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Involvement of N-methyl-D-aspartate glutamate receptor and nitric oxide in cardiovascular responses to dynamic exercise in rats

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ABSTRACT

Dynamic exercise evokes sustained cardiovascular responses, which are characterized by arterial pressure and heart rate increases. Although it is well accepted that there is central nervous system mediation of cardiovascular adjustments during exercise, information on the role of neural pathways and signaling mechanisms is limited. It has been reported that glutamate, by acting on NMDA receptors, evokes the release of nitric oxide through activation of neuronal nitric oxide synthase (nNOS) in the brain. In the present study, we tested the hypothesis that NMDA receptors and nNOS are involved in cardiovascular responses evoked by an acute bout of exercise on a rodent treadmill. Moreover, we investigated possible central sites mediating control of responses to exercise through the NMDA receptor–nitric oxide pathway. Intraperitoneal administration of the selective NMDA glutamate receptor antagonist dizocilpine maleate (MK-801) reduced both the arterial pressure and heart rate increase evoked by dynamic exercise. Intraperitoneal treatment with the preferential nNOS inhibitor 7-nitroindazole reduced exercise-evoked tachycardiac response without affecting the pressor response. Moreover, treadmill running increased NO formation in the medial prefrontal cortex (MPFC), bed nucleus of the stria teminalis (BNST) and periaqueductal gray (PAG), and this effect was inhibited by systemic pretreatment with MK-801. Our findings demonstrate that NMDA receptors and nNOS mediate the tachycardiac response to dynamic exercise, possibly through an NMDA receptor–NO signaling mechanism. However, NMDA receptors, but not nNOS, mediate the exercise-evoked pressor response. The present results also provide evidence that MPFC, BNST and PAG may modulate physiological adjustments during dynamic exercise through NMDA receptor–NO signaling.

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1. Introduction

Nitric oxide (NO) is a small, relatively unstable molecule that modulates many aspects of physiological functions (Bredt, 1999). It is synthesized from L-arginine by three cell-specific nitric oxide synthase (NOS) isoforms: neuronal nitric oxide synthase (nNOS) and endothelial nitric oxide synthase (eNOS) are constitutively expressed enzymes, whose activities are stimulated by increase in intracellular Ca^{+2}, whereas inducible nitric oxide synthase (iNOS) is calcium-independent and mediates immune functions for NO (Alderton et al., 2001). The NO acts in the central nervous system (CNS) as a signaling molecule and has been considered an atypical neurotransmitter (Prast and Philippu, 2001; Garthwaite, 2008; Zhou and Zhu, 2009). Garthwaite et al. (1988) demonstrated that activation of NMDA receptors present in postsynaptic neurons in the CNS results in the formation of NO, through a mechanism dependent on Ca^{+2}. Therefore, it has been proposed that the nNOS enzyme in the brain is activated in response to Ca^{+2} influx following the activation of NMDA receptors by glutamate (Garthwaite, 2008; Zhou and Zhu, 2009).

It has been demonstrated that dynamic exercise activates numerous supra-medullary structures involved in control of cardiovascular function, such as the medial prefrontal cortex (MPFC), bed nucleus of the stria terminalis (BNST), paraventricular nucleus of the hypothalamus (PVN) and periaqueductal gray (PAG) (Timofeeva et al., 2003). Moreover, functional results have supported a role of these structures in cardiovascular adjustments to

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exercise (Li, 2004; Crestani et al., 2010; Alves et al., 2011; Mastelari et al., 2011). NMDA glutamate receptor and nNOS interaction is an important signaling mechanism involved in control of autonomic and cardiovascular function in several CNS regions (Wu et al., 2000; Li et al., 2001; Ryu et al., 2001; Resstel and Correa, 2006; Karlsson et al., 2007; Tavares et al., 2007; Alves et al., 2009; Busnardo et al., 2010). Furthermore, acute exercise by contractions of the tibialis anterior muscle increased nitrite, a stable metabolite of NO, in the brain (Paula et al., 2005). Although there are the above cited results, the involvement of NMDA glutamate receptors and nNOS mechanisms in cardiovascular adjustment during dynamic exercise has never been investigated.

Therefore, in the present study we tested the hypothesis that NMDA glutamate receptors and the nNOS enzyme are involved in cardiovascular responses observed during dynamic exercise. To test this hypothesis, we investigated the effect of treatment with a selective NMDA glutamate receptor antagonist or a preferential nNOS inhibitor on both pressor and tachycardiac responses elicited by an acute bout of exercise on a rodent treadmill. Moreover, in order to investigate possible central sites mediating control of cardiovascular responses to dynamic exercise through NMDA receptor–NO signaling mechanisms, we evaluated the effect of an acute bout of exercise on NO formation in supra-medullary structures involved in cardiovascular control. A possible involvement of the activation of NMDA glutamate receptors in central NO production was also studied.

2. Materials and methods

2.1. Animals

Eighty-five male Wistar rats weighing 190–210 g at the beginning of the experiments were used in the present experiments. Animals were housed in plastic cages in a temperature-controlled room (24 ± 1 °C) under standard laboratory conditions with free access to food and water and a 12 h light/dark cycle (lights on at 06:00 am). Procedures were conducted in conformity with the Brazilian Society of Neuroscience and Behavior guidelines for the care and use of laboratory animals, which are in compliance with international laws and politics. The Institution's Animal Ethics Committee approved the housing conditions and experimental procedures.

