Hypertension and Exercise Training Differentially Affect Oxytocin and Oxytocin Receptor Expression in the Brain

Adriano S. Martins, Alessandra Crescenzi, Javier E. Stern, Silvana Bordin, Lisete C. Michelini

Abstract—We have previously shown that exercise training activates nucleus tractus solitarii (NTS) oxytocinergic projections, resulting in blunted exercise tachycardia. The objective of this study was to determine the effects of hypertension and training on oxytocin (OT) and OT receptor expression in the hypothalamic paraventricular nucleus (PVN) and projection areas (dorsal brain stem [DBS]). Male, normotensive, Wistar-Kyoto (WKY) and spontaneously hypertensive (SHR) rats were trained (55% maximal exercise capacity, 3 months) or kept sedentary, and pressure was measured weekly. DBS sections were processed for immunohistochemistry (polyclonal guinea pig anti-OT) or in situ hybridization for OT and OT receptor (35S-oligonucleotide probes). Other groups of rats had brains removed and frozen to isolate the DBS and PVN; samples were processed for OT and OT receptor cDNA reverse transcription–polymerase chain reaction amplification with β-actin as the housekeeping gene. Training was equally effective in improving running distance in both groups, with pressure reduction only in SHR (−10%, \( P<0.05 \)). In trained WKY, baseline bradycardia (\( P<0.05 \)) occurred simultaneously with increased NTS OT immunostaining and mRNA expression (+3.5-fold), without any change in OT receptor mRNA expression. PVN OT mRNA and DBS OT receptor mRNA expressions were significantly lower in SHR versus WKY (−39% and −56%, respectively). Training did not alter DBS OT receptor density in the SHR group but increased OT mRNA in both PVN and DBS areas (+78% and +45%, respectively). Our results show a marked hypertension-induced reduction in OT receptor mRNA expression, not altered by training. In contrast, training increased OT mRNA expression in sedentary and hypertensive rats, which may facilitate training-induced cardiac performance. (Hypertension. 2005;46[part 2]:1004-1009.)

Key Words: hypothalamus • exercise • rats, spontaneously hypertensive • neurotransmitter • genetics • autonomic nervous system • immunohistochemistry

Hypertension is a highly prevalent disease and a common risk factor for different cardiovascular diseases, with a major impact on morbidity and mortality.\(^1\) Hypertensive individuals present with a series of functional and anatomic deficits, such as increased vascular resistance, vessel rarefaction, increased heart energy expenditure, increased stroke work, impaired baroreceptor reflex control, hormonal imbalance with overactivation of the renin-angiotensin system, and increased insulin resistance. Most of these effects contribute to increased sympathetic activity and depressed cardiovascular function.\(^2\) On the other hand, exercise training has been associated with a variety of beneficial cardiovascular adjustments in hypertensive individuals, such as eutrophic remodeling of arterioles causing wall-lumen ratio normalization,\(^2,3\) capillary angiogenesis and venule neoformation in exercised muscles resulting in increased vascular capacity and \( O_2 \) extraction,\(^4,7\) and remodeling of the heart with simultaneous stroke volume increase and heart rate decrease.\(^8,9\) Although there are some observations suggesting decreased sympathetic activity after exercise training,\(^10,11\) very little is known about the potential benefits of repetitive physical activity on central mechanisms controlling cardiovascular functions.

Previous studies by our group have shown that vasopressin and oxytocin (OT) projections from parvocellular neurons of the hypothalamic paraventricular nucleus (PVN) to the nucleus tractus solitarii (NTS) are involved in both reflex control of the heart and heart rate adjustments during dynamic exercise. For example, whereas vasopressin resets baroreceptor reflex control of the heart toward higher heart rate values and improves exercise tachycardia,\(^12,13\) OT improves reflex bradycardia and blunts exercise tachycardia.\(^14,15\) Furthermore, we showed that vasopressinergic and oxytocinergic modulation of cardiovascular control in normotensive rats is improved after training, resulting in occlusion of reflex bradycardia and thus facilitating tachycardic response and increased cardiac output during dynamic exercise.\(^13,16,17\) There is no information, however, on the efficiency of vasopressin and OT modulation of cardiovascular control in hypertensive subjects.

