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Peripheral pre-synaptic pathway reduces the heart rate response to sympathetic activation following exercise training: role of NO

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Abstract

Objectives: We tested the hypothesis that the attenuated heart rate (HR) response to sympathetic activation following swim training in the guinea pig (*Cavia porcellus*) results from a peripheral modulation of pacemaking by nitric oxide (NO). **Methods:** Nitric oxide synthase (NOS) inhibition on the increase in heart rate with sympathetic nerve stimulation (SNS) was investigated in the isolated guinea pig double atrial/right stellate ganglion preparation from exercise trained (6-weeks swimming, $n=20$) and sedentary animals ($n=20$). Western blot analysis for neuronal nitric oxide synthase (nNOS) was performed on the stellate ganglion from both groups. **Results:** Relative to the control group, the exercise group demonstrated typical exercise adaptations of increased ventricular weight/body weight ratio, enhanced skeletal muscle citrate synthase activity and higher concentrations of [^{3}H]ouabain binding sites in both skeletal and cardiac tissue ($P<0.05$). The increase in heart rate (bpm) with SNS significantly decreased in the exercise group ($n=16$) compared to the sedentary group ($n=16$) from 30±5 to 17±3 bpm at 1 Hz; 67±7 to 47±4 bpm at 3 Hz; 85±9 to 63±4 bpm at 5 Hz and 101±9 to 78±5 bpm at 7 Hz stimulation ($P<0.05$). The increase in heart rate with cumulative doses (0.1–10 μM) or a single dose (0.1 μM) of bath-applied norepinephrine expressed as the effective doses at which the HR response was 50% of the maximum response (EC_{50}) were similar in both exercise (EC_{50} −6.08±0.16 M, $n=8$) and sedentary groups (EC_{50} −6.18±0.07 M, $n=7$). Trained animals had significantly more nNOS protein in left stellate ganglion compared to the sedentary group. In the exercise group, the non-isoform selective NOS inhibitor, *N*-ω nitro-L-arginine (L-NA,100 μM) caused a small but significant increase in the heart rate response to SNS. However, the positive chronotropic response to sympathetic nerve stimulation remained significantly attenuated in the exercise group compared to the sedentary group during NOS inhibition ($P<0.05$). **Conclusions:** Our results indicate that there is a significant peripheral pre-synaptic component reducing the HR response to sympathetic activation following training, although NO does not play a dominant role in this response.

Keywords: Autonomic nervous system; Heart rate (variability); Nitric oxide

1. Introduction

Aerobic exercise training reduces the heart rate response to sub-maximal work rates [2,9,28]. Whether this occurs via a peripheral modulation of sympathetic activity, a reduction in cardiac β-adrenoceptor density and agonist affinity or a reduction in central sympathetic outflow is unclear [11,33]. Exercise training is associated with enhanced endothelial NO synthase (eNOS) gene expression [31,42] and endothelial production of NO in coronary microvessels and skeletal muscle arteries [16,22]. There is also considerable evidence indicating that NO exerts an inhibitory effect on the peripheral sympathetic control of cardiac function. Inhibition of endogenous NO production with non-isoform specific and neuronal NOS (nNOS) inhibitors increases both the release of norepinephrine

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(NE) during cardiac sympathetic nerve stimulation (SNS) in the isolated rat heart [29] and the heart rate response to SNS in-vivo [12,30]. These findings suggest that enhanced NO production could contribute to the reduction in cardiac sympathetic activity following exercise training.

Therefore, the aims of this study were to investigate whether exercise training modulates the peripheral sympathetic control of heart rate in the guinea-pig atria and whether NO affects the HR response to peripheral sympathetic activation following training. Some of these findings have been presented in abstract form [5].

2. Methods

2.1. Animal care

The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985) and was performed in accordance with Home Office license requirements (PPL 30/1133, Queen Anne's Gate, London, UK) and the Animals (Scientific Procedures) Act 1986 (UK). Forty male guinea pigs (Dunkin Hartley) initially weighing between 125 and 150 g were housed in a temperature controlled room (20±1°C) with a 12-h light/dark cycle. Chow and water were provided ad libitum. At least 3 days after arrival from the breeder, animals were randomly assigned into either an exercise ($n=20$) or sedentary ($n=20$) group.

2.2. Exercise training protocol

Animals from the exercise group spent at least 10–15 min over 2 days being familiarised to a swimming tank [50 cm (L)×30 cm (W)×30 cm (H)] maintained at a constant water temperature of 37±0.1°C (Grant Instruments Water Heater, Cambridge, UK). The animals from the exercise group were manually supported below their thorax, placed in the water tank and allowed to swim (in groups of 2). Daily training periods ranged from 60 to 90 min/day for 6 weeks (weeks 1–2, 75 min/day for weeks 3–4, and 90 min/day for weeks 5–6). Animals from the sedentary group were placed simultaneously into a tank with shallow water (37±0.1°C) for the same time and duration as the training period. All animals were weighed on training days prior to the experimental period.