2.2. Surgical preparation

One day before the trial (De Angelis et al., 2006; Higa-Taniguchi et al., 2009; Alves et al., 2011), rats were anesthetized with tribromoethanol (250 mg/kg, i.p.) and a catheter was inserted into the abdominal aorta through the femoral artery for cardiovascular recording. Catheters consisted of a 4 cm piece of PE-10 heat-bound to a 13 cm piece of PE-50 (Clay Adams, Parsippany, New Jersey, USA) and were tunneled under the skin and exteriorized on the animal's dorsum. After surgery, the animals received a poly-antibiotic (Pentabiotico®, Fort Dodge, Cotia, SP, Brazil), with streptomycins and penicillins, to prevent infection and the non-steroidal anti-inflammatory flunixine meglumine (Banamine®, Schering Plough, Campinas, SP, Brazil) for post-operation analgesia.

2.3. Measurement of cardiovascular responses

The arterial cannula was connected to a pressure transducer (DPT100, Utah Medical Products Inc., Midvale, UT, USA) and the pulsate arterial pressure was recorded using an amplifier (Quad Bridge Amp, ML224, ADInstruments, NSW, Australia) and an acquisition board (PowerLab 4/30, ML866/P, ADInstruments, NSW, Australia) connected to a personal computer. Both mean arterial pressure (MAP) and HR values were derived from pulsate arterial pressure recordings.

2.4. Measurement of nitrogen oxides (NO_x)

Immediately after an acute bout of exercise on the rodent treadmill (0.8 km/h for 6 min), rats were decapitated and the brains immediately removed. The medial prefrontal cortex (MPFC), bed nucleus of the stria terminalis (BNST), paraventricular nucleus of the hypothalamus (PVN), dorsal and ventral hippocampus and periaqueductal gray (PAG) were bilaterally dissected, homogenized in ice-cold lysis buffer (Tris–HCl 20 mM pH 7.6, glycerol 10%, NaCl 137 mM) and stored at −80 °C. The nitrogen oxide (NO_x) assay was adapted from that described previously (Bories and Bories, 1995). Briefly, before the NO_x assay, homogenates were centrifuged at 20,000*g* for 10 min at 4 °C. After centrifugation, the supernatant was removed and incubated overnight with 0.5 mg/ml β-NADPH (Sigma-Aldrich, St. Louis, MO, USA) and 0.2 U/ml nitrate reductase (Sigma-Aldrich) in KH_2PO_4 buffer (0.2 M, pH 7.6) at 37 °C for reduction of nitrate to nitrite. Nitrite level was determined by adding the Griess reagent (N-(1-naphthyl)ethylenediamine dihydrochloride and sulfanilic acid) (Molecular Probes, Eugene, OR, USA) to the samples, according to the manufacturer's instructions. After 10 min of incubation at room temperature, the absorbance at 540 nm was determined and nitrite concentrations were calculated from the sodium nitrite (Sigma-Aldrich, St. Louis, MO, USA) standard curve. The content of protein in individual samples was measured using the Bradford reagent (Sigma-Aldrich, St. Louis, MO, USA) using serum albumin as standard (Biorad, Wien, Austria). All measurements were performed in triplicate and results were expressed as μM NO_x/μg protein.

2.5. Drugs

Dizocilpine maleate (MK-801) (Sigma-Aldrich, St. Louis, MO, USA), a selective and non-competitive NMDA receptor antagonist, and the anesthetic tribromoethanol (Sigma-Aldrich) were dissolved in saline (0.9% NaCl). The preferential nNOS inhibitor 7-nitroindazole (7-NI) (Sigma-Aldrich) was dissolved in dimethyl sulfoxide (DMSO). Flunixine meglumine (Banamine®, Schering Plough, Campinas, SP, Brazil) and a poly-antibiotic preparation of streptomycins and penicillins (Pentabiotico®, Fort Dodge, Cotia, SP, Brazil) were used as provided.

2.6. Experimental protocols

Before surgical preparation all animals were familiarized with exercise on the rodent treadmill (Insight, Ribeirão Preto, SP, Brazil) for at least one week. During the familiarization period, animals ran daily on the treadmill at a speed of 0.3–0.8 km/h and 0% grade for 10 min. No electrical stimulation was used to induce them to run.

On the trial day, animals were brought to the experimental room in their home cages. Animals were allowed one hour to adapt to the conditions of the experimental room, such as sound and illumination, before starting experiments. The experimental room had controlled temperature (24 °C) and was acoustically isolated from the main laboratory.

2.6.1. Effect of treatment with the selective NMDA receptor antagonist MK-801 or the preferential nNOS inhibitor 7-NI on cardiovascular changes induced by dynamic exercise

Rats received an intraperitoneal injection of the selective and non-competitive NMDA receptor antagonist MK-801 (0.03, 0.1 or 0.3 mg/kg), the preferential nNOS inhibitor 7-nitroindazole (7-NI) (15, 30 or 45 mg/kg) or the respective vehicles. The solutions were administrated in a volume of 1 ml/kg. Thirty minutes after treatment, the animals were submitted to an acute exercise test on the rodent treadmill. The test consisted of exercise at 0.8 km/h for 6 min (Higa-Taniguchi et al., 2009; Alves et al., 2011), which corresponds to about 70% of the rats' maximum running capacity on the treadmill. Each animal received only one injection. An untreated group, which did not receive any treatment, was also included in the study.

2.6.2. Effect of dynamic exercise and/or treatment with the selective NMDA receptor antagonist MK-801 on NO_x levels in the central nervous system

A different set of animals was used in this protocol. Animals were divided into four groups: (1) vehicle rest (saline, $n=7$); (2) MK-801 rest (0.1 mg/kg, $n=7$); (3) vehicle exercise ($n=7$); and MK-801 exercise ($n=7$). The solutions were administrated intraperitoneally in a volume of 1 ml/kg. Dose of MK-801 was based on the results obtained in experiment 1. Thirty minutes after treatment, the animals in groups "vehicle exercise" and "MK-801 exercise" were submitted to an acute exercise test on the rodent treadmill (0.8 km/h for 6 min). The animals in groups "vehicle rest" and "MK-801 rest" were placed on the treadmill without being submitted to exercise. Immediately after the acute bout of exercise, rats were decapitated and the brains immediately removed and dissected for NO_x assay.