OT has been shown to stimulate autonomic neurons in the NTS, thereby improving vagal outflow and augmenting reflex bradycardia.\(^15,18-20\) effects found to be blunted in most

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models of hypertension. Interestingly, OT-mediated effects within the NTS were improved by training.\textsuperscript{14,16} On the basis of the aforementioned information, we sought to investigate whether functional responses observed in trained, normotensive rats could be correlated with plastic changes in OT neurons projecting to the NTS. Furthermore, we determined the differential effects of training on peptide mRNA expression in biosynthetic and target areas and on OT receptor mRNA expression in the NTS of normotensive and hypertensive rats.

Methods

Animals and Training Protocol

Two-month-old, male, Wistar-Kyoto (WKY) and spontaneously hypertensive (SHR) rats were housed in Plexiglas cages on a 12-hour/12-hour light/dark schedule and allowed free access to food and water. Rats were initially preselected for their ability to walk on a treadmill (Inbramed, KT-300; 5 to 10 sessions, 0.3 to 0.6 km/h, 0% grade, 10 min/d). Before starting the protocols (week 0), active rats were subjected to a maximal exercise test (graded exercise on the treadmill, starting at 0.3 km/h with increments of 0.3 km/h every 3 minutes up to the maximal intensity attained) to determine individual exercise capacities and to assign rats with equivalent capability to trained (T) or sedentary (S) groups. Low-intensity training was performed 5 d/wk, 1 d/3 for 3 months, as described previously.\textsuperscript{2,3,5,12,14} Exercise intensity was increased progressively by a combination of time and speed to attain 50% to 60% of maximal exercise capacity, as determined by exercise tests on the treadmill. Individual maximal exercise tests were repeated at weeks 6 and 13 to adjust training intensity and compare the efficacy of the training protocol, respectively. Rats allocated to S groups were handled every day. Body weight was measured weekly. All protocols and surgical procedures used are in accordance with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation and were approved by the Institutional Animal Care and Use Committee.

Functional Measurements

Pressure was measured indirectly (tail or systolic pressure, weekly during S and T protocols) and directly at the end of the protocols, as described previously.\textsuperscript{2,3,5,12,14} In brief, a catheter was placed in the femoral artery and exteriorized on the back of the neck under anesthesia. After a 24-hour recovery period, the catheter was connected to the recording system, and the rat was allowed to rest for stabilization of cardiovascular parameters: basal values of arterial pressure (AP) and heart rate (HR) were then recorded in conscious, freely moving rats for \textasciitilde30 minutes. Rats were deeply anesthetized (sodium pentobarbital, 60 mg/kg IP) and submitted, immediately after respiratory arrest, to thoracotomy and transection of the obex up to 300 to 400 \textmu m (pixels/window) were made on the acquired dark-field images (Image Pro Plus, Media Cybernetics), and values were converted to pixels/mm\textsuperscript{2}. Measurements were made in the commissural NTS from the obex up to 300 to 400 \mu m rostral and corresponded to the average of 3 windows/slice (central area, as showed in Figure 1, plus left and right windows). Background measurements were taken from adjacent areas showing no labeling.

