the reductions in this immediate early gene in the present study without the need of artificially elevating the gene level by administering peripherally acting vasodilators. The latter approach was followed in normotensive animals (El-Mas and Abdel-Rahman, 2000), which may have confounded the data interpretation. Nonetheless, we felt it important that the impact of lowering blood pressure with a peripherally acting drug (hydralazine) on brainstem *c-jun* gene expression be investigated in the model system used in the present study. Hydralazine evoked hypotension was associated with enhanced expression of *c-jun* mRNA in the NTS and rostral ventrolateral medulla. This finding agrees with reported findings with other peripheral vasodilators in normotensive animals (Minson et al., 1996a) except that the magnitude of the enhancement of *c-jun* gene expression was much less in the SHRs perhaps due to the attenuated baroreflex sensitivity in the SHRs (Wang and Abdel-Rahman, 2004).

The finding that α-methylnorepinephrine, a highly selective α_2-adrenoceptor agonist (Allen and Guyenet, 1993), similarly reduced *c-jun* mRNA expression in the NTS and rostral ventrolateral medulla, suggests that α_2-adrenoceptors modulate *c-jun* gene expression in brain areas implicated in autonomic control of blood pressure and cardiac reflexes. This notion is supported by the observations that another α_2-adrenoceptor agonist medetomidine suppresses another immediate early gene, Fos, expression in the spinal cord (Pertovaara et al., 1993) and the α_2-adrenoceptor antagonist yohimbine increases *c-fos* mRNA expression in the brain (Tsujino et al., 1992). It is imperative to note, however, that the reductions in NTS *c-jun* mRNA and blood pressure might constitute dissociated events because: (i) in spite of the presence of the α_{2A} receptor in the NTS (Nicholas et al., 1993; Milner and Pickel, 2003), intra-NTS clonidine causes little or no reduction in blood pressure (Zandberg et al., 1979); (ii) clonidine-evoked hypotension was associated with reductions in NTS *c-jun* mRNA in the present study and others (El-Mas and Abdel-Rahman, 2000). We corroborated the notion that the reduction in *c-jun* mRNA is α_2-mediated by demonstrating, for the first time, that the highly selective α_2 agonist α-methylnorepinephrine, but not the selective imidazoline I_1 receptor agonist rilmenidine, suppressed NTS *c-jun* mRNA at equihypotensive doses. Together, these findings seem to suggest that the suppression of *c-jun* gene expression in the NTS may not constitute an important neuronal event for the associated hypotensive response elicited by clonidine. It is notable, however, that at equihypotensive response, intracisternal α-methylnorepinephrine caused greater reduction in *c-jun* gene expression in the NTS than did clonidine. Further, microinjection of α-methylnorepinephrine into the NTS lowers blood pressure (Zandberg et al., 1979). These finding may suggest a role for the reduced *c-jun* expression in the NTS in the hypotension elicited by the first generation centrally acting drugs such as alpha-methyldopa. On the other hand, the neurobiological relevance of the reduced NTS *c-jun* gene expression by clonidine needs to be determined. It is possible that the clonidine evoked reduction in NTS *c-jun* gene expression contributes, at least partly, to clonidine enhancement of baroreflex sensitivity. The latter has been reported in many clinical and experimental studies (Elghozi et al., 1991; Tank et al., 2004a,b). In support of this notion are the findings that a reciprocal relationship exists between *c-fos* and *c-jun* mRNA level in the NTS and BRS (Chan et al., 2002; Wang and Abdel-Rahman, 2004) and that antisense-induced inhibition of neuronal *c-fos* expression in the NTS results in the enhancement of BRS in the SHRs (Chan et al., 1998).