2.7. Data analysis

The results were expressed as mean ± S.E.M. Basal values of MAP and HR were compared using one-way ANOVA. Time-course curves of MAP and HR changes were compared using two-way ANOVA with treatment as independent factor and time as repeated measurement. The effect of dynamic exercise on NO_x levels was evaluated using two-way ANOVA with treatment and exercise as independent factors. When interactions between the factors were observed, one-way ANOVA followed by Bonferroni's *post-hoc* test was used to compare the effect of the treatments on the exercise-evoked MAP, HR and NO_x level changes. Moreover, nonlinear regression analysis was performed to investigate the dose–effect relationship of treatment with crescents doses of MK-801 and 7-NI on cardiovascular responses to dynamic exercise. Differences were considered significant at $P<0.05$ level.

3. Results

3.1. Effect of treatment with the selective NMDA receptor antagonist MK-801 or the preferential nNOS inhibitor 7-NI on cardiovascular changes induced by dynamic exercise

Vehicles—Systemic administration of either saline or DMSO did not affect basal values of either MAP or HR, when compared with the untreated group (Table 1). Exercise on the treadmill caused a marked and sustained increase of both MAP (time factor: $F_{(18,247)}=45$, $P<0.0001$) and HR (time factor: $F_{(18,247)}=160$, $P<0.0001$). Changes in MAP (treatment factor: $F_{(2,247)}=1$, $P>0.05$) and HR (treatment factor: $F_{(2,247)}=3$, $P>0.05$) induced by exercise in animals that received saline or DMSO were not significantly different from those of the untreated group.

Table 1
Basal values of mean arterial pressure (MAP) and heart rate (HR).

Group		MAP (mmHg)	HR (bpm)
Untreated	$n=5$	96 ± 2	366 ± 11
Saline	$n=6$	101 ± 3	371 ± 11
DMSO	$n=5$	102 ± 2	368 ± 7
		$F_{(2,15)}=1.5$, $P>0.05$	$F_{(2,15)}=0.1$, $P>0.05$
Vehicle (saline)	$n=6$	101 ± 3	371 ± 11
MK-801 0.03 mg/kg	$n=5$	102 ± 3	360 ± 8
MK-801 0.1 mg/kg	$n=5$	95 ± 2	398 ± 14
MK-801 0.3 mg/kg	$n=5$	106 ± 2	408 ± 14
		$F_{(3,20)}=1.8$, $P>0.05$	$F_{(3,20)}=2.8$, $P>0.05$
Vehicle (DMSO)	$n=5$	102 ± 2	368 ± 7
7-NI 15 mg/kg	$n=5$	101 ± 3	349 ± 12
7-NI 30 mg/kg	$n=5$	99 ± 2	363 ± 6
7-NI 45 mg/kg	$n=5$	99 ± 3	363 ± 9
		$F_{(3,19)}=0.3$, $P>0.05$	$F_{(3,19)}=0.6$, $P>0.05$

The values are means ± S.E.M., One-way ANOVA.

MK-801—Systemic administration of different doses (0.03, 0.1 or 0.3 mg/kg) of the selective and non-competitive NMDA receptor antagonist MK-801 did not affect either MAP or HR baseline (Table 1). Exercise on the treadmill caused a significant increase in MAP (time factor: $F_{(18,323)}=36$, $P<0.0001$) and HR (time factor: $F_{(18,323)}=78$, $P<0.0001$) (Fig. 1). Administration of MK-801 (0.1 and 0.3 mg/kg) reduced both the MAP (treatment factor: $F_{(3,323)}=66$, $P<0.0001$) and HR (treatment factor: $F_{(3,323)}=72$, $P<0.0001$) increase induced by dynamic exercise (Fig. 1). Moreover, there was a significant interaction between treatment and time for the MAP ($F_{(54,323)}=3$, $P<0.0001$) and HR ($F_{(54,323)}=3$, $P<0.0001$) responses. Nonlinear regression analysis revealed that MK-801 effects on exercise-evoked cardiovascular responses were dose-dependent, showing a significant association between drug doses and MAP ($df=13$, $r^2=0.62$, $P<0.05$) and HR ($df=13$, $r^2=0.59$, $P<0.05$) increase (Fig. 1). Representative experimental recordings showing effects of MK-801 treatment on cardiovascular responses induced by exercise on the rodent treadmill are presented in Fig. 2.

7-Nitroindazole—Systemic administration of different doses (15, 30 or 45 mg/kg) of the preferential nNOS inhibitor 7-NI did not affect either MAP or HR baseline (Table 1). Exercise on the treadmill caused a significant increase in both MAP (time factor: $F_{(18,304)}=72$, $P<0.0001$) and HR (time factor: $F_{(18,304)}=69$, $P<0.0001$) (Fig. 3). Treatment with 7-NI (30 and 45 mg/kg) reduced the exercise-evoked HR increase (treatment factor: $F_{(3,304)}=120$, $P<0.0001$) without significantly affecting the MAP response (treatment factor: $F_{(3,304)}=2$, $P>0.05$) (Fig. 3). Moreover, there was a significant interaction between treatment and time for the HR response (HR: $F_{(54,304)}=5$, $P<0.0001$ and MAP: $F_{(54,304)}=1$, $P>0.05$). Nonlinear regression analysis confirmed that 7-NI effects on exercise-evoked tachycardiac responses were dose-dependent, showing a significant association between drug doses and HR response (HR: $df=13$, $r^2=0.78$, $P<0.05$; MAP: $r^2=0$, $P>0.05$) (Fig. 3). Representative experimental recordings showing the effects of 7-NI treatment on cardiovascular responses induced by exercise on the treadmill are presented in Fig. 4.

