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Original Article

Determining the cardiovascular effects of nitric oxide in the dorsolateral Periaqueductal Gray (dlPAG) in anaesthetised rats

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الملخص

أهداف البحث: المادة الرمادية حول القناة الظهرية الجانبية هي منطقة تقع في جذع الدماغ لديها مجموعة من الوظائف تتضمن تنظيم القلب والأوعية الدموية. نظرا لوجود أكسيد النيتريك في هذه المنطقة، تم التحقيق في تأثيرها على جهاز القلب والأوعية الدموية.

طرق البحث: تم تقسيم الفئران إلى أربع مجموعات ١- التحكم ،٢- إل-أرجينين (إل-أرجين، هو مقدمة لأكسيد النيتريك، ٦٠ نانومول)، ٣- إل —نيم (ن اوميغا-نيترو —إل- أرجينين ميثيل استر، مثبط سينثاس أكسيد النيتريك، ٩٠ نانومول)، ٤- نيتروبروسيد الصوديوم (نيتروبروسيد الصوديوم، هو أكسيد النيتريك المانح، ٢٧ نانومول). بعد التخدير، تم تثبيت الفئران على جهاز تجسيمي وتم حقن الأدوية مجهريا في المادة الرمادية حول القناة الظهرية الجانبية. وتم تسجيل معلمات القلب والأوعية الدموية بشكل مستمر من خلال نظام مختبر الطاقة المتصل بشريان الفخذ المقنن عبر محول الضغط. كما تم حساب التغيرات في ضغط الدم الانقباضي، ومتوسط ضغط الشرايين ومعدل ضربات القلب في أوقات مختلفة مقارنة بمجموعة التحكم.

النتائج: في مجموعة إل —نيم، لم يتأثر كل من التغيرات في ضغط الدم الانقباضي، ومتوسط ضغط الشرايين ومعدل ضربات القلب بشكل ملحوظ مقارنة بمجموعة التحكم. وفي مجموعة إل — أرجنين، زاد تغير ضغط الدم الانقباضي ومتوسط ضغط الشرايين، ولكن فقط زيادة ضغط الدم الانقباضي كانت كبيرا مقارنة بمجموعة التجكم. بنما في مجموعة نيتروبروسيد الصوديوم تأثر ضغط الدم الانقباضي ومتوسط ضغط الشرايين كثيرا مقارنة بمجموعة التحكم. بالإضافة لذلك، انخفض تغير معدل ضربات القلب في كل من مجموعة إل-أرجنين ونيتروبروسيد الصوديوم ولكن كان التغير مهما فقط في مجموعة إل-أرجينين.

الاستنتاجات: أظهرت الدراسة أن أكسيد النيتريك للمادة الرمادية حول القناة الظهرية الجانبية له تأثير ضاغط ويخفف من بطء القلب المنعكس. ومع ذلك ، فإن تأثير الضغط هو أكثر أهمية.

الكلمات المفتاحيه: المادة الرمادية حول القناة الظهرية الجانبية؛ إل- أرجينين؛ إل- نيم؛ نيتروبروسيد الصوديوم؛ ضغط الدم؛ معلمات القلب والأوعية الدموية

Abstract

Objective: The dorsolateral periaqueductal gray (dlPAG) is an area located in the brain stem that performs a host of functions including cardiovascular regulation. Owing to the presence of nitric oxide (NO) in this area, we investigated its effect on the cardiovascular system.

Methods: We divided rats into four groups: 1) control; 2) L-arginine (L-Arg, a precursor for nitric oxide, 60 nmol); 3) L-NAME (N omega-nitro-L-arginine methyl ester, a nitric oxide synthase inhibitor, 90 nmol); and 4) sodium nitroprusside (SNP, a nitric oxide donor, 27 nmol). After anaesthesia, the rats were mounted on a stereotaxic apparatus and the drugs were microinjected into the dlPAG. Cardiovascular parameters were continuously recorded by a PowerLab system connected to the cannulated femoral artery via a pressure transducer. The changes (Δ) of systolic blood pressure (SBP), mean arterial pressure (MAP), and heart rate (HR) were calculated at different times as compared to the control group.