After the 6-week training period, exercised and sedentary animals were killed by cervical dislocation followed by exsanguination. The thorax and mediastinum were rapidly removed and placed in Tyrode's solution aerated with 95% O_2/5% CO_2. The left stellate ganglion and samples from the left ventricle and right latissimus dorsi (primary swimming muscle) were dissected free and frozen at −70°C.

2.3. Indices of exercise training

Oxidative enzyme activity [2,35] and skeletal muscle Na^+/K^+ pump concentration [8,20] are increased with physical training, therefore these were chosen as markers of a training effect.

2.3.1. Citrate synthase activity

Latissimus dorsi and ventricular tissue samples were analysed for mitochondrial citrate synthase from whole muscle homogenates using the methodology previously described [1,32] to assess the change in muscle oxidative capacity following training. The assay medium contained 100 mM Tris/HCl, 0.4 mM 5,5-dithiobos-(2-nitrobenzoic acid) and 2 mM acetyl-CoA to which 100 μl of muscle homogenate was added. The assay was initiated by addition of oxaloacetate (4 mM, pH=7). The final volume of the cuvette was 2 ml. Citrate synthase was assayed by following the rate of change of E_{412} in a spectrophotometer at 25°C. All activities are expressed as mmol of substrate utilised per minute gramme of fresh weight of muscle.

2.3.2. [^{3}H]Ouabain binding in vitro

Samples of the left ventricle and, latissimus dorsi were analysed for [^{3}H]ouabain binding site concentrations using the vanadate-facilitated binding methodology [24]. This procedure quantifies all functional Na^+/K^+ pumps in skeletal and cardiac muscle [20], using a high affinity [^{3}H]ouabain binding assay. In the present study, specimens of 5 mg each were taken from each muscle for the determination of [^{3}H]ouabain binding site concentration. Based on the mean value of each measurement performed on the two muscles each from nine exercise and nine sedentary animals, the total concentrations of [^{3}H]ouabain binding sites were expressed in pmol per gramme of wet weight of tissue.

2.4. Guinea-pig double atrial/right stellate ganglion preparation

Immediately after removal of the mediastinum and thorax, the ventricles were perfused with heparinised Tyrode's solution (heparin=1000 U ml^{-1}), excised and weighed. The atria and right stellate ganglion were dissected free and loose sutures (Ethicon 6/0 silk) were placed on the auricles of both atria. The preparation was then transferred to a preheated (37±0.1°C), water-jacketed bath containing 100 ml of oxygenated guinea-pig Tyrode solution. The atria were vertically mounted with the suture in the left atrium connected to a stainless steel hook, and the suture in the right atrium attached to an isometric force transducer (Harvard Apparatus, Model 60-2997, USA). The right stellate ganglion was placed through a pair of circular platinum ring electrodes connected to a stimulator. Data were collected on a Power Macintosh 7500 computer

(Apple Systems, USA) using a Biopac MP100 acquisition system and Acqknowledge software (Biopac Systems Inc., USA). Heart Rate (bpm) was triggered from the upstroke of each contraction, and signals were displayed in real time. Data were stored on CD Rom for offline analysis.

2.5. Solutions and drugs

The guinea-pig Tyrode's solution contained (mM) NaCl 120, KCl 4, $MgCl_2$ 2, NaH_2PO_4 0.1, $NaHCO_3$ 25, $CaCl_2$ 2, and glucose 11. It was aerated with carbogen (95%O_2 and 5%CO_2), adjusted to pH 7.4, and its temperature was maintained at 37±0.1°C (Grant Instruments Water Heater, Cambridge, UK). All pharmacological chemicals and biochemical assay reagents were obtained from Sigma Aldrich (UK). Norepinephrine and L-arginine were added from 1-mM stock solution, made up prior to the experiment using reagent grade water from an Elga water purification system. A stock solution (10 mM) of N^{ω}-nitro-L-arginine (L-NA) was made up in Tyrode's solution on the day of the experiment.

2.6. Experimental protocols

The atria were equilibrated in Tyrode's solution for 45–90 min until the heart rate did not alter by 10 bpm over a 20 min period. Following this equilibration period, the stellate ganglion was stimulated at 3 Hz, 10 V, 1 ms pulse width for 30 s at 2–3 min intervals. Experimental protocols commenced after three consistent positive chronotropic responses to ganglion stimulation were achieved. The change in heart rate with SNS was calculated by difference in 5-s averages in heart rate taken prior to the onset and cessation of a stimulation period.