3.2. Effect of the dynamic exercise and/or treatment with the selective NMDA receptor antagonist MK-801 on NO_x levels in the central nervous system

Medial prefrontal cortex—Dynamic exercise on the treadmill caused a significant increase in NO_x levels in the MPFC (exercise

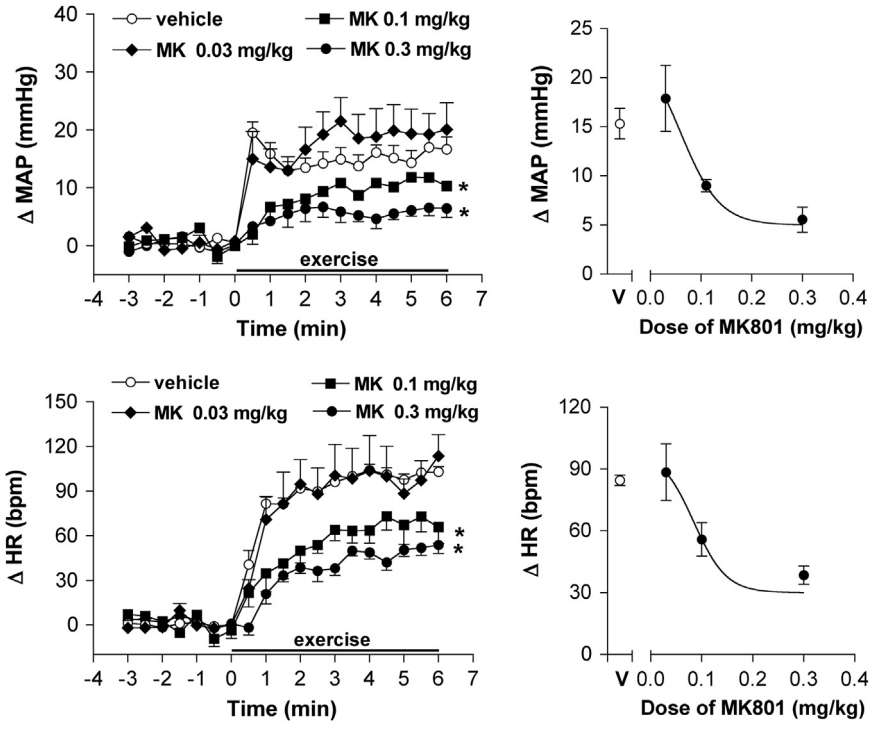

Fig. 1. (**Left**) Time-course of changes in mean arterial pressure (ΔMAP) and heart rate (ΔHR) during dynamic exercise on the treadmill (0.8 km/h for 6 min) in rats treated intraperitoneally with vehicle (saline, 1 ml/kg, $n=6$) or different doses (0.03, 0.1 and 0.3 mg/kg, $n=5$/group) of the selective and non-competitive NMDA receptor antagonist MK-801. The onset of exercise was at $t=0$. Circles represent the mean and bars the S.E.M. *$P<0.05$ indicates a significant difference over the whole exercise period compared to vehicle treated animals; ANOVA followed by Bonferroni's *post test*. (**Right**) Mean arterial pressure (ΔMAP) and heart rate (ΔHR) changes during exercise in rats treated with increasing doses of MK-801 (0.03, 0.1 and 0.3 mg/kg, $n=5$/group). V: vehicle (saline, 1 ml/kg). Dose–effect curves were generated by nonlinear regression analysis. Data shown represent the means ± S.E.M. of the variation of MAP and HR during the 6 min of exercise.

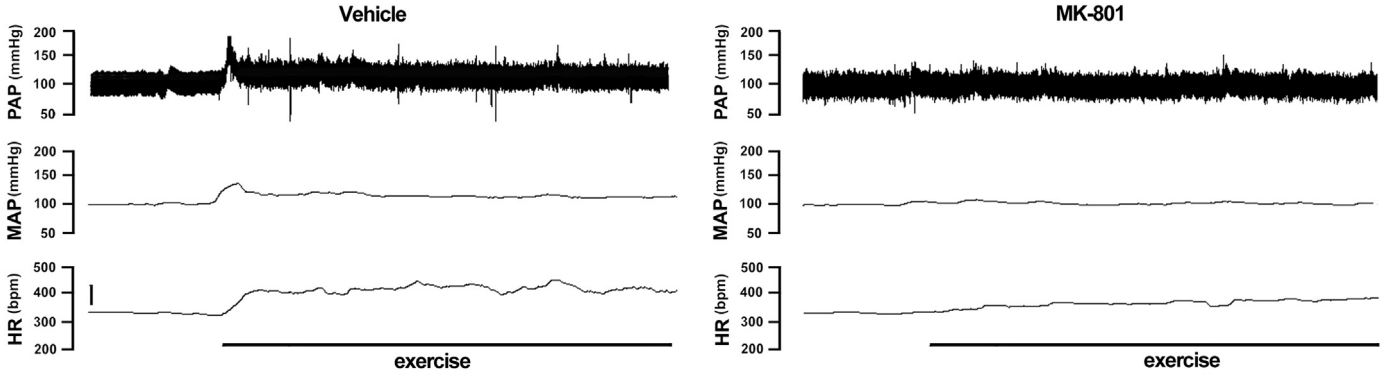

Fig. 2. Recording from individual representative animals illustrating changes in pulsatile arterial pressure (PAP), mean arterial pressure (MAP) and heart rate (HR) observed during dynamic exercise on the treadmill after intraperitoneal treatment with vehicle (saline, 1 ml/kg) or the selective and non-competitive NMDA receptor antagonist MK-801 (0.1 mg/kg). Note the decrease in both MAP and HR response to exercise in the animal that received MK-801.

factor: $F_{(1,24)}=9$, $P<0.008$) (Fig. 5). However, analysis did not indicate a significant effect of systemic treatment with MK-801 (treatment factor: $F_{(1,24)}=0.2$, $P>0.05$) (Fig. 5). Also, there was no interaction between treatment and exercise factors ($F_{(1,24)}=0.1$, $P>0.05$).