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Results: In the L-NAME group, the ΔSBP, ΔMAP, and ΔHR were not significantly affected compared to the control group. In the L-Arg group, ΔSBP and ΔMAP increased; however, only SBP showed a significant increase compared to the control group. In the SNP group, SBP and MAP were significantly affected in comparison to the controls. Additionally, ΔHR decreased in both L-Arg and SNP, but was only significant in L-Arg.

Conclusion: Our study showed that NO of dlPAG has a pressor effect and attenuates baroreflex bradycardia. However, its pressor effect is more significant.

Keywords: Blood pressure; Cardiovascular parameters; Dorsolateral periaqueductal gray; L-arginine; L-NAME; Sodium nitroprusside

Introduction

The dorsolateral periaqueductal gray (dlPAG) is a mesencephalic area located around the cerebral aqueduct.[1] This area is mainly involved in defence responses and stress mechanisms. The dlPAG has connections to numerous parts of the brain including the cortex, hypothalamus, and spinal cord.[2] The dlPAG also has bidirectional connections to the hypothalamus nuclei,[3] such as the dorsomedial hypothalamus (DMH),[4] especially with its rostral segment.[5] This area also has a major projection to the cuneiform nucleus (CnF).[3]

Nitric oxide (NO) is a highly soluble gas, which acts as an important neurotransmitter in the brain. It mediates the production of cyclic guanosine monophosphate (cGMP) through the activation of the enzyme guanylate cyclase. NO is synthesised by the enzyme nitric oxide synthase (NOS), which has three isoforms (eNOS, iNOS, and nNOS),[6] and has been found in cardiovascular control centres.[7,8] NOS catalyses a reaction, in which the amino acid L-arginine (L-Arg) acts as a precursor, resulting in the release of citrulline and NO.[9]

Nitric oxide is an essential neurotransmitter in the dlPAG and is involved in the modulation of autonomic responses during defence responses and stress. Its interaction with the GABAergic and glutamatergic systems of dlPAG has previously been determined to increase miniature inhibitory post-synaptic currents in all neurons.[10] Additionally, NO has also been identified as an important neurotransmitter in other parts of the rat brain such as the rostral ventrolateral medulla (RVLM) nucleus,[11] which plays an essential role in cardiovascular function.[12,13] Microinjection of a NO donor into RVLM significantly decreased the blood pressure, while the inhibition of NOS increased it.[7,14] Such inhibitory effects of NO on the cardiovascular system have also been documented in both CnF[15] and pedunculopontine tegmental (PPT) nuclei.[16] Inhibition of NOS in the paraventricular nucleus (PVN) increased blood pressure and heart rate, which were attenuated by L-Arg.[17] In the dlPAG, NO has also been shown to attenuate the cardiovascular effects of glutamate during thermal and mechanical stimulation.[18] Since NO present in the dlPAG is an important neurotransmitter in the brain that exerts central cardiovascular effects via interactions with numerous neurotransmitters, this study is an initiative to investigate the possible role of NO in the dlPAG on the cardiovascular system.

Materials and Methods

Animals

Twenty male Wistar rats weighing 240 ± 20 g were used in this experiment. The animals were maintained under a 12:12 h light−dark cycle and had free access to food and water.

Selection of drugs

The drugs used in this study were urethane (anaesthesia), L-Arg (an NO precursor), NG-nitro-L-arginine methyl ester (L-NAME, a nitric oxide synthase inhibitor), and sodium nitroprusside (SNP, an NO donor). All drugs used in this experiment were procured from Sigma, USA.

Experiment groups

Rats were divided into four groups based on different drug microinjections administered, as follows: 1) Control group (n = 5): Microinjection of saline into the dlPAG; 2) L-Arg group (n = 5): Microinjection of L-Arg (60 nmol[15]) into the dlPAG; 3) L-NAME group (n = 5): Microinjection of L-NAME (90 nmol[15]) into the dlPAG; 4) SNP group (n = 5): Microinjection of SNP (27 nmol[15]) into the dlPAG. Volume injection was 100−150 nl in all groups.