2.6.1. The effect of exercise training on the increase in heart rate with SNS

The right stellate ganglion was stimulated at 1, 3, 5, 7 and 10 Hz (10 V, 1 ms pulse width, 30 s duration) to evaluate whether exercise training altered the heart rate response to SNS, in the isolated double atrial preparations from sedentary (n=16) and exercised (n=16) animals. A minimum time of 120 s was left between consecutive stimulations.

2.6.2. The effect of exercise training on the increase in heart rate with bath-applied norepinephrine

The increase in heart rate with bath-applied norepinephrine (cumulative addition; 0.1–10 μM) was measured in atria from exercised (n=8) and sedentary (n=7) animals to determine whether training altered the heart rate response to sympathetic stimulation via a pre- or post-synaptic mechanism. Following wash-out of the Tyrode's solution and a 10 min recovery period, the heart rate response to a single dose of norepinephrine (1 μM) was measured (exercised n=5, sedentary n=4). The response to the single dose of norepinephrine was later compared with the same concentration of norepinephrine in the dose response curve to enable assessment of the extent to which β-adrenoceptor desensitisation may have occurred during the cumulative addition of norepinephrine.

2.6.3. Role of NO in the positive chronotropic response to SNS in atria from sedentary and trained animals

We evaluated the effects of the NOS inhibitor, Nω-nitro-L-arginine (L-NA, 100 μM) on the increase in heart rate with SNS at 3 Hz (sedentary, n=14; exercised, n=13) and 5 Hz (sedentary n=15; exercised, n=13). This single drug concentration (100 μM L-NA) has been shown to enhance evoked norepinephrine release [30] and the heart rate response to sympathetic nerve stimulation [6]. The right stellate ganglion was stimulated alternately at 3 and 5 Hz (10 V, 1ms pulse width) for a total of three cycles. Fresh Tyrode's solution was then placed in the organ bath and L-NA (100 μM) was equilibrated with the tissue for 20 min. A second period of three cycles of alternate 3- and 5-Hz sympathetic stimulation was completed in the presence of L-NA. Following the addition of fresh Tyrode's solution L-NA (100 μM) and L-arginine (1 mM) (the substrate for NOS) was applied to the preparation. After an equilibration period of 20–25 min, a final three cycles of 3- and 5-Hz sympathetic stimulation was completed. The increases in heart rate with SNS were averaged for control, L-NA and L-NA+L-arginine at 3-Hz and 5-Hz stimulation.

2.7. Effect of exercise training on nNOS protein levels in the stellate ganglion from exercised and sedentary guinea-pigs

The stellate ganglia from four sedentary and four trained guinea pigs and a sample of forebrain and intestinal endothelium from a control guinea pig were lysed in buffer containing 50 mM Tris–HCl pH 7.4, 1mM EDTA, 0.5% Triton X-100, and a cocktail of protease inhibitors (Complete, Roche Diagnostics, UK). Protein from the sedentary and trained stellate ganglia were pooled into two separate tubes and protein concentrations measured using the Bio-Rad DC protein assay kit. Two hundred microgrammes total protein was then separated on 12% SDS–polyacrylamide (SDS–PAGE) gels [21] at a constant 70 V for 12 h and resolved proteins then transferred to MSI PVDF membrane (GRI, UK) using a semi-dry transfer cell following manufacturer's protocols (Bio-Rad, UK). Membranes were blocked for 12 h in 3% dried milk in PBS/0.1% Tween-20 (PBST) at 4°C, washed twice in PBST and incubated for 12 h in PBST containing 1% dried milk powder and primary antibody at 4°C. Blots were washed three times for 10 min in PBST and incubated with the appropriate secondary HRP-conjugated antibody for 30 min and then washed as before. Unless specified, all incubations were done at room temperature. Antibody-bound proteins were detected using luminol-based

chemiluminescent detection reagents (Renaissance, NEN Life Science, UK) and exposure to chemiluminescence autoradiography film (Fugi MI-NP Imaging film, GRI, UK) for 12 h. Autoradiographs were analysed by densitometry using NIH-Image software and a optical density (OD) step tablet.

2.7.1. Antibodies

Purified polyclonal anti-eNOS (N30030) and monoclonal anti-nNOS (N31020) both obtained from Transduction Labs via Affiniti UK) were used as the primary antibodies. Secondary horseradish peroxidase(HRP)-conjugated anti-mouse IgG was used for Western blot analysis (Amersham Plc, UK).