Bed nucleus of the stria terminalis—Dynamic exercise on the treadmill caused a significant increase in NO_x levels in the BNST (exercise factor: $F_{(1,24)}=8$, $P<0.01$) (Fig. 5). Analysis did not show a significant effect of treatment (treatment factor: $F_{(1,24)}=2$, $P>0.05$), but indicated interaction between treatment and exercise factors ($F_{(1,24)}=6$, $P<0.03$) (Fig. 5). *Post-hoc* analysis revealed that treatment with MK-801 inhibited the exercise-evoked increase in NO_x levels in the BNST ($P<0.05$).

Paraventricular nucleus of the hypothalamus—Analysis did not indicate a significant effect of either dynamic exercise (exercise factor: $F_{(1,24)}=2$, $P>0.05$) or treatment with MK-801 (treatment factor: $F_{(1,24)}=1$, $P>0.05$) on NO_x levels in the PVN (Fig. 5).

Dorsal and ventral hippocampus—Analysis did not indicate a significant effect of either dynamic exercise (exercise factor: $F_{(1,24)}=0.1$, $P>0.05$) or treatment with MK-801 (treatment factor: $F_{(1,24)}=0.6$, $P>0.05$) on NO_x levels in the dorsal hippocampus (DH) (Fig. 5). Analysis also did not indicate a significant effect of either dynamic exercise on the treadmill (exercise factor: $F_{(1,24)}=0.3$, $P>0.05$) or treatment with MK-801 (treatment factor: $F_{(1,24)}=3$, $P>0.05$) on NO_x levels in the ventral hippocampus (VH) (Fig. 5).

Periaqueductal gray—Dynamic exercise on the treadmill caused a significant increase in NO_x levels in the periaqueductal gray (PAG) (exercise factor: $F_{(1,24)}=12$, $P<0.002$) (Fig. 5). Analysis also indicated a significant effect of treatment with MK-801

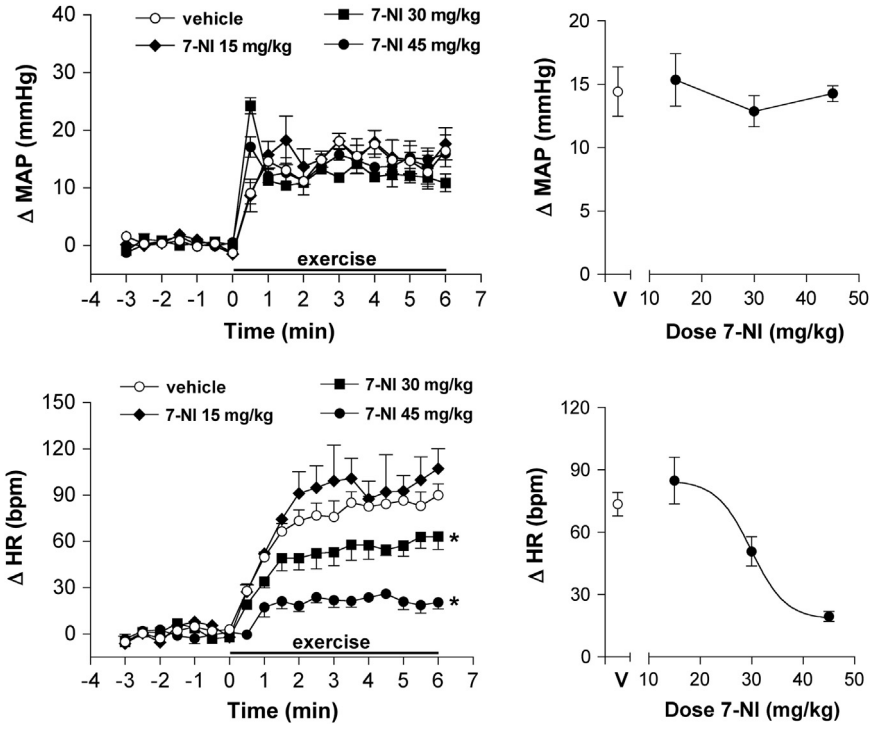

Fig. 3. (**Left**) Time-course of changes in mean arterial pressure (ΔMAP) and heart rate (ΔHR) during dynamic exercise on the treadmill (0.8 km/h for 6 min) in rats treated intraperitoneally with vehicle (DMSO, 1 ml/kg, $n=5$) or different doses (15, 30 and 45 mg/kg, $n=5$/group) of the preferential nNOS inhibitor 7-nitroindazole (7-NI). The onset of exercise was at $t=0$. Circles represent the mean and bars the S.E.M. *$P<0.05$ indicates a significant difference over the whole exercise period compared to vehicle treated animals; ANOVA followed by Bonferroni's *post test*. (**Right**) Mean arterial pressure (ΔMAP) and heart rate (ΔHR) changes during dynamic exercise in rats treated with increasing doses of 7-NI (15, 30 and 45 mg/kg, $n=5$/group). V: vehicle (saline, 1 ml/kg). Dose–effect curves were generated by nonlinear regression analysis. Data shown represent the means ± S.E.M. of the variation of MAP and HR during the 6 min of exercise.