Experiment protocol

The rats were anaesthetised before the experiment with urethane (1.4 g/kg, ip).[19] The left femoral artery was then cannulated using a blue angiocath filled with heparinised saline. This angiocath was connected to a PowerLab system (ADInstruments, Bella Vista, NSW, Australia) via a pressure transducer to record cardiovascular parameters.[20] For microinjection of the drugs, the rats were mounted on a stereotaxic apparatus, and the skull was surgically exposed. A hole was then drilled through the skull directly over the dlPAG in accordance with the rat brain atlas of Paxinos and Watson (AP: 6.8 mm caudal to bregma, L: 0.7 mm lateral to the midline; H: 5 mm).[21] After a stabilising period (10 min), the drugs were microinjected, and cardiovascular responses were recorded continuously for 15 min thereafter.

Statistical analysis

The cardiovascular parameters were recorded before and after the injections. A trend of change (Δ) in the systolic

blood pressure (ΔSBP), mean arterial pressure (ΔMAP) and heart rate (ΔHR) was obtained before, and 5, 10, and 15 min after drug injection and compared with the control group. The data were expressed as the mean ± SEM and comparisons were performed by repeated measures of analysis of variance (ANOVA) (GraphPad InStat version 3.10), and a P-value of <0.05 indicated significance.

Histological analysis

After the experiment, the rats were sacrificed under urethane (1.5 g/kg, i.p) anaesthesia, their brains were removed, and stored in a 10% formalin solution for at least 48 h. Serial sections (60 μm) were then obtained using a vibrator microtome. The injection site was observed under a light microscope, and the injection site was verified according to the Paxinos and Watson rat brain atlas (Figure 1).[22]

Results

Cardiovascular responses evoked after microinjection of saline into the dlPAG

In this experiment, saline was microinjected into the dlPAG. The recorded baseline values for HR, SBP, and MAP were 340 ± 8 beats/min, 130 ± 5 mmHg, and 110 ± 3 mmHg, respectively. After the microinjection of saline, these parameters were recorded as follows: ΔHR: 351 ± 10 beats/min, ΔSBP: 136 ± 6.5 mmHg, and ΔMAP: 115 ± 4.9 mmHg, which were not significant compared to pre-injection.

Cardiovascular responses evoked after microinjection of L-Arg into the dlPAG

When L-Arg was microinjected into the dlPAG, the SBP and MAP increased, whereas HR decreased (Figure 2A and B). Time-course changes in cardiovascular responses are indicated in Figure 3A and B. Increase in ΔSBP and ΔMAP were significant compared to the control over time ($P < 0.05$, repeated measures ANOVA). Peak changes in ΔSBP and ΔMAP were also significant compared to that in the control ($P < 0.01$ and $P < 0.05$, respectively; Figure 3A and B). The ΔHR was not significant compared to the control group over time; however, the peak change was significant compared to the control group ($P < 0.05$; Figure 3C).

Cardiovascular responses evoked after microinjection of L-NAME into the dlPAG

When L-NAME was microinjected into the dlPAG, the SBP, MAP, and HR slightly increased. As shown in Figures 3 and 4, time-course changes in all responses were not significant when compared to the control over time. Moreover, peak changes in all parameters were not significant with respect to the saline group.

Cardiovascular responses evoked after microinjection of SNP into the dlPAG

Microinjection of SNP into the dlPAG increased SBP and MAP but decreased HR (Figure 4 C). Time-course changes showed that ΔSBP and ΔMAP significantly increased over time ($P < 0.001$-$P<0.01$, respectively; Figure 3A and B). Peak changes in ΔSBP and ΔMAP also significantly increased compared to those of the control groups ($P < 0.001$; Figure 4A and B). The mean changes of ΔHR were not significant when compared to that of the control group over time (Figure 3C). The peak change in ΔHR was not significant with respect to the control group (Figure 4C).