RT-PCR Studies

For reverse transcription–polymerase chain reaction (RT-PCR) analysis, WKY\textsubscript{S}, WKY\textsubscript{T}, SHR\textsubscript{S}, and SHR\textsubscript{T} were perfused with 0.1 mol/L PBS treated with diethylpyrocarbonate (0.01%), pH 7.2, and decapitated to remove the brains, which were quickly transferred to a dry-ice box. Slices (~1000 \mu m) were taken at the obex and hypothalamic levels to isolate target areas: dorsal brain stem (DBS, containing the NTS and surrounding structures, such as the dorsal motor nucleus of the vagus, area postrema, and part of the gracilis and hypoglossal nuclei) and hypothalamic PVN (medial plus caudal parts of the nucleus). DBS and PVN areas were obtained by needle punches on the thick frozen slices. For each group, PVN and DBS tissue samples were pooled (5 rats/sample) and immediately frozen in L\textsubscript{N}\textsubscript{2}. Total RNAs were extracted with Trizol reagent, according to the manufacturer’s instructions (Invitrogen). The RNAs used for RT-PCR analysis were obtained from 3 independent experiments. RT was done with 2 \mu g of total RNA with Moloney murine leukemia virus reverse transcriptase (Superscript II) and random hexamers. The cDNA was diluted in a 1:2 ratio, and 1 \mu L was used for PCR. RT-PCR assays were done in quadruplicate with recombinant Taq DNA polymerase (Invitrogen, EUA) and 10 pmol of each primer in a master mix of 100 \mu L. Gene-specific primers were designed according to GenBank sequences encoding OT (NM_012996), OT receptor (NM_012872), and \beta-actin (NM_031144). Primers used for RT-PCR analysis with their respective melting points and product lengths were as follows: OT, 5'-ATCTCCGACAGTGAACACCAACG-3' (sense) and 5'-AGGGCAGGTGCTTCCCTCC-3' (antisense), 57°C, 380 cpm/100 \mu L, and stored at −80°C until processing. For immunohistochemical studies (n=8, 4 S and 4 T), serial brainstem sections were cut (20 \mu m), Leica cryostat CM3050), transferred to 0.01 mol/L PBS, and incubated in a solution of 0.01 mol/L PBS with 0.01% Triton X-100 and 10% normal goat serum for 1 hour. Sections were then incubated overnight in a monoclonal mouse antibody raised against OT neurophysin (PS41, 1:1000 dilution; kindly donated by Dr Harold Gainer, National Institutes of Health, Bethesda, Md) or a polyclonal guinea pig anti-OT (Bachem, 1:100 000 dilution). Reactions in primary antibodies were followed by 2-hour incubation in the presence of donkey anti-mouse or anti-rabbit Cy3-labeled secondary antibodies (1:400 dilution).