2.8. Statistical analysis

Data reported are presented as the mean±SEM unless otherwise specified. For the physical (e.g. body weight) and biochemical markers (e.g. citrate synthase activity) of exercise training, unpaired *t*-tests were used to evaluate the difference between exercise and sedentary groups. For the double atrial/right stellate ganglion preparations, the difference in the positive chronotropic response to SNS at 1, 3, 5, 7 and 10 Hz between exercised and sedentary groups were compared using unpaired *t*-tests. A one-way repeated measures ANOVA followed by a Student Newman-Keuls test for pairwise comparison was used to examine the effects of L-NA and L-NA+L-arginine on the increase in heart rate with sympathetic activation in both exercise and sedentary groups. Differences in heart rate at Control, L-NA, L-NA+L-arginine between exercise and sedentary groups were evaluated by using an unpaired t tests were used. Statistical significance was accepted at $P<0.05$.

3. Results

3.1. Markers of the training effect

Body weights of sedentary (585±20 g; $n=20$) and exercised (594±28 g; $n=20$) animals were similar at the end of the 6 week training period. However, the ventricular weight/body weight ratio was significantly higher in the exercised group compared with the sedentary group ($P<0.05$, Table 1). In addition, citrate synthase activity in the latissimus dorsi and the total concentration of [^{3}H]ouabain binding sites in the latissimus dorsi and ventricles were significantly higher in the exercised group ($P<0.05$, see Table 1).

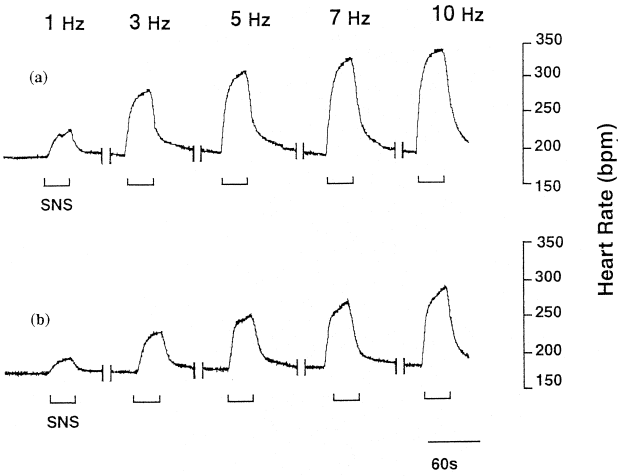

Fig. 1. Representative raw data traces showing the effects of cardiac SNS (1, 3, 5, 7 and 10 Hz, 10 V, 1ms pulse width) on heart rate (bpm) in a double atrial/right stellate ganglion preparation from a sedentary (Fig. 1a) and an exercise trained (6 weeks swimming training; Fig. 1b) animal. The right stellate ganglion was stimulated for 30 s, with 2–3 min between each stimulation period. The time calibration bar refers to both traces.

3.2. Effect of exercise training on the increase in heart rate with SNS

There was no difference in the baseline heart rate in the isolated atria from exercised (165±5 bpm, $n=16$) and sedentary (171±6 bpm; $n=16$) animals. Fig. 1 shows representative raw data traces for the effect of sympathetic stimulation (1, 3, 5, 7 and 10 Hz) on heart rate (bpm) in isolated atrial preparations from a sedentary (Fig. 1a) and an exercised (Fig. 1b) animal. The increase in heart rate with sympathetic stimulation was significantly reduced in atria from exercised animals at 1, 3, 5, and 7 Hz ($P<0.05$). Fig. 2 shows frequency response curves for the mean positive chronotropic response to SNS (1, 3, 5, 7 and 10 Hz) in sedentary ($n=16$) and exercised ($n=16$) groups.

Table 1
Indices of exercise training: ventricular/body weight ratio, latissimus dorsi citrate synthase activity and Na^+ pump content of latissimus dorsi and ventricular tissue from sedentary and exercise trained guinea-pigs

	Ventricular weight/ body weight (mg g^{-1})	Citrate synthase activity (μmol mg^{-1} min^{-1})[a] latissimus dorsi	^{3}[H] Ouabain binding	
			Latissimus dorsi	Ventricle
Sedentary	2.6±0.20 ($n=18$)	0.18±0.03	534±21	1054±23
Exercised	3.0±0.09 ($n=19$)*	0.33±0.05*	696±24*	1152±19*

[a] $n=9$ animals in each group.
* $P<0.05$ sedentary vs. exercise, unpaired *t*-test.