Comparison of peak changes in cardiovascular responses in the experimental groups

The MAP and SBP increased after microinjection of SNP and L-Arg into dlPAG; however, the microinjection of L-NAME had no significant effect (Figure 2). As has been shown in Figure 3A and B, peak changes in SBP

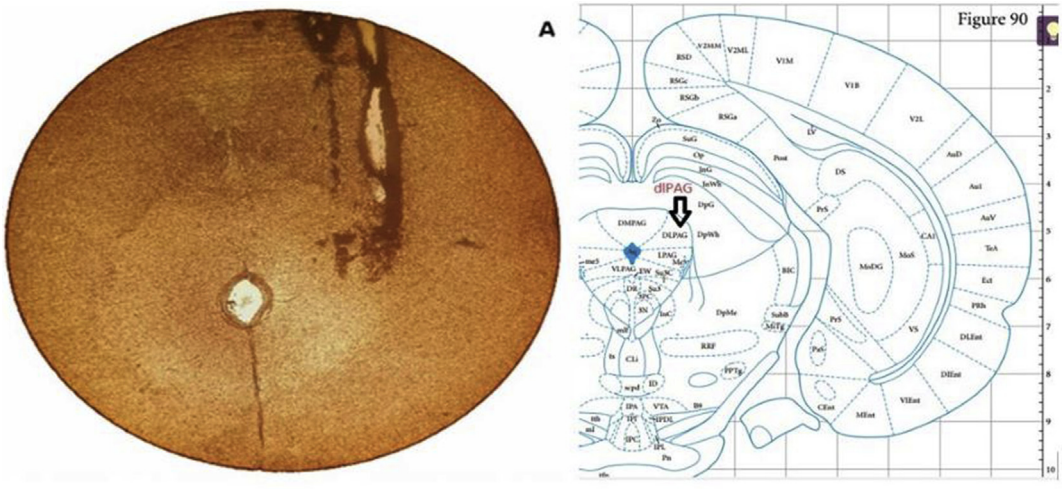

Figure 1: Sample of brain section after microinjection of the drug into the dlPAG (A). Coordinates of injection adopted from the Paxinos atlas (B).

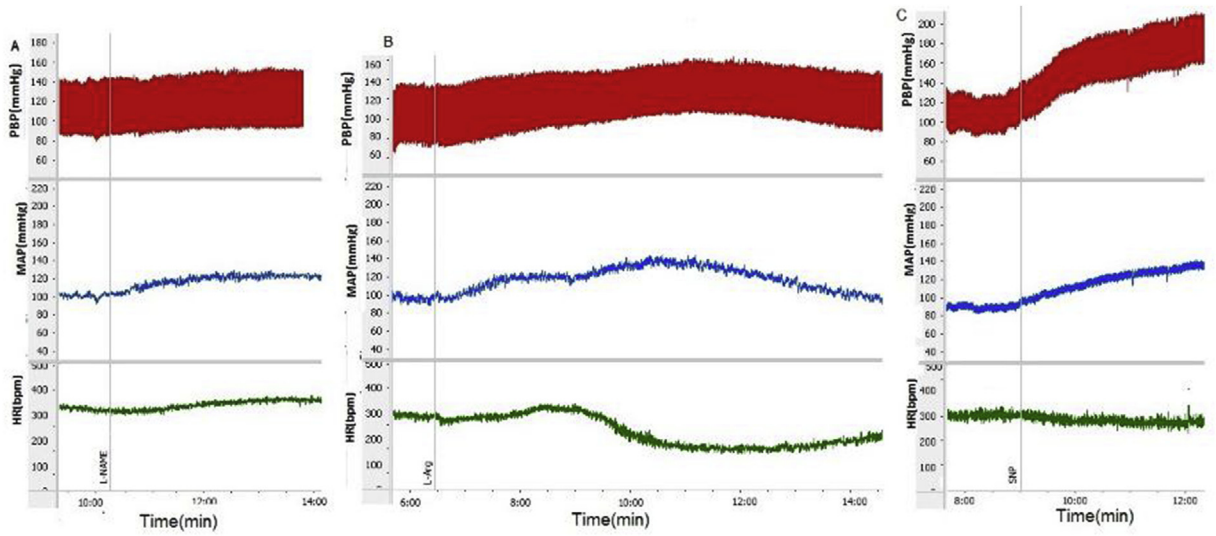

Figure 2: Recorded samples of the changes after microinjection of L-Arg (A), L-NAME (B), and SNP (C) into the dlPAG. PBP, pulsative blood pressure; MAP, mean arterial pressure; HR, heart rate.