In situ hybridization experiments were conducted in normotensive WKY only (n=8, 4 S and 4 T). Brainstem coronal slices (20 \mu m) were cut and collected in 8 tissue-culture wells with 0.01 mol/L PBS at 4°C. For each rat, collected slices were mounted on 8 slides covered with polylysine (Sigma) and dried overnight. Hybridization experiments were performed according to the technique described by Key et al.\textsuperscript{21} Oligodeoxynucleotide probes complementary to rat nucleotide sequences 3177 to 3206\textsuperscript{22} and 3250 to 3276\textsuperscript{23} for OT and OT receptor, respectively, were labeled with 35S-dATP (NEN) with terminal deoxynucleotidyl transferase (Life Technologies). After nonincorporated radioactivity was removed, probes were diluted in hybridization buffer to yield \textasciitilde10\textsuperscript{6} cpm/\mu L and stored (−20°C). For the hybridization reaction, slides were washed (0.01 mol/L PBS followed by 2× saline sodium citrate [SSC] at room temperature) and probes were diluted further to final concentration of 0.3 to 0.5×10\textsuperscript{6} cpm/100 \mu L. Experiments were conducted in duplicates: for each rat, 2 slides were hybridized with antisense for OT, 2 with antisense for the OT receptor, and the other 4 slides with sense 35S-oligonucleotides for OT and OT receptor (negative controls). Probe was applied on the top of each slice (30 \mu L/slide), and slides were incubated for 20 to 24 hours at 37°C in a humidified box. Posthybridization treatment included high-stringency washes (1× SSC for 2 hours at room temperature plus 1× SSC for 30 minutes at 50°C). Autoradiography was used to localize probes in brain areas and to quantify the amount of T and S per area in trained and control rats. In a darkroom, slides were dipped in NTB-2 liquid emulsion (Kodak), dried and stored in appropriate black boxes, and kept in a refrigerator for 20 (OT probes) or 40 (OT receptor probes) days. After development (D-19 Kodak developer, F-24 fixative), sections were counterstained with cresyl violet.\textsuperscript{21} Brainstem areas were identified by bright-field examination (Leica DMLB). After the area to be quantified was selected, dark-field images (87 500×-mm\textsuperscript{2}) were obtained at 200× magnification. Density measurements (pixel/1000 mm\textsuperscript{2}) were made on the acquired dark-field images (Image Pro Plus, Media Cybernetics), and values were converted to pixels/mm\textsuperscript{2}. Measurements were made in the commissural NTS from the obex up to 300 to 400 \mu m rostral and corresponded to the average of 3 windows/slice (central area, as showed in Figure 1, plus left and right windows). Background measurements were taken from adjacent areas showing no labeling.
bp; OT receptor, GTTCCTGGCTCTGAGTGGCAAC-3’ (sense) and 5’-ACAATGTAGACGGCGAGCGTG-3’ (antisense), 57°C, 487 bp; and β-actin, 5’-ATGAAGATCCTGACCGAGCGTG-3’ (sense) and 5’-CTTGCTGATCCACATCTGCTGG-3’ (antisense), 58°C, 510 bp. For each gene, the number of cycles was within the logarithmic phase of amplification and defined through standard curves with 20 to 42 cycles. PCR products were separated on 1.2% ethidium bromide–agarose gels, and the intensities of bands were determined by digital scanning and quantified by means of Scion Image analysis software (Scion Corp). The results were expressed as a ratio of target gene to β-actin signal.

Statistical Analysis
Results are expressed as mean±SEM. Differences between groups (SHR and WKY) and conditions (T and S) were analyzed by 2-way ANOVA, followed by a Student-Newman-Keuls multiple-comparison test. For studies in normotensive rats, differences between groups (T and S) were analyzed by Student t test. Differences were considered significant at P<0.05.

Results

Studies on Normotensive Rats
MAP and HR recordings in resting WKY T versus WKY S (8 to 10 rats in each group) showed that training did not change mean AP (116±3 vs 112±4 mm Hg) but caused significant bradycardia (310±7 vs 337±9 bpm, respectively, P<0.05). Immunohistochemical analysis of the DBS revealed that training-induced bradycardia was accompanied by a marked increase in OT-like immunoreactivity in the commissural NTS (Figure 1). Semiquantitative analysis performed by in situ hybridization of the DBS revealed that training increased OT mRNA expression in the commissural NTS (from 4456±787 to 15787±1099 pixels/mm², a 3.5 fold increment, P<0.05, Figure 2), without changing OT receptor mRNA expression in the same area (WKY S=7348±930; WKY T=9732±1739 pixels/mm², P>0.05, Figure 2).

Comparative Studies in Normotensive and Hypertensive Rats
To confirm these results and uncover the central effects of training on hypertensive rats, we compare WKY and SHR groups submitted to training or kept sedentary (5 rats in each group). Training did not change systolic tail-cuff pressure of normotensive groups (average means of 121±3 vs 125±4 mm Hg for WKY T versus WKY S during the 3-month period) but caused a small, significant fall in hypertensive rats, observed since the ninth week of training (average of 195±4 vs 209±4 mm Hg for SHR T versus SHR S in the last month of protocols, P<0.05). RT-PCR studies revealed that hypertension caused opposite effects on OT mRNA expression in biosynthetic and target areas: there was a 39% decrease in hypothalamic content (0.59±0.08 vs 0.97±0.14 arbitrary units for SHR S versus WKY S, P<0.05, Figure 3A), whereas a
2-fold increase was observed in DBS (0.75±0.04 vs 0.36±0.02 arbitrary units for SHR vs WKY, *P<0.05, Figure 3B). Hypertension was also accompanied by a marked decrease in OT receptor mRNA expression in DBS (from 1.19±0.13 in WKY to 0.57±0.06 arbitrary units in SHR, *P<0.05 for group factor, Figure 3C). Training was not effective in changing DBS OT receptor mRNA expression in both SHR and WKY (Figure 3C). On the other hand, training caused significant increases in peptide mRNA expression in both areas of both groups: SHRT = 1.05±0.12, WKYT = 1.56±0.14, corresponding to increases of 78% and 61% over respective S controls, *P<0.05, in the PVN (Figure 3A); SHRt = 1.09±0.20, WKYt = 1.26±0.02, corresponding to increases of 45% and 3.5-fold over respective S controls, *P<0.05, in the DBS (Figure 3B). Interestingly the relative exercise-induced increment in peptide mRNA expression in the DBS of normotensive rats (Figure 3B) was similar to that measured in the NTS by the in situ hybridization technique (Figure 2).