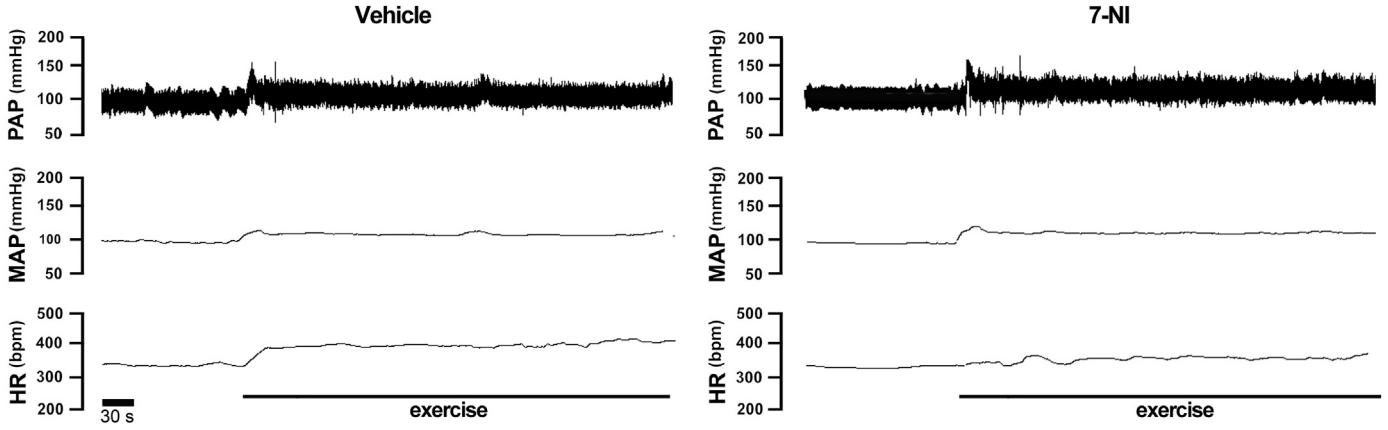

Fig. 4. Recording from individual representative animals illustrating changes in pulsatile arterial pressure (PAP), mean arterial pressure (MAP) and heart rate (HR) observed during dynamic exercise on the treadmill after intraperitoneal treatment with vehicle (DMSO, 1 ml/kg) or the preferential nNOS inhibitor 7-nitroindazole (7-NI) (30 mg/kg). Note the decrease in the HR response to exercise in the animal that received 7-NI.

(treatment factor: $F_{(1,24)}=11$, $P<0.004$) and interaction between treatment and exercise ($F_{(1,24)}=12$, $P<0.003$). *Post-hoc* analysis revealed that treatment with MK-801 inhibited the exercise-evoked increase of NO_x levels in the PAG ($P<0.001$) (Fig. 5).

4. Discussion

Our findings provide the first direct evidence for the involvement of NMDA glutamate receptors and nNOS in cardiovascular adjustments during dynamic exercise. We observed that treatment with the selective and non-competitive NMDA receptor antagonist MK-801 reduced both the MAP and HR increase induced by exercise in a dose-dependent manner. Treatment with the preferential nNOS inhibitor 7-NI dose-dependently decreased HR response to exercise without affecting pressor response. Moreover, treadmill running increased NO_x levels in supra-medullary structures involved in control of cardiovascular function (MPFC, BNST and PAG), and this effect was inhibited after blockade of NMDA glutamate receptors.

The nNOS isoform was first found in neurons (Bredt et al., 1990). However, further studies have also identified neuronal synthesis of NO in skeletal, cardiac and smooth muscles, lung epithelial cells and skin (Asano et al., 1994; Kobzik et al., 1994; Schwarz et al., 1999; Xu et al., 1999; Guix et al., 2005). An important role has been described for nNOS-derived NO in regulation of kidneys, adrenal, coronary and liver blood flow at rest (Ichihara et al., 1998; Wakefield et al., 2003; Seddon et al.,