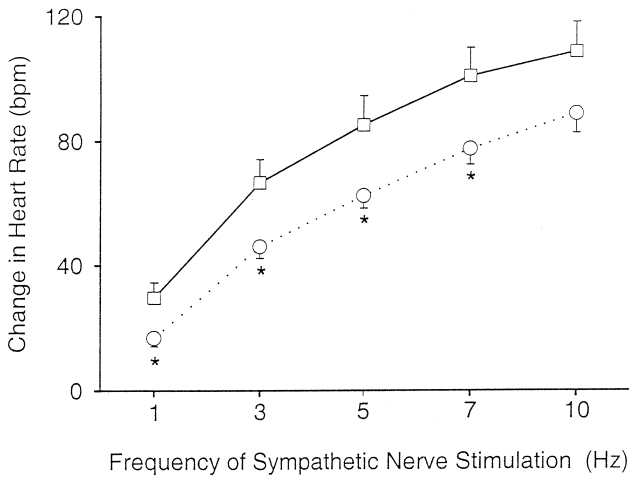

Fig. 2. Frequency response curves for the increase in heart rate (bpm) with SNS (1, 3, 5, 7 and 10 Hz, 10 V, 1 ms pulse width, 30 s duration) in atria from sedentary ($n=16$; empty squares) and exercised ($n=16$; empty circles) animals. The positive chronotropic response to sympathetic stimulation at 1, 3, 5 and 7 Hz was significantly attenuated in exercised atria (*, $P<0.05$).

3.3. Effect of exercise training on the increase in heart rate with bath-applied norepinephrine

The EC_{50} for the positive chronotropic effect of the cumulative addition of norepinephrine (0.1–10 μM) was not significantly different between exercised (−6.08±0.16 M, $n=8$) and sedentary groups (−6.18±0.07 M, $n=7$) animals (see Fig. 3). In addition, there was no significant difference between the positive chronotropic response to this single dose of norepinephrine (0.1 μM) or the equivalent dose in the norepinephrine dose response curve (0.1 μM), for either the exercise or control groups.

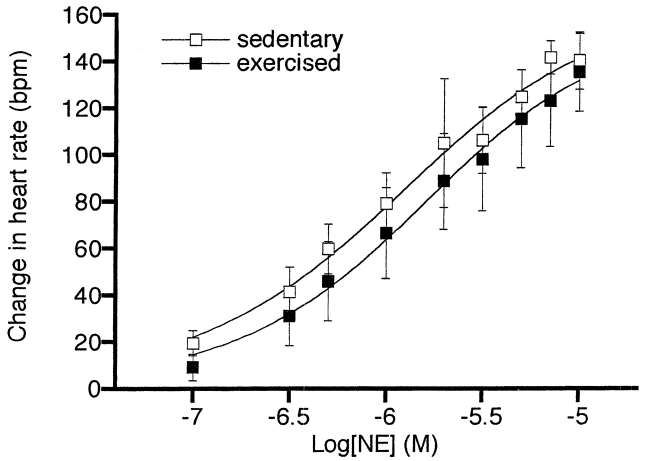

Fig. 3. Dose response curves for the increase in heart rate (bpm) with bath-applied norepinephrine (0.1–10 μM) in atria from sedentary ($n=7$; $EC_{50}=-6.18\pm0.15$ M empty squares) and exercised ($n=8$; $EC_{50}=-6.08\pm0.17$ M, filled squares) animals.

3.4. Role of NO on the positive chronotropic response to SNS

The non-isoform selective NOS inhibitor L-NA (100 μM) and the NO substrate L-arginine (1 mM) did not significantly alter baseline heart rate in either group. The raw data traces in Fig. 4 show the effect of NOS inhibition L-NA (100 μM) on the heart rate response to 3 Hz sympathetic nerve stimulation in atria from trained animals. In the exercised group, L-NA (100 μM) caused a small but significant ($P<0.05$) increase in the magnitude of the positive chronotropic response to sympathetic nerve stimulation at both 3 Hz (Fig. 5a) and 5 Hz (Fig. 5b). The effect was significantly reversed with excess L-arginine (100 μM L-NA+1 mM L-arginine). A similar trend was observed in the sedentary animals, although this did not reach statistical significance.

The positive chronotropic response evoked by sympathetic stimulation was significantly attenuated in the trained compared to the sedentary group during NOS inhibition with L-NA and excess L-arginine (3 and 5 Hz stimulation, $P<0.05$; Fig. 5). However, for the control protocol (prior to the addition of L-NA) the heart rate response to SNS in the exercise group was significantly attenuated only at 5 Hz stimulation (Fig. 5).