Efficacy of Training Protocol

Low-intensity training was equally effective at increasing treadmill performance in all groups analyzed. Three-month training increased the effective running distance by 3.6-fold in WKY groups used for immunohistochemistry and in situ hybridization studies (WKYt = 320±22 vs WKYS = 89±9 m, as measured during maximal exercise tests at the end of protocols, *P<0.05, n=10 in each group). Similar training-induced improvement was observed in the groups used for RT-PCR studies. Although SHR treadmill performance was higher than that of the WKY group since the beginning of protocols (141±21 vs 92±11 m, respectively, at week 0, n=10 for each group), effective running distance was equally improved by training in both groups: SHRt = 520±61, WKYt = 298±30 m at the end of protocols, corresponding to 4.0- and 3.6-fold increases over respective S controls (*P<0.05).

Discussion

Several new observations arise from the present set of data: (1) decreased baseline HR (an important adjustment to training) is accompanied by increased peptide content and OT mRNA expression in the NTS, as revealed by immunohistochemistry, in situ hybridization, and RT-PCR studies; (2) NTS OT receptor density is markedly reduced in SHR compared with WKY and not changed by training; (3) hypertensive rats have decreased OT mRNA expression in the biosynthetic area (magnocellular and parvocellular PVN neurons) but an increased expression in a target area (DBS, parvocellular projections); and (4) training is effective in increasing OT mRNA expression within the PVN and DBS of both WKY and SHR, with larger changes being observed in the DBS of the WKY group. Altogether these results indicate that the central OT system is differentially affected by hypertension and training.

The role of OT in cardiovascular control has been the subject of previous investigations. It has been described as a weak vasoconstrictor with significant effects on vascular tone, blood pressure, and renal function.24–26 A dense core of OT projections arising from the PVN has been shown to
project to brainstem areas controlling cardiovascular function.27–29 Brainstem administrations of OT or OT receptor antagonist did change local neuronal activity18–19 and the autonomic control of the heart.15,20 Functional experiments in conscious, normotensive rats showed that NTS oxytocinergic projections exert a tonic effect on HR control, facilitating vagal outflow and reflex bradycardia during baroreceptors loading.15 We also demonstrated in normotensive, trained rats running on a treadmill that the increased OT release within the DBS was the main determinant of the reduced tachycardic response observed after training.14,16 The present results showing enhanced OT mRNA expression and OT immunostaining in the NTS of trained, normotensive rats provide complimentary anatomic data supporting our previous functional data. Thus, we proposed that the increased peptide release within the NTS is an important mechanism underlying the smaller exercise tachycardia of trained individuals.