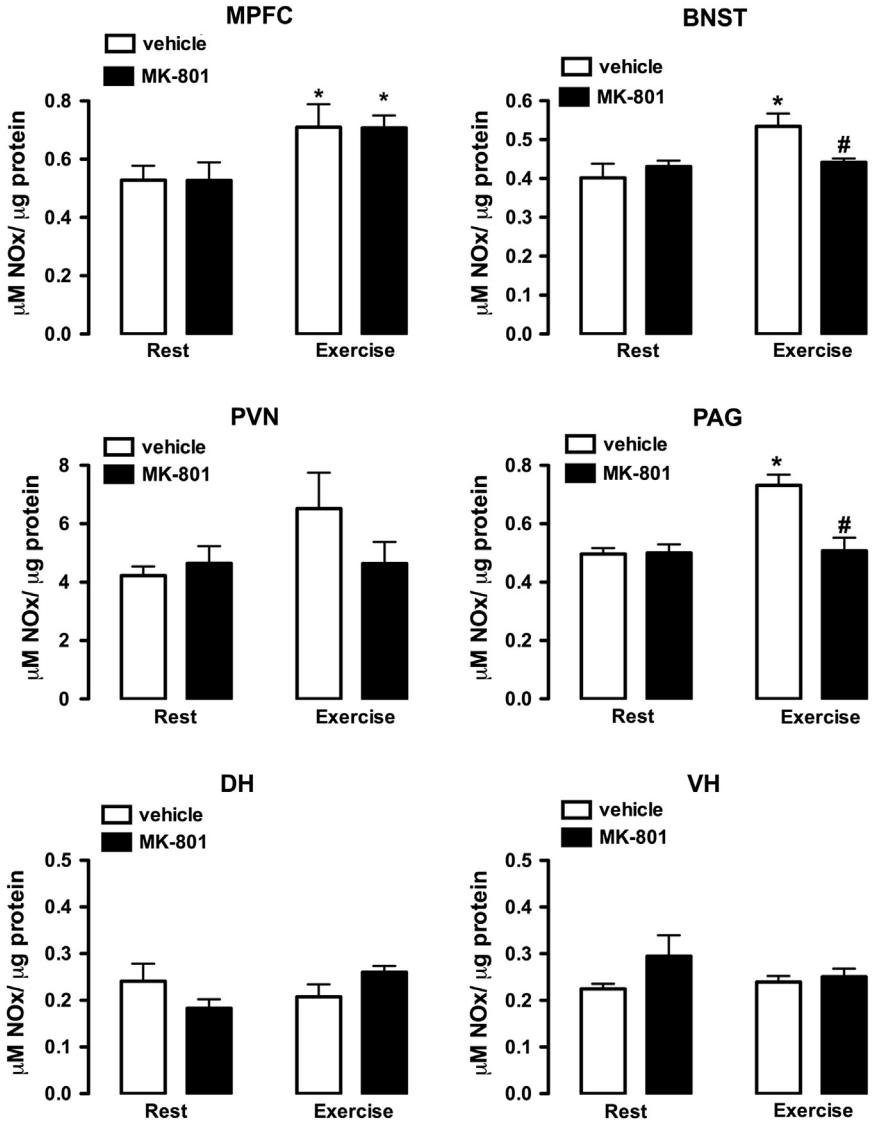

Fig. 5. Effect of dynamic exercise on the treadmill (0.8 km/h for 6 min) on NO_x levels in the medial prefrontal cortex (MPFC), bed nucleus of the stria terminalis (BNST), paraventricular nucleus of the hypothalamus (PVN), ventral (VH) and dorsal (DH) hippocampus and periaqueductal gray (PAG) in animals treated intraperitoneally with vehicle (saline, 1 ml/kg) or the selective and non-competitive NMDA receptor antagonist MK-801 (0.1 mg/kg). Columns represent the mean and bars the S.E.M. $^*P < 0.05$ compared with the same treatment at rest, $\#P < 0.05$ compared with the vehicle group under the same conditions; ANOVA followed by Bonferroni's *post test*.

2009; Copp et al., 2010). Moreover, nNOS seems to be an important regulator of visceral and muscle blood flow during exercise (Lai et al., 2009; Copp et al., 2010). Redistribution of blood flow is an important mechanism involved in the arterial pressure response to exercise (Waldrop et al., 1996). However, blockade of nNOS by systemic treatment with 7-NI reduced the HR increase from exercise without affecting the pressor response. The increase of HR during exercise is mediated by neural mechanisms (*i.e.*, changes in autonomic activity) (Goldsmith et al., 2000). Therefore, our findings suggest that changes in cardiac response to exercise reported in the present study are mainly due to a central blockade of nNOS in the brain. Moreover, our results are similar to those demonstrating that a reduction in HR responses does not affect the exercise-induced arterial pressure increase (Overton, 1993).

A recent study in rats showed that systemic treatment with the selective nNOS inhibitor S-metil-L-tiocitruline (SMLT) did not affect either arterial pressure, HR or hyperemic responses to dynamic exercise on a treadmill (Copp et al., 2010). The reasons for the discrepancy with our findings are not clear. *In vitro* studies have demonstrated that the selectivity of SMLT for nNOS is higher than that of 7-NI (Furfine et al., 1994; Alderton et al., 2001). However, it has been proposed that the selectivity of NOS inhibitors *in vivo* may be influenced by other factors, such as a cell-type specificity effect (e.g., neuronal *vs* vascular) and metabolism (Alderton et al., 2001). Several studies have demonstrated that systemic administration of 7-NI inhibits nNOS activity in the CNS (Babbedge et al., 1993; MacKenzie et al., 1994; Ryu et al., 2001; Zhou et al., 2007). A preferential blockade of nNOS in the CNS has been further evidenced by the failure of 7-NI to increase arterial pressure (Babbedge et al., 1993; Moore et al., 1993), an effect thought to be mediated by inhibition of the vascular action of NO (Alderton et al., 2001). Moreover, 7-NI does not affect skin blood flow in rats (Kajekar et al., 1995). Most previous studies investigating the role of nNOS-derived NO in blood flow regulation have used SMLT (Ichihara et al., 1998; Wakefield et al., 2003; Seddon et al., 2008, 2009; Copp et al., 2010). Contrary to 7-NI, systemic administration of SMLT increases baseline arterial pressure (Wakefield et al., 2003; Patterson et al., 2008; Copp et al., 2010). These results suggest a reduced influence of 7-NI in the vascular action of NO, when compared to SMLT. Therefore, differences in nNOS inhibitor may explain the differences between our findings and those of Copp et al. (2010). Moreover, the above

evidence reinforces the idea that changes in exercise-evoked tachycardiac response following 7-NI administration are possibly mediated by blockade of nNOS in the brain.

The nNOS enzyme in the CNS is activated in response to Ca^{+2} influx following activation of NMDA receptors (Garthwaite, 2008; Zhou and Zhu, 2009). Despite reports of the expression of other NOS isoforms in the brain (Dinerman et al., 1994; Amitai, 2010), nNOS seems to be the major isoform involved in NO synthesis in the CNS (Garthwaite, 2008; Zhou and Zhu, 2009). Indeed, nitric oxide synthase catalytic activity in the brain of animals deficient for nNOS ($nNOS^{-/-}$) was reduced by more than 90% (Huang et al., 1993). Therefore, since both MK-801 and 7-NI reduced the tachycardiac response observed during exercise, our results suggest that the cardiac response during dynamic exercise is possibly mediated by a NMDA receptor–NO signaling mechanism. However, arterial pressure response to exercise seems to be mediated by NMDA receptors through a mechanism independent of nNOS activation.