and MAP in the SNP group were significant compared to the L-Arg ($P < 0.05$ to $P < 0.001$, respectively) and L-NAME groups ($P < 0.01$ in both parameters). Peak ΔSBP and ΔMAP in the L-Arg group were not significant compared to those in the L-NAME group. The HR in the L-NAME group increased but those in the L-Arg and SNP groups decreased (Figure 2C). As shown in Figure 4, peak ΔHR in the L-NAME group was significant compared to that of SNP ($P < 0.01$) and L-Arg ($P < 0.05$) groups. Moreover, there was no significant difference between the peak changes of L-Arg and SNP groups (Figure 4C).

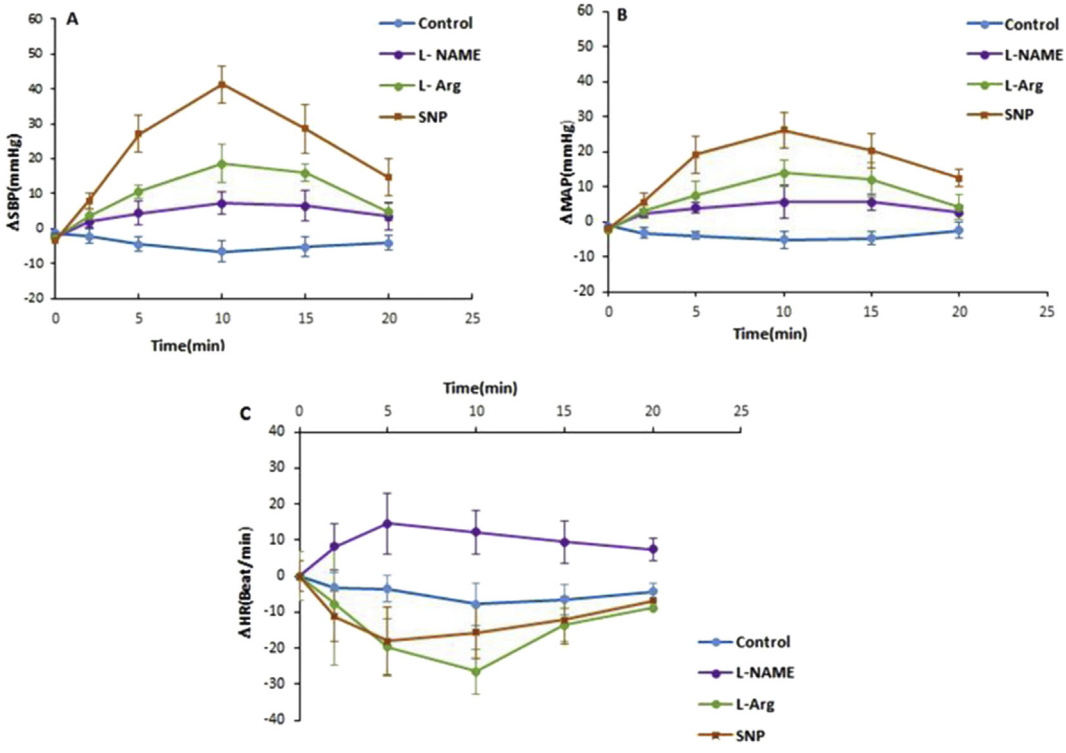

Figure 3: Time-course for changes in systolic blood pressure (ΔSBP) (A), mean arterial pressure (ΔMAP) (B) and heart rate (ΔHR) (C) following the injection L-NAME, SNP, and L-Arg into the dlPAG. ΔSBP and ΔMAP only in L-Arg ($P < 0.05$) and SNP ($P < 0.001$ and $P < 0.01$, respectively) significantly increased compared to control over time. In all groups, ΔHR was not significant than control over time. No significance was found for microinjection with L-NAME when compared to control. Statistical analysis: Repeated measures ANOVA; $n = 5$. L-NAME: N^G-nitro-L-arginine methyl ester; a NOS inhibitor, L-Arg: L- Arginine, a NO precursor, SNP: sodium nitroprusside, a NO donor.