3.5. Identification of NOS Isoforms by western blot

Fig. 6 shows the results of Western blot analysis of nNOS protein expression, using a specific monoclonal antibody, in the stellate ganglia from sedentary and exercise-trained animals. A 155 kDa protein band compatible with nNOS [4] was identified in the total protein

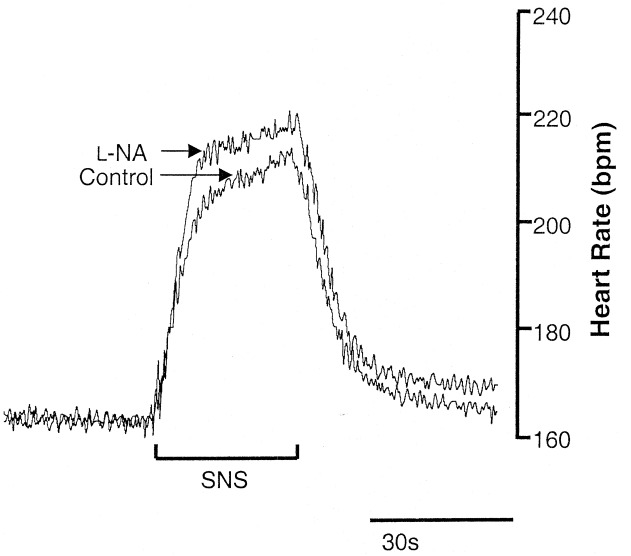

Fig. 4. Superimposed raw data traces from an exercised atria showing that NOS inhibition with L-NA (100 μM) enlarged the increase in heart rate with SNS (5 Hz, 10 V, 1 ms pulse width, 30 s duration).

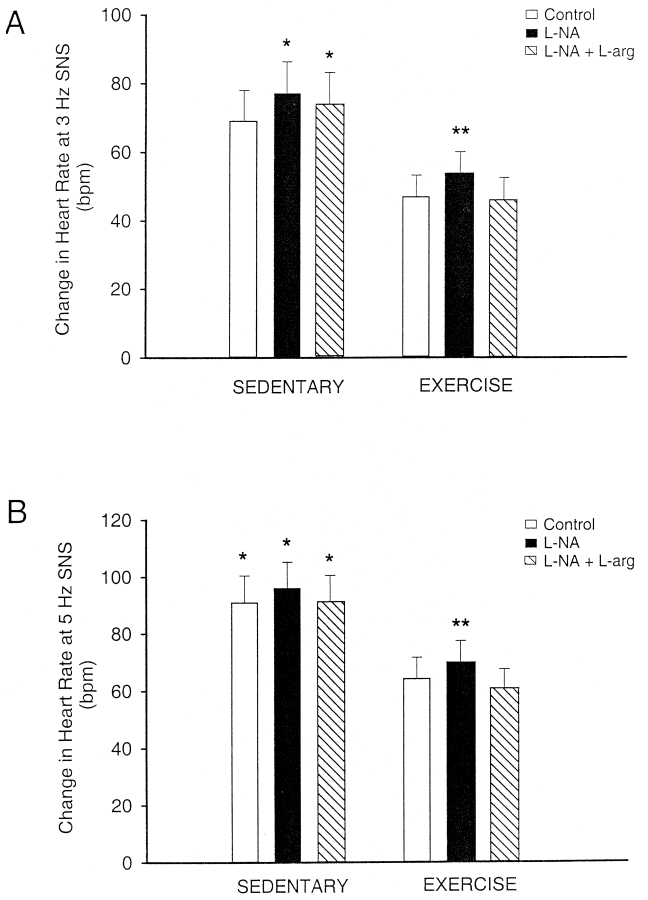

Fig. 5. The effect NOS inhibition with L-NA (100 μM) and its reversal with L-arginine (1 mM) on the increase in heart rate with right stellate ganglion stimulation (10 V, 1 ms pulse width, 30 s duration) at 3 Hz (a, $n=14$ sedentary, $n=13$ exercised) and 5 Hz (b, $n=15$ sedentary, $n=13$ exercised). L-NA significantly enhanced the magnitude of the positive chronotropic response to sympathetic activation in exercised atria at 3 and 5 Hz stimulation and this effect was significantly attenuated with L-arginine (*, $P<0.05$). Note that the heart rate response to sympathetic stimulation remained significantly attenuated during NOS inhibition (**, $P<0.05$).

sample from both groups and also in a sample of guinea pig brain tissue. The level of nNOS protein was significantly higher (83% increase) in the exercised group compared to the sedentary group (control, 6.60 ± 1.63 OD, $n=3$; exercised, 12.07 ± 1.93 OD, $n=4$). This protein was not detected in guinea pig small intestinal endothelium, where eNOS is the main isoform of NOS present. There was no evidence of eNOS in the stellate from either group.