NO is an unstable gas that undergoes various reactions in biological fluids resulting in its rapid degradation (Guix et al., 2005). Therefore, measurement of stable metabolites of NO, such as nitrite and nitrate, has been used to evaluate NOS catalytic activity and NO formation (Marzinzig et al., 1997). We observed in the present study that treadmill running increased NO_x levels in the MPFC, BNST and PAG. Unexpectedly, treadmill running did not significantly increase NO_x in the PVN. Previous results have supported a role for the PVN in cardiovascular adjustments associated with exercise (Jackson et al., 2005; de Abreu et al., 2009; Stern et al., 2012). Moreover, some pieces of evidence have suggested that nitrergic mechanisms within the PVN are associated with cardiovascular adaptations to exercise training. For example, nonselective inhibition of NO synthesis within the PVN partly reverted adaptation in HR variability (increase in high-frequency oscillations and decrease in low-frequency oscillations) induced by exercise training (Mastelari et al., 2011). Moreover, exercise training restored the altered control of autonomic and cardiovascular functions by nitrergic mechanisms within the PVN in heart failure rats (Zheng et al., 2005). Therefore, we cannot exclude the possibility that PVN control of responses to dynamic exercise is mediated by local formation of NO. The reasons for the absence of a NO_x increase in the PVN are not clear. A possible explanation could be differences in NO inactivation in different brain regions. Indeed, the brain has a very active mechanism of NO inactivation (Hall and Garthwaite, 2006), but it has been proposed that its activity may vary between brain regions (Garthwaite, 2008). Moreover, we cannot exclude the possibility that exercise intensity or duration was insufficient.

Interestingly, pretreatment with MK-801 inhibited exercise-evoked increase in NO_x levels in the BNST and PAG, thus suggesting an important role of NMDA receptor activation in exercise-evoked formation of NO in these structures. However, the increase in NO_x level in the MPFC was not affected by systemic treatment with MK-801. Three mechanisms may explain the findings in the MPFC. Firstly, nNOS activation in the MPFC could be mediated by a mechanism independent of NMDA receptor activation. In addition to regulation by the Ca^{+2}/calmodullin complex, the main known mechanism in which Ca^{+2} influx following activation of NMDA receptors activates nNOS, some studies have demonstrated that nNOS possesses sites for phosphorylation and its activity may be modulated by the action of protein kinases (Garthwaite, 2008). Indeed, although controversial (Bredt et al., 1992), a moderate increase in enzyme activity was identified after phosphorylation by protein kinase C (Nakane et al., 1991). Furthermore, recent studies have demonstrated that nNOS can bind to serotonin transporters in the plasma membrane, which in turn increase the nNOS enzymatic activity through a mechanism independent of Ca^{+2} influx (Chanrion et al., 2007; Garthwaite, 2007). A second possibility is that a mechanism other than nNOS activation may be involved in exercise-induced NO formation in the MPFC. Although nNOS is the most expressed NOS isoform in the brain (Garthwaite, 2008), there are reports that neurons in the CNS also contain eNOS and iNOS (Dinerman et al., 1994; Amitai, 2010). Finally, we cannot exclude the possibility that access of MK-801 may differ between different sites in the CNS, and consequently the dose of the antagonist was ineffective in blocking the NMDA receptor in the MPFC. Therefore, further studies are necessary to clarify the mechanisms involved in NO formation in the MPFC during dynamic exercise. However, considered overall, the present results provide initial evidence that the MPFC, BNST and PAG may modulate responses to exercise through NMDA receptor and NO signaling mechanisms. However, since the NMDA receptor–NO pathway is an important signaling mechanism in other medullary and supra-medullary structures involved in cardiovascular control (Martins-Pinge et al., 2007; Lin, 2009), effects observed after systemic pharmacological treatment may also be mediated by action on other sites. Therefore, further studies are necessary to clarify CNS sites controlling cardiovascular responses to dynamic exercise through NMDA receptor and NO signaling.

Both the sympathetic and the parasympathetic branches of the autonomic nervous system are involved in the control of cardiovascular activity during exercise. Blockade of cardiac parasympathetic activity reveals that most of the initial cardiac response to exercise is attributable to the withdrawal of tonic vagal activity, whereas β-adrenergic blockade reveals the importance of augmented cardiac sympathetic activity during exercise (Overton, 1993; Goldsmith et al., 2000). Moreover, sympathetic-mediated vasoconstriction diverts blood away from the kidneys and splanchnic beds to active muscles, and is an important mechanism involved in arterial pressure increase during exercise (O'Hagan et al., 1993; Waldrop et al., 1996). Interaction between NMDA receptors and nNOS is involved in tonic control of sympathetic and parasympathetic activity in medullary and supra-medullary structures (Martins-Pinge et al., 2007; Lin, 2009; Busnardo et al., 2010). Therefore, it is possible that NMDA receptors and nNOS could modulate the cardiac response during exercise by inhibiting vagal neurons and/or by stimulating sympathetic neurons. Also, facilitation of sympathetic neurons controlling vascular activity may mediate NMDA receptor control of arterial pressure responses during exercise.

In summary, our results suggest that NMDA receptors and nNOS mediate the tachycardiac response to dynamic exercise, possibly through a NMDA receptor–NO signaling mechanism. On the other hand, NMDA receptors, but not nNOS, mediate the exercise-evoked pressor response. Present results also provide initial evidence that MPFC, BNST and PAG may modulate responses to exercise through NMDA receptor–NO signaling.

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