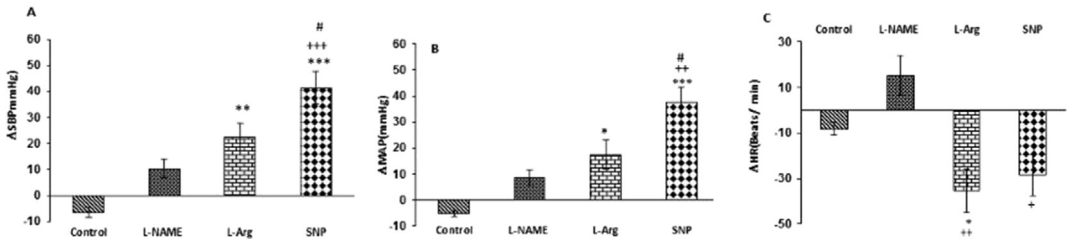

Figure 4: Peak changes in systolic blood pressure (ΔSBP) (A), mean arterial pressure (ΔMAP) (B), and heart rate (ΔHR) (C), after microinjection of L-NAME, SNP, and L-Arg into the dlPAG in separate groups. Statistical analysis: One-way ANOVA, followed by Tukey's post hoc test; n = 5. *: $P < 0.05$, **: $P < 0.01$, and ***: $P < 0.001$ (*vs.* control). +: $P < 0.05$, ++: $P < 0.01$, and +++: $P < 0.001$ (*vs.* L-NAME). #$P < 0.05$ (comparison of L-Arg and SNP, significant for ΔSBP and ΔMAP).

Discussion

The results showed that both SNP (a NO donor) and L-Arg (a NO precursor), when microinjected into the dlPAG, increased SBP and MAP, while L-NAME had no significant effect on these parameters. Only L-Arg had a significant effect on HR.

This study shows that NO in the dlPAG is involved in cardiovascular regulation, and this effect contrasts the cardiovascular effect of NO in other areas of the brain such as the RVLM, CnF, and PPT.[14–16] The effects of L-Arg confirmed the presence of NOS enzyme in the dlPAG, in accordance with previous studies.[23–25] In addition, the cardiovascular effects of both SNP and L-Arg showed the existence of NO receptors within the dlPAG in accordance with previous experiments.[10,26]

In this study, the microinjection of L-NAME did not significantly change the cardiovascular parameters. A possible explanation is that there is no substrate for the enzyme to utilise; therefore, it produces no effect when inhibited. Based on these results, we suggested that in anaesthetised rats, the substrate for NOS was not available in the dlPAG, and therefore NO was not synthesised.

The mechanism for the cardiovascular effect of NO in the dlPAG is unknown, and is suggested to be the result of projections from this area to various other regions involved in cardiovascular regulation. Direct projections from dlPAG to RVLM, an essential area for cardiovascular regulation, have not been reported, and indirect projections are possibly involved in this effect. The dlPAG has two principal projections to the CnF[27] and lateral parabrachial subnucleus (LPB).[28] A minor projection to the DMH nucleus of the hypothalamus also exists.[4,5] These connections project to the RVLM, and probably precipitate the cardiovascular control of dlPAG.[29] One important projection of dlPAG is to the CnF.[29] The CnF is a symphatoexcitatory area that, via its glutamatergic neurons, can evoke cardiovascular responses.[19] We suggest the presence of an excitatory projection from dlPAG to CnF, which, through NO, increases blood pressure.