Results of the present study, demonstrate for the first time, that the selective imidazoline I_1 receptor agonist rilmenidine elicited significant reduction in *c-jun* mRNA expression in the rostral ventrolateral medulla, but not in the NTS. This finding suggests site- and/or receptor selectivity for rilmenidine action on *c-jun* gene expression. There were two issues that needed to be addressed before a conclusion is accepted that the gene expression response in the rostral ventrolateral medulla was imidazoline I_1 receptor -mediated and contributes to the hypotensive response. First, although recognized as a selective imidazoline I_1 receptor agonist (Bricca et al., 1989; Head et al., 1997; Bousquet et al., 2003), rilmenidine exhibits α_2 agonist activity (Mao et al., 2003). Microinjection of high doses of rilmenidine into the NTS of rabbits lowered blood pressure (Head et al., 1998a), which seems to be mediated via the α_2-adrenoceptor because functional imidazoline I_1 receptor does not seem to be existent in the NTS (Ernsberger and Haxhiu, 1997). Second, the rostral ventrolateral medulla contains α_2-

adrenoceptor (Nicholas et al., 1993) whose activation leads to a reduction in *c-jun* gene expression as we have demonstrated with α-methylnorepinephrine (Fig. 2). Therefore, the lack of a reduction in NTS *c-jun* gene expression following intracisternal rilmenidine may not unequivocally rule out a role for the α_2-adrenoceptor in the reduced *c-jun* gene expression in the rostral ventrolateral medulla. Third, although still a matter of debate (Szabo, 2002), some reported studies suggest that imidazoline I_1 receptor activation leads to downstream activation of α_2-adrenoceptor and the latter contributes significantly to the neurobiological effects of imidazoline I_1 receptor agonists (Head et al., 1998b). Notably, our previous finding that clonidine reduced *c-jun* mRNA in the rostral ventrolateral medulla (El-Mas and Abdel-Rahman, 2001), the major anatomical site for the imidazoline I_1 receptor mediated hypotension (Head et al., 1997), inferred that the reduction in *c-jun* is imidazoline I_1 receptor mediated.

It was important, however, to bolster our conclusion by undertaking a study where rilmenidine was directly applied to the rostral ventrolateral medulla neurons in the absence and presence of selective imidazoline I_1 receptor or α_2 antagonist. Our finding that intra-rostral ventrolateral medulla rilmenidine suppressed neuronal c-Jun expression suggests neuroinhibitory action for rilmenidine within the rostral ventrolateral medulla that is independent of other brain areas. Further, the hypotensive response elicited by intra-rostral ventrolateral medulla rilmenidine, which agrees with reported findings (Head et al., 1998a; Zhang and Abdel-Rahman, 2002), raises the interesting possibility that the associated neuronal c-Jun response may contribute, at least partly, to the hypotensive response. Indeed, the findings that I_1- but not α_2-adrenoceptor receptor blockade within the rostral ventrolateral medulla abolished the neuronal (c-Jun) and hypotensive responses elicited by intra-rostral ventrolateral medulla rilmenidine suggest that the imidazoline I_1 receptor in the rostral ventrolateral medulla mediates these responses. The present findings are consistent with the view that the neuronal c-Jun in the rostral ventrolateral medulla may serve a functional role in modulating sympathetic neural activity in conscious animals (Dampney and Horiuchi, 2003).

We report, for the first time, findings that seem to support a functional role for c-Jun in the rostral ventrolateral medulla in imidazoline I_1 receptor-mediated hypotension. Intra-rostral ventrolateral medulla efaroxan, abolished the reductions in neuronal c-Jun and blood pressure caused by intra-rostral ventrolateral medulla rilmenidine. Further, this conclusion is supported by the lack of effect of intra-rostral ventrolateral medulla blockade of α_2-adrenoceptor in the RVLM had no effect on local c-Jun and blood pressure responses elicited by rilmenidine in the present study and on the hypotensive response elicited by another imidazoline I_1 receptor agonist (Haxhiu et al., 1994). Together, the present and reported findings suggest that rilmenidine acts primarily on imidazoline I_1 receptors, located in the rostral ventrolateral medulla, to reduce c-Jun expression and blood pressure independent of α_2-adrenoceptor.

In conclusion, the present investigation provides evidence to suggest: (1) α_2-adrenoceptor suppression of the activity of the *c-jun* containing neurons in the NTS and rostral ventrolateral medulla is implicated in the hypotension elicited by first generation centrally acting antihypertensive agents; (2) selective suppression of the activity of *c-jun* gene containing neurons in the rostral ventrolateral medulla by imidazoline I_1 receptor activation contributes to the centrally mediated hypotension by the second generation drugs; (3) the imidazoline I_1 receptor mediated reductions in *c-jun*/c-Jun expression in the rostral ventrolateral medulla and the associated hypotension occur independent of the α_2-adrenoceptor.

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