4. Discussion

We show for the first time that there is a significant peripheral component to the reduced heart rate response to cardiac SNS with training, and that this may involve a pre-synaptic mechanism since there was no difference in the HR response to bath applied NE. In the trained group, NOS inhibition significantly increased the HR response to SNS, an affect not seen in the sedentary group. In addition, the level of nNOS protein from the stellate ganglion was higher in the trained animals. These findings suggest that NO may be partly responsible for the reduction in the cardiac sympathetic response following training. The overall effect of NO however, appears to be small since the positive chronotropic response to SNS was still significantly reduced in the trained group during NOS inhibition when compared to the control response in untrained animals.

4.1. Efficacy of training

It is generally accepted that physical training improves oxidative enzyme activity [2,35], induces cardiac hypertrophy [28], and upregulates skeletal muscle Na^+/K^+ pump concentration [8,20]. In the present study exercise training increased latissimus dorsi citrate synthase activity, ventricular weight/body weight ratio and Na^+/K^+ pump concentration in skeletal and cardiac muscle. The signifi-

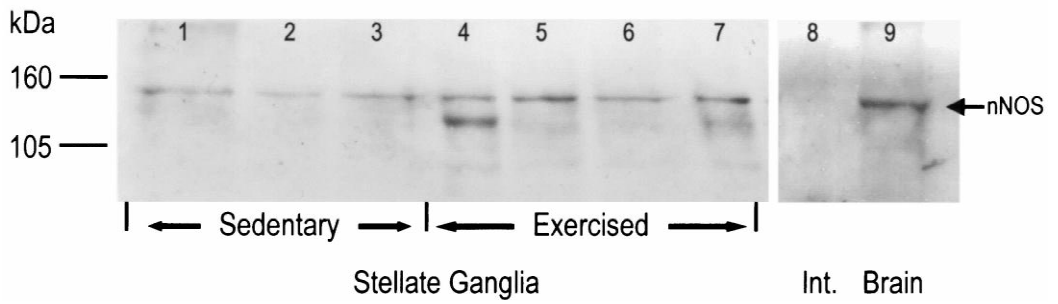

Fig. 6. Western blot showing mature nNOS (~155 kDa) protein levels in right stellate ganglia isolated from 3 sedentary (lanes 1–3) and 4 exercised (lanes 4–7) guinea pigs and from isolated guinea pig small intestine (negative control, lane 8) and guinea pig fore-brain (positive control, lane 9). Equal amounts of protein (50 μg) were loaded into each lane.

cance of elevated Na^+/K^+ pump concentration in the heart and its potential correlation to physical performance remains unclear. Functionally, increased Na^+/K^+ pump concentration following training may favour skeletal muscle performance as it enables more efficient K^+ clearance of the exercise-induced hyperkalemia [7].

The effect of chronic exercise on myocardial mass varies according to training modality, age of animals, gender, training duration and/or intensity [21]. Data from longitudinal swimming and treadmill running studies in rats report moderate degrees of cardiac hypertrophy defined by an increase in the heart weight/body weight ratio [14,25,27,28]. The present study reports a 15% increase in ventricular weight/body weight ratio in the exercised group relative to the sedentary group, which is within the range for exercise-induced ventricular hypertrophy [21]. In the current study, development of ventricular hypertrophy in response to training was not associated with concomitant reduction in intrinsic heart rate.

4.2. Exercise training attenuates the heart rate response to sympathetic activation in-vitro via a pre-synaptic mechanism

The attenuated heart rate response to sub-maximal exercise following physical training is well established [2,28], however the mechanisms underlying the training effect on HR are controversial. Some studies report that physical training reduces both the density of β-adrenoceptors and affinity of β-adrenoceptors agonists [23,39] in cardiac tissue with a subsequent reduction in the chronotropic responsiveness to exercise and isoproterenol [17]. Others however, have shown a reduction in circulating catecholamines during exercise following training [10,18,38]. In the present study, physical training significantly reduced the heart rate response to peripheral SNS. This effect could result from a pre-synaptic mechanism because the heart rate responses to bath-applied norepinephrine were not different between the exercise and sedentary groups. These findings are consistent with studies that have shown that cardiac β-adrenoceptor number and agonist affinity are unaffected by physical training [40,41].