Another projection of dlPAG is to the PB complex. This area comprises 13 subnuclei, including the lateral parabrachial nucleus (lPB),[30] to which the dlPAG exclusively projects.[28] Previous experiments have shown that a stimulation of the lPB by glutamate increases blood pressure.[31] We suggest that this pathway also precipitates in the cardiovascular effect of NO. The dlPAG also projects to the DMH, both directly and indirectly through the CnF[32] and superior lateral BP nucleus.[30] The DMH has connections to various other nuclei containing sympathetic outflow neurons, including the RVLM[33] and the raphe pallidus.[34] It has also been shown that the activation or disinhibition of the DMH provokes an excitatory cardiovascular response.[35]

In the dlPAG nucleus, NO is shown to act as an inhibitory neurotransmitter, acting on the GABAergic system via presynaptic sites, which facilitates the release of GABA and suppresses the neuronal activity within the nucleus.[29] However, our results showed that L-Arg and SNP increased blood pressure. The mechanism(s) of this opposite effect has not been determined. However, it has been reported that NO donors in the PAG could increase both excitatory and inhibitory synapses that could have a different effect on neuronal populations.[36] The dlPAG has many neurons including excitatory (glutamatergic neurons) and inhibitory (GABAergic neurons)[2,37]; therefore, the interaction of NO with these neuronal population may explain this contradictory effect.

The NOS has three isoforms (eNOS, iNOS, and nNOS), and the effects of each isoform are different. For example, in RVLM, NO synthesised by nNOS causes sympathoexcitation via glutamate receptors, and NO driven by iNOS have been shown sympathoinhibition via GABA receptors.[38] We suggest that the dlPAG also has different isoforms of NOS, and its excitatory effect is higher than the inhibitory effect. In support of this opinion, the dlPAG is involved in autonomic responses during stress, during which, the expression of nNOS in dlPAG is increased.[39] Based on these results, the increase in nNOS induced by L-Arg and SNP could increase blood pressure. However, further studies are needed to confirm this hypothesis.

In addition, our results indicated that L-NAME slightly increased HR; however, SNP and L-Arg decreased HR. This bradycardia may be mediated by baroreflex activity. In our results, bradycardia after microinjection of L-Arg was higher than that after SNP microinjection, while the effect of SNP on blood pressure was higher than that of L-Arg. We suggest that NO within the dlPAG may attenuate baroreflex bradycardia in response to elevated blood pressure. Moreover, since SNP induced a stronger response, baroreflex was highly inhibited, which led to a reduction in the heart rate. This effect is possibly due to the NTS, by way of the CnF nucleus.[40] The dlPAG is a critical area involved in autonomic

responses in stress and defence responses. These responses are mediated by the interaction of several neurotransmitters. Therefore, the effect of NO alone may differ when several neurotransmitters interact with each other. Thus, we propose that future studies focus on the interaction of NO with other neurotransmitters.

An important strength of this study is the investigation of all aspects of NO in the nucleus. There are some limitations to this current study. First, we did not investigate the baroreflex sensitivity. Second, we did not perform bilateral microinjection due to stereotaxic limitations. Third, we did not focus on other neurotransmitters and their roles in cardiovascular responses. We hope that future studies in this area address these limitations.

Conclusion

In summary, direct microinjection SNP and L-Arg significantly increased MAP and SBP blood pressure, while L-NAME had no significant effect on any recorded parameter. In addition, HR was decreased by SNP and L-Arg microinjection, and this effect was only significant in the L-Arg group.

The current results indicate that NO synthesis in the dlPAG produces pressor responses and attenuates baroreflex bradycardia, but its pressor effect is more significant.

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Conflict of interest

The authors have no conflict of interest to declare.

Ethical approval

All experimental procedures were approved by the ethical committee of Mashhad University of Medical Sciences (IR.MUMS.MEDICAL.REC.1398.248, dated 09 April 2019).

Authors contributions

MNS and RM conceived and designed the study, conducted experiments, provided research materials, and collected and organised the data. RM and RNSA analysed and interpreted the data. ASZ and RNSA wrote the initial and final draft of the article and provided logistic support. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

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