Interestingly, our results showed that training increased OT mRNA expression in both biosynthetic and target areas. Although it has been traditionally believed that OT (and vasopressin) are synthesized in neuronal somata and then transported with the carrier neurophysin to their axonal terminals, an increasing number of studies in the last decade have challenged this concept. Recent studies showed not only that transport of mRNAs occurs to extrasomata locations in the central and peripheral nervous system but also that peripheral domains of neurons have been shown to possess the ability to synthesize proteins independent of the cell body.30–32 Dendrites are known to contain the operative translation machinery (ribosomes, rough endoplasmic reticulum, Golgi compartment), as well as cytoskeletal elements involved in mRNA transport.31,33 For example, mRNA sorting with dendritic protein synthesis has been observed in oxytocinergic and vasopressinergic neurons.31 Other studies have also reported mRNA sorting to axons of mammalian nerve cells. Vasopressin mRNA and vasopressin transcripts are detected in axons of embryonic rat magnocellular neurons, suggesting that axonal mRNA plays a role in development and neuronal differentiation.32,34,35 Furthermore, continuous axonal transport of mRNAs in mature magnocellular neurons has also been reported.13 Our present findings showed a huge training-induced enhancement of OT mRNA levels both within the PVN (neuronal cell bodies) and in the DBS (axonal terminals) in WKY and SHR groups. Whether increased DBS mRNA indicates true peptide synthesis within the axonal compartment or alternatively reflects leakage of excess somata mRNA remains unknown at the present.

Another original observation of the present study is the hypertension-associated deficit in central OT pathways: both OT receptor density in projection areas and PVN OT mRNA expression (magnocellular and parvocellular) were found to be depressed in SHR. Interestingly, we observed that DBS OT mRNA expression was increased in SHRsr, an effect that could represent a compensatory adjustment to the reduced OT receptor density. Opposite effects on brainstem (reduced) and on neurohypophysis vasopressin content (increased) have been previously reported in SHR when compared with WKY.36 The present set of data showing reduced hypothalamic OT content in SHR (magnocellular plus parvocellular neuronal synthesis) along with increased DBS OT mRNA density (a specific parvocellular target area) suggests differential effects of training on magnocellular (inhibition) and parvocellular (activation) OT pathways. Indeed, a previous study from our laboratory showed reduced plasma OT levels simultaneously with increased DBS OT content in trained rats running on a treadmill.14

It is important to note that training was unable to correct the reduced OT receptor density observed in the DBS of hypertensive rats. On the other hand, training partially compensated for the hypertension-associated deficit in OT modulation, most likely by increasing central peptide content. Considering that activation of the central OT system both changes neuronal activity in brainstem areas18,19 and facilitates vagal outflow and slowing of the heart,14–16,20 it is reasonable to speculate that training-induced effects are beneficial to hypertensive individuals, who have high sympathetic activity and high HR levels. The effectiveness of training on OT modulatory effects, however, seems to be larger in normotensive than hypertensive individuals. It is important to note that SHR exhibited a better treadmill performance than age-matched WKY both before and after the training protocol,3 but the efficacy of training was similar between groups.

In conclusion, our data show hypertension-associated and training-induced plastic changes in oxytocinergic pathways from the PVN to the DBS. These changes support previous functional studies, confirming both the abnormal HR control in hypertensive individuals (reduced DBS OT receptor density) and the improvement in cardiovascular control observed after training (increased DBS OT density). In addition, the present results suggest that changes in peptide content (but not receptor expression) within the NTS may constitute a functional mechanism by which training improves cardiovascular control in normotensive and hypertensive individuals.

Perspectives
In the last decade, several reports showing beneficial effects of exercise on cardiovascular effectors have suggested the use of physical training as an additional therapeutic tool to control pressure levels in hypertensive patients. Findings from the present study, showing that beneficial effects of training also occur on neural mechanisms controlling cardiovascular function, extend our knowledge of the ability of training to improve blood pressure control, not only in hypertensive but also in normotensive individuals. It is important to note that although training is unable to correct the hypertension-induced deficit in OT receptor expression, it improves cardiovascular control by increasing peptide expression and peptide content in cardiovascular controlling areas. The present observations open the possibility that other peptidergic pathways could present similar adjustments to training and/or other behavior to offset modulatory deficits in homeostatic control.

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