4.3. NO inhibits the sympathetic control of cardiac excitability

Extensive experimental evidence supports a NO mediated inhibition of peripheral cardiac sympathetic neurotransmission in vitro and in vivo [6,13,29,30,34]. In isolated perfused rat hearts NOS inhibition with L-NA significantly enhanced NE overflow with cardiac SNS [29]. This effect persisted following selective damage of the endothelium, suggesting that the source of NO may be neuronal. Similarly, NOS inhibition with L-NAME (N^G-nitro-L-methylester) enhanced NE levels in the coronary sinus and increased inotropic responsiveness of the left ventricle to sub-maximal stimulation of the left stellate ganglion in anesthetized and vagotomised dogs [34]. Conversely, Fei et al. [13] found that L-arginine (the substrate for NOS) reduced plasma norepinephrine spillover and the shortening of the effective refractory period during ansae subclaviae stimulation in autonomically denervated dogs. In the same preparation, Elvan and coworkers [12] have shown that inhibition of NOS with the non-isoform selective NOS inhibitor L-NMMA significantly enhanced the effects of ansae subclaviae and isoproterenol infusion on sinus cycle length and atrioventricular conduction time.

Recent studies provide evidence of a pre-synaptic inhibitory action of NO on cardiac sympathetic nerve activity. The selective neuronal NOS inhibitor TRIM (1-(2-Trifluoromethylphenyl-)imidazole) enhanced the magnitude of the positive chronotropic response to stimulation of the right stellate ganglion, but not to infused isoprenaline in the cardiac sympathectomized and vagotomized anesthetized rabbit [30]. Similarly, the selective neuronal NOS inhibitor 7-NiNa (7-nitroindazole) increased the heart rate response with cardiac sympathetic stimulation, but not with bath-applied NE, in the isolated guinea-pig atria [6]. Taken together, these data are consistent with the idea that NO, synthesized from nNOS within the cardiac sympathetic innervation, inhibits cardiac sympathetic transmission.

In the current study, the NOS inhibitor L-NA, enhanced the increase heart rate with cardiac SNS, by 11% and 5% at 3 Hz and 5 Hz respectively in sedentary animals, however, these effects did not reach statistical significance. L-NA has previously been shown to enhance the positive chronotropic response to SNS in the isolated guinea-pig atria [6]. The discrepancy between these findings may reflect the different ages at which the animals were studied since NOS levels may decrease during development [36,43]. Young and Ciampoli [43] found that the number of NOS-containing neurons in the mouse submucous plexus decreased from 50% at postnatal day 0 to 3% in adult mice. In the fascia dentata of the rat hippocampus the density of NADPH-diaphorase (a histochemical marker for nNOS) positive neurons decreased by 69% between 6- and 12-month old animals [36]. Our previous findings were obtained in animals that were 2–4 weeks old (pre-pubescent; 125–150 g), whereas in the present study the animals were 8–10 weeks old (adult; 550–600 g) when they were studied following 6 weeks training.

4.4. The role of NO in the reduced heart rate response to sympathetic stimulation with exercise training

While there is extensive evidence on the role of NO in vascular control following exercise training [15,19], its role in the sympathetic modulation of heart rate following exercise training is unknown. In the present study, NOS

inhibition significantly enhanced the effect of cardiac SNS (3 and 5 Hz) on atrial beating rate in the exercised (but not the sedentary) group. We also show that nNOS protein is increased in the stellate ganglia from the trained group. The mechanism behind this is unclear. However, potential sites that regulate nNOS mRNA and transcription of the nNOS gene are emerging [3]. Regulation of nNOS expression has been correlated with changes in neurotransmitters, hormones, metabolites, chronic electrical stimulation of skeletal muscle [27] and the co-induction of transcription factors (such as c-fos and c-jun) [3]. To our knowledge, there is no information about the effect of training on regulation of nNOS promotor activity. Other studies have shown that exercise training enhances eNOS gene expression [31,42] in coronary and skeletal muscle arteries, an event probably related to increased shear stress [31].

In addition to NO, our results suggest that other factors are involved in the reduced positive chronotropic response to peripheral SNS with training since the heart rate response to sympathetic activation in trained (versus sedentary) animals remained significantly attenuated after NOS inhibition. These factors could conceivably involve upregulation of pre-synaptic inhibitory α_2-adrenoceptors, inhibition of neuronal calcium channels or increased activation of the norepinephrine uptake-1 transporter. Recently, raised atrial natriuretic peptide (ANP) has been linked with concomitant elevations of nitrate and cGMP following long term exercise training [26]. Furthermore, ANP inhibits norepinephrine (NE) biosynthesis by increasing neuronal NE uptake and reducing basal and evoked neuronal NE release [37]. Therefore, we cannot exclude the possibility that in our study, elevated ANP levels following exercise training contributed to the reduced heart response to sympathetic stimulation. Our results also do not rule out the possibility that NO may decrease central sympathetic activation following training, since NO has been reported to reduce central sympathetic excitability in anesthetised pigs [